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UNIVERSITÉ DE MONTRÉAL

PHYSICOCHEMICAL CHARACTERIZATION AND BIOCOMPATIBILITY OF ALGINATE-POLYCATION MICROCAPSULES DESIGNED FOR ISLET TRANSPLANTATION

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Cette thèse intitulée :

PHYSICOCHEMICAL CHARACTERIZATION AND BIOCOMPATIBILITY OF ALGINATE-POLYCATION MICROCAPSULES DESIGNED FOR ISLET TRANSPLANTATION

présentée par : TAM Susan Kimberly

en vue de l'obtention du diplôme de : Philosophiae Doctor

a été dûment acceptée par le jury d'examen constitué de :

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This work is dedicated to all those that are facing the struggle of dealing with type 1 diabetes, particularly the brave parents of diabetic children that I've had the good fortune to meet.

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Last but not least, I am forever grateful to my family for your incredible support over these past many years : Odette, for having kept me on the « engineering » track, and for sticking with me through my ups and downs of student life. Lisa, for inspiring me to do what I do because I choose to. Mom and Dad for your never-ending support and for having always pushed me to be the best I can be. Axel et Matéo, pour votre amour et votre soutien tous les jours depuis qu'on s'est rencontré, pour votre croyance en moi, et pour me rappeler ce qui sont les choses les plus importantes dans la vie.

PREFACE

This thesis is submitted in partial fulfillment of the requirements for the attainment of a Philosophiae Doctor (Ph.D.) degree in Biomedical Engineering at the École Polytechnique de Montréal (EPM). The work was principally carried out at the EPM and the Centre de Recherche de l'Hôpital-Maisonneuve Rosemont (CRHMR), under the direction of D^{r} L'Hocine Yahia and co-direction of D^{r} Jean-Pierre Hallé. Some of this work was also completed at the University Medical Centre Groningen (UMCG) under the supervision of D^{r} Paul de Vos, during a 6-month internship.

The majority of the results presented in this thesis are the basis of three original articles that have been published or submitted for publication. These have been included in this thesis as separate chapters. During my doctoral studies, I also contributed to a number of other published works that have either been included as an *Appendix* or have not been included at all. These contributions are described in **Table P.1**.

Table P.1 List of publications written during my doctoral studies (2005-2010)

Original articles (first author)		
☑ Chapter 3	Contributed 70%	**Tam SK**, de Haan B, Faas MM, Hallé JP, Yahia L'H, de Vos P. *Adsorption of human immunoglobulin to implantable alginate-poly-L-lysine microcapsules: Effect of microcapsule composition*. J Biomed Mater Res A (Jun 2009) 89(3): 609-15.
☑ Chapter 5	Contributed 85%	**Tam SK**, Dusseault J, Bilodeau S, Langlois G, Hallé JP, Yahia L'H. *Factors influencing alginate gel biocompatibility*. J Biomed Mater Res A. [revised manuscript submitted for publication]
☑ Chapter 6	Contributed 85%	**Tam SK**, Bilodeau S, Dusseault J, Langlois G, Hallé JP, Yahia L'H. *Biocompatibility and physicochemical characteristics of alginate-polycation microcapsules*. Acta Biomater (2010). [in press]

☑ included in thesis ; ☒ not included in thesis

Table P.1 (continued). List of publications written during my doctoral studies (2005-2010)

Original articles (first author)		
☒ *Based on master's research*	Contributed 40%	**Tam SK**, Dusseault J, Polizu S, Ménard M, Hallé JP, Yahia L. *Impact of residual contamination on the biofunctional properties of purified alginates used for cell encapsulation*. Biomaterials (Mar 2006) 27(8): 1296-1305.
☒ *Based on master's research*	Contributed 80%	**Tam SK**, Dusseault J, Polizu S, Ménard M, Hallé JP, Yahia L. *Physicochemical model of alginate-poly-l-lysine microcapsules defined at the micrometric/nanometric scale using ATR-FTIR, XPS, and ToF-SIMS*. Biomaterials (Dec 2005) 26(34): 6950-6961.
Original articles (not first author)		
☒ *Not main contributor*	Contributed 4%	Langlois G, Dusseault J, Bilodeau S, **Tam SK**, Hallé JP. *Effects of the co-encapsulation of duct cells with islets on the viability and function of islets*. PLoS ONE [submitted for publication]
☒ *Not main contributor*	Contributed 15%	Bilodeau S, **Tam SK**, Langlois G, Hallé JP. *Improved ability of rat islet cell aggregates to resist hypoxia*. Transplant Proc [submitted for publication]
☒ *Not main contributor*	Contributed 2.5%	Ménard M, Dusseault J, Langlois G, Baille WE, **Tam SK**, Yahia L'H, Zhu XX, Hallé JP. *Role of protein contaminants in the immunogenicity of alginates*. J Biomed Mater Res B Appl Biomater (May 2010) 93(2): 333-40.
☒ *Not main contributor*	contributed 5%	Langlois G, Dusseault J, Bilodeau S, **Tam SK**, Magassouba D, Hallé JP. *Direct effect of alginate purification on the survival of islets immobilised in alginate-based microcapsules*. Acta Biomater (Nov 2009) 5(9): 3433-40.
☒ *Based on master's research*	contributed 30%	Dusseault J, **Tam SK**, Ménard M, Polizu S, Jourdan G, Yahia L'H, Hallé JP. *Evaluation of alginate purification methods: effect on polyphenol, endotoxin and protein contamination*. J Biomed Mater Res A (Feb 2006) 76(2): 243-51.
Review articles		
☒ *Not original works*	contributed 20%	Orive G, **Tam SK**, Pedraz JL, Hallé JP. *Biocompatibility of Biomaterials for Cell Encapsulation.* Biomaterials (Jul 2006) 27(20): 3691-3700.
☒ *Not original works*	contributed 5%	de Vos P, Andersson A, **Tam SK**, Faas MM, Hallé JP. *Advances and barriers in mammalian cell encapsulation for treatment of diabetes*. Curr Med Chem (Apr 2006) 6(2): 139-153.

☑ included in thesis ; ☒ not included in thesis

Table P.1 (continued). List of publications written during my doctoral studies (2005-2010)

Book Chapters		
☑ Appendix II	contributed 90%	**Tam SK**, Hallé JP, Yahia L'H. *Ch 11 La microencapsulation pour la thérapie cellulaire*. In: Microencapsulation : des sciences aux technologies (2007) Coord. Vandamme TF, Poncelet D and Subra-Paternault P. Lavoisier, Paris. pp 149-174.
☑ Appendix III	contributed 40%	Poncelet D, **Tam SK**. *Ch 3 Microencapsulation technologies for a bioartificial endocrine pancreas*. In: The Bioartificial Pancreas and Other Biohybrid Therapies (2009) Ed. Hallé JP, de Vos P and Rosenberg L. Research Signpost Publishing, India. pp 37-50.
☑ Appendix IV	contributed 90%	**Tam SK**, Hallé JP, Yahia L'H. *Ch 7 Physical and chemical analysis of the microcapsule surface*. In: The Bioartificial Pancreas and Other Biohybrid Therapies (2009) Ed. Hallé JP, de Vos P and Rosenberg L. Research Signpost Publishing, India. pp 97-136.

☑ included in thesis ; ☒ not included in thesis

RÉSUMÉ

La microencapsulation représente une stratégie visant à protéger les cellules ou les tissus thérapeutiques du rejet de greffe à l'aide d'une barrière physique. Cette approche est avantageuse puisqu'elle ne nécessite pas l'administration d'immunosuppresseurs à long terme et qu'elle permet l'option d'exploiter des sources de cellules non-cadavériques (ex. les cellules d'animaux). Les microcapsules que nous étudions sont conçues pour l'immunoprotection des îlots de Langerhans (qui sont responsables de sécréter l'insuline) dans le but de traiter le diabète insulino-dépendant.

La transplantation d'îlots microencapsulés n'est pas encore utilisée régulièrement en clinique parce que la survie et le fonctionnement des cellules greffées restent limités. Un facteur qui contribue à l'échec de la greffe est la biocompatibilité inadéquate des microcapsules elles-mêmes. Dans ce cas, les cellules immunitaires adhèrent à la surface du dispositif et sécrètent des substances cytotoxiques pouvant pénétrer la barrière protectrice et endommager les cellules à l'intérieur. Ensuite, du tissu fibrotique se développe autour de l'implant, ce qui peut obstruer ou limiter la diffusion des nutriments, de l'oxygène, du glucose et de l'insuline à travers la membrane et ultimement mener au dysfonctionnement et/ou la mort des cellules encapsulées.

Au moins deux groupes de recherche ont démontré la faisabilité, sous conditions optimales, de fabriquer des microcapsules d'alginate-polycation biocompatibles. Cependant, la plupart des laboratoires ont de la difficulté à reproduire de tels résultats. Ceci souligne notre manque de connaissances à propos des paramètres importants qui déterminent la biocompatibilité de la microcapsule. Cette situation est fortement reliée au fait qu'aucun standard n'existe pouvant nous guider dans la fabrication des microcapsules afin d'atteindre une biocompatibilité et une bioperformance optimales.

A l'aide des techniques d'analyses physicochimiques, cette recherche cherchait à comprendre quelles propriétés de la microcapsule sont importantes pour déterminer sa biocompatibilité. L'objectif de ce travail était d'élucider les corrélations entre la structure chimique, les propriétés physicochimiques, et la biocompatibilité *in vivo* des microcapsules à base d'alginate. Ces informations aideront la communauté scientifique à comprendre les facteurs

qui doivent être contrôlés afin d'optimiser la biocompatibilité. Notre approche est basée sur l'hypothèse que la réponse immunitaire est déterminée par, et donc peut être contrôlée par, des propriétés physicochimiques spécifiques de la microcapsule et de ses composantes matérielles.

Le travail expérimental a été divisé en cinq phases, chacune ayant un but spécifique: (1) Prouver que les immunoglobulines s'adsorbent sur la surface des microcapsules d'alginate-polycation, et corréler cette adsorption avec la chimie de la microcapsule. (2) Tester la reproductibilité interlaboratoire de la fabrication des microcapsules biocompatibles, et déterminer si nos matériaux et protocoles de fabrication sont appropriés pour des études subséquentes. (3) Déterminer quelles propriétés physicochimiques des alginates influencent la biocompatibilité *in vivo* de leur gel. (4) Déterminer quelles propriétés physicochimiques des microcapsules d'alginate-polycation sont les plus importantes pour leur biocompatibilité *in vivo*. (5) Déterminer si une membrane légèrement immunogénique augmente ou diminue la capacité d'une microcapsule d'immunoprotéger des xénogreffes d'îlots dans des souris diabétiques. Afin d'atteindre ces buts, une gamme d'analyses physicochimiques a été employée pour la caractérisation des alginates et des microcapsules. Les propriétés des alginates qui ont été étudiées incluent la pureté (essai LAL, essai microBCA), la composition chimique (la résonance magnétique nucléaire, NMR), la composition élémentaire (la spectroscopie de photoélectrons avec rayons X, XPS), et l'hydrophilicité (l'angle de contact). Quant aux microcapsules, la composition chimique de leur surface (XPS) et l'hydrophilicité, ainsi que les interactions entre l'alginate et le polycation (la spectroscopie infrarouge à transformée de Fourier, FTIR) et la résistance de la membrane (le gonflement osmotique) ont aussi été évaluées.

Les résultats de cette recherche ont mené à plusieurs conclusions importantes à propos de la biocompatibilité des alginates et des microcapsules à base d'alginate. D'abord, la purification de l'alginate ne garantit pas qu'elle sera biocompatible. Effectivement, nous avons démontré que la composition chimique de l'alginate (i.e. le contenu relatif de mannuronate et guluronate) et sa viscosité intrinsèque influencent le degré d'adhésion des cellules immunitaires à surface des billes d'alginate. En employant un alginate biocompatible, nous avons démontré que la membrane polycationique des microcapsules compromet la biocompatibilité de la microcapsule. Cette membrane était responsable de l'adsorption des protéines opsonisantes *in vitro* ainsi que l'adhésion des cellules immunitaires *in vivo*. Cela dit, la sévérité de la réponse

inflammatoire contre la membrane peut se varier, et celle-ci dépendait de la structure de la microcapsule, incluant le choix de l'alginate et du polycation. Les résultats de nos analyses physicochimiques ont suggéré que le facteur le plus important pour la biocompatibilité est la capacité du polycation de diffuser dans le gel d'alginate et de le lier. De plus, le fait d'ajouter une couche d'alginate à la microcapsule n'a pas contrecarré les effets de la membrane polycationique sur des différentes propriétés des microcapsules (la composition de sa surface, son hydrophobicité, sa stabilité), ni réduit son immunogénicité.

Même si nous avons fourni de nombreuses preuves que la membrane de la microcapsule est le problème principal du manque de biocompatibilité, nous avons aussi démontré que la gravité de ce problème peut varier selon les procédés de fabrication. Ce résultat est important à noter puisqu'il confirme la possibilité d'atteindre une biocompatibilité optimale *si* les interactions entre alginate et polycation sont idéales. Néanmoins, les résultats de notre étude sur la survie et le fonctionnement des îlots encapsulés, qui ont démontré un échec presque total des microcapsules contrairement aux billes d'alginate sans membrane, soulèvent la question suivante : Vaut-il la peine d'essayer d'optimiser la biocompatibilité de la membrane si celle-ci n'est peut-être pas nécessaire à l'immunoprotection des cellules thérapeutiques transplantées?

ABSTRACT

Microencapsulation represents a method for immunoprotecting transplanted therapeutic cells or tissues from graft rejection using a physical barrier. This approach is advantageous in that it eliminates the need to induce long-term immunosuppression and allows the option of transplanting non-cadaveric cell sources, such as animal cells and stem cell-derived tissues. The microcapsules that we have investigated are designed to immunoprotect islets of Langerhans (i.e. clusters of insulin-secreting cells), with the goal of treating insulin-dependent diabetes.

Microencapsulated islet transplantation has not yet reached regular clinical application because graft survival and function remains limited and variable. One of the main factors that contribute to graft failure is an inadequate biocompatibility of the microcapsule itself. Upon recognition of the microcapsule, host immune cells adhere to the device and secrete cytotoxic substances that are small enough to penetrate the protective barrier and potentially harm the cells within. As the inflammatory response persists, fibrotic tissue develops around the implant and can hinder the diffusion of cell nutrients, oxygen, glucose and insulin into and out of the microcapsule, thereby leading to encapsulated cell dysfunction and death.

At least two research groups have demonstrated the feasibility of producing alginate-polycation microcapsules that are biocompatible. However, most labs have had difficulty reproducing such results. This underlines our lack of understanding about the parameters that are important for determining the biocompatibility of the microcapsule. This situation is intimately related to the fact that no standards currently exist to guide the fabrication process of microcapsules in order to achieve optimal biocompatibility and bioperformance.

With the aid of techniques for physicochemical analysis, this research focused on understanding which properties of the microcapsule are the most important for determining its biocompatibility. The objective of this work was to elucidate correlations between the chemical make-up, physicochemical properties, and *in vivo* biocompatibility of alginate-based microcapsules. This information is expected to help the research community understand what factors must be controlled and standardized in order to achieve optimal biocompatibility. Our approach was based on the hypothesis that the immune response to the microcapsules is

governed by, and can therefore be controlled by, specific physicochemical properties of the microcapsule and its material components.

The experimental work was divided into five phases, each associated with a specific aim : (1) To prove that immunoglobulins adsorb to the surface of alginate-polycation microcapsules, and to correlate this adsorption with the microcapsule chemistry. (2) To test interlaboratory reproducibility in making biocompatible microcapsules, and evaluate the suitability of our materials and fabrication protocols for subsequent studies. (3) To determine which physicochemical properties of alginates affect the *in vivo* biocompatibility of their gels. (4) To determine which physiochemical properties of alginate-polycation microcapsules are most important for determining their *in vivo* biocompatibility (5) To determine whether a modestly immunogenic membrane hinders or helps the ability of the microcapsule to immunoprotect islet xenografts in diabetic mice. To achieve these aims, extensive physicochemical analyses of the alginates and microcapsules were carried out. Among the properties of the alginates that were investigated include their purity (LAL assay, microBCA), chemical composition (nuclear magnetic resonance, NMR), elemental composition (x-ray photoelectron spectroscopy, XPS), and hydrophilicity (contact angle technique). As for the microcapsules, we also examined their surface chemical composition (XPS), hydrophilicity, as well as alginate-polycation interactions (Fourier transform infrared spectroscopy, FTIR), and membrane strength (osmotic swelling).

The results of this research led to a number of important conclusions about the biocompatibility of alginates and alginate-based microcapsules. First of all, purifying an alginate does not guarantee its biocompatibility. Indeed, we provided evidence that both the alginate chemical composition (i.e. relative content of mannuronate and guluronate) and its intrinsic viscosity influence the extent of host cell adhesion to alginate gel beads. Using a biocompatible alginate, we then provided evidence that microcapsule biocompatibility is greatly compromised by its polycationic membrane. We showed that this membrane is responsible for the adsorption of opsonizing proteins *in vitro* and the adhesion of immune cells *in vivo*. That said, the severity of inflammatory response to the membrane can vary, and this depended on the microcapsule design, including the choice of alginate and polycation type. Results of our physicochemical analyses suggested that the most important factor determining biocompatibility is the ability of the polycation to diffuse into, and subsequently bind to, the alginate gel core. Moreover, adding

a final coating of alginate had no significant effect on reversing the effects of the membrane on various microcapsule properties (surface composition, hydrophobicity, stability), nor did this coating reduce its immunogenicity.

Although we repeatedly provided evidence that the microcapsule membrane is the main problem for biocompatibility, we also demonstrated that the severity of this problem can vary according to the fabrication details. This is an important note, as it confirms the possibility of achieving optimal microcapsule biocompatibility *if* the interactions between the alginate and polycation are ideal. Nevertheless, the results of our study on the survival and function of encapsulated islets, which demonstrated an almost complete failure of the microcapsules in contrast to alginate beads without a membrane, forces us to raise the following question : Is it worth trying to optimize the biocompatibility of the membrane when this membrane may not even be necessary for the immunoprotection of transplanted therapeutic cells?

CONDENSÉ EN FRANÇAIS

INTRODUCTION

La microencapsulation pour l'immunoprotection

La microencapsulation représente une stratégie pour protéger des cellules et des tissus thérapeutiques contre le rejet de greffe à l'aide d'une barrière physique. La majorité des microcapsules conçues pour l'immunoprotection des cellules sont à base d'alginate, avec une membrane polycationique semi-perméable. En principe, les composantes de haut poids moléculaire du système immunitaire (les cellules et les anticorps) sont bloquées par la membrane, tandis que les nutriments, l'oxygène, et le produit thérapeutique (que les cellules encapsulées sécrètent) peuvent diffuser librement à travers la membrane afin d'assurer le bon fonctionnement et la survie des cellules encapsulées.

Les principaux avantages de l'immunoprotection par la microencapsulation sont que le patient n'a pas besoin de prendre des immunosuppresseurs à long terme et que la microcapsule permet l'utilisation de source de cellules non-cadavériques, telles que les cellules d'animaux, les cellules souches ou les cellules modifiées génétiquement. Dans notre laboratoire, nous travaillons avec des microcapsules à base d'alginate conçues pour l'immunoprotection des îlots de Langerhans (des groupements de cellules qui sécrètent l'insuline) dans le but de traiter le diabète insulino-dépendant. Autour du monde, des premiers essais cliniques de transplantation d'îlots encapsulés sont en cours.

La problématique

La période de survie et de fonctionnement des îlots encapsulés *in vivo* reste toujours limitée et variable (de quelques jours à quelques années). Un des facteurs qui contribuent au rejet de la greffe est une biocompatibilité inadéquate des microcapsules. En effet, si une microcapsule n'est pas suffisamment biocompatible, des cellules immunitaires adhèrent à sa surface et sécrètent des agents cytotoxiques de faibles poids moléculaire qui peuvent diffuser dans la membrane de la microcapsule et endommager les cellules à l'intérieur. Si la réponse

inflammatoire persiste, un tissu fibrotique se développe autour de l'implant, ce qui peut obstruer la diffusion des nutriments, de l'oxygène, de glucose, et de l'insuline à travers la membrane et donc mener au dysfonctionnement et/ou la mort des cellules encapsulées.

Même si quelques groupes de recherche ont démontré la faisabilité, sous conditions optimales, de fabriquer une microcapsule d'alginate-polycation biocompatible, d'autres laboratoires ont eu de la difficulté à reproduire ces résultats. Ceci souligne notre manque de connaissances concernant les facteurs déterminant la biocompatibilité des microcapsules. Cette situation est fortement liée au fait qu'aucun standard n'existe pouvant nous guider dans la fabrication des microcapsules afin d'atteindre une biocompatibilité et une bioperformance optimales.

Notre objectif général

Le but de tous les chercheurs dans ce domaine est d'optimiser le système d'îlots encapsulés afin que la greffe survive et reste fonctionnelle tout au long de la vie du patient diabétique.

Le but de cette recherche est de contribuer à l'optimisation d'un aspect de ce système : la biocompatibilité des microcapsules. La question que nous visons à répondre est *pourquoi* une microcapsule est biocompatible tandis qu'une autre ne l'est pas, malgré leurs structures similaires. Notre objectif est donc d'élucider les corrélations entre la structure chimique, les propriétés physicochimiques, et la biocompatibilité *in vivo* des microcapsules.

Notre hypothèse

Ce travail est basé sur l'hypothèse que la réponse immunitaire contre les microcapsules, incluant l'adsorption des protéines et la réponse cellulaire, est gouvernée par, et donc peut être contrôlée par, les propriétés physicochimiques spécifiques de la microcapsule et ses composantes matérielles.

Nos buts spécifiques

La section expérimentale de cette thèse a été divisée en cinq phases qui suivent le processus de notre recherche. Chacune de ces phases a été dirigée par un but de recherche spécifique :

1) Prouver que les immunoglobulines s'adsorbent à la surface des microcapsules d'alginate-polycation, et corréler cette adsorption à la chimie des microcapsules.

2) Évaluer la reproductibilité interlaboratoire de la fabrication des microcapsules alginate-poly-L-lysine biocompatibles, et déterminer si nos matériaux et notre protocole pour fabrication sont appropriés pour des études subséquentes.

3) Déterminer quelles propriétés physicochimiques des alginates influencent la biocompatibilité *in vivo* de leur gel.

4) Déterminer quelles propriétés physicochimiques des microcapsules d'alginate-polycation sont les plus importantes pour leur biocompatibilité *in vivo*.

5) Déterminer si la membrane légèrement immunogénique des microcapsules d'alginate-poly-L-ornithine augmente ou diminue la capacité de la microcapsule à prolonger la survie et le fonctionnement des xénogreffes d'îlots dans des souris diabétiques.

RÉSUMÉS DES RÉSULTATS

Partie I

L'adsorption de l'immunoglobuline humaine sur les microcapsules implantables à base d'alginate-poly-L-lysine : L'effet de la composition de la microcapsule

PUBLIÉ : Tam SK, de Haan BJ, Faas MM, Hallé JP, Yahia L, de Vos P. Adsorption of human immunoglobulin to implantable alginate-poly-L-lysine microcapsules: Effect of microcapsule composition. J Biomed Mater Res A 2009 Jun; 89(3): 609-15.

Les microcapsules d'alginate-poly-L-lysine-alginate (APA) continuent d'être les dispositifs les plus communément étudiés pour l'immunoprotection des cellules thérapeutiques transplantées. La fabrication des microcapsules APA qui ont une biocompatibilité de haut niveau et reproductible demande une connaissance des mécanismes de la réponse immunitaire contre les implants. Ici, nous investiguons l'adsorption des immunoglobulines (IgG, IgM et IgA) sur la surface des microcapsules APA *in vitro* après que celles-ci aient été exposées au sérum et au liquide péritonéal humain. Les immunoglobulines (Ig) sont des protéines opsonisantes, donc ils ont une tendance à faciliter l'inflammation quand ils s'adsorbent aux surfaces étrangères. L'adsorption d'Ig a été évaluée avec la technique d'immunofluorescence directe. La quantité d'Ig qui a adhéré à la surface de la microcapsule n'a pas été influencée significativement par le contenu d'acide guluronique (G), ni par la pureté de l'alginate. Pourtant, les microcapsules à base d'alginate pure et ayant un contenu intermédiaire de G ont corrélé avec le niveau le plus faible d'adsorption. L'adsorption d'Ig était négligeable quand la poly-L-lysine était omise, ce qui suggère que les charges positives à la surface de la microcapsule sont responsables de la liaison d'Ig.

Partie II

Une comparaison interlaboratoire des alginates et des microcapsules APA (Groningen vs Montréal)

PAS SOUMIS POUR PUBLICATION

Le laboratoire de Groningen est connu pour sa capacité de fabriquer des microcapsules alginate-poly-L-lysine-alginate (APA) qui sont biocompatibles. Nous avons essayé de reproduire leurs résultats en suivant leur protocole de fabrication des microcapsules, mais en utilisant les matériaux et les équipements qui sont disponibles au laboratoire de Montréal. L'alginate que nous avons acheté et purifié nous-mêmes était légèrement contaminé, mais similaire en composition à l'alginate fourni par le labo de Groningen. Néanmoins, les deux alginates étaient biocompatibles *in vivo*. Les microcapsules APA produites avec les deux alginates avaient des immunogénicités comparables. En utilisant l'alginate de Montréal, nous avons réussit à fabriquer des billes de gel parfaitement biocompatibles, indépendamment du protocole de fabrication de billes employé. Cependant, notre version du protocole de Groningen pour la fabrication des microcapsules APA a mené à des microcapsules plus immunogéniques ($p < 0,01$) que celles qui étaient fabriquées en suivant le protocole de Montréal. Cette discordance a été attribuée aux modifications apportées au protocole original de Groningen, comme la méthode de génération des gouttelettes et le choix de modèle animal. Les résultats de cette étude démontrent la difficulté d'atteindre la reproductibilité interlaboratoire. Finalement, les protocoles de Montréal de purification de l'alginate et de la fabrication des billes et des microcapsules APA peuvent être considérés comme étant satisfaisants pour les études subséquentes d'implantation.

Partie III

Les facteurs qui influencent la biocompatibilité des gels d'alginate

SOUMIS POUR PUBLICATION APRÈS 2e RÉVISION (J Biomed Mater Res A): Tam SK, Dusseault J, Bilodeau S, Langlois G, Hallé JP, Yahia L. Factors influencing alginate gel biocompatibility.

L'alginate reste toujours le polymère le plus populaire pour l'encapsulation des cellules, pourtant sa biocompatibilité est inconsistante. Deux alginates commerciaux ont été comparés, l'un avec 71% guluronate (HiG), et l'autre avec 44% (IntG). Les deux alginates ont été purifiés, et leur pureté a été vérifiée. Après deux jours dans la cavité péritonéale des souris C57BL/6J, les billes d'alginate IntG (gélifiées avec du baryum (Ba) et du calcium (Ca)) étaient propres, tandis que les cellules de l'hôte ont adhéré aux billes d'alginate HiG. Les billes de gel IntG, cependant, ont démontré de la fragmentation *in vivo*, tandis que les billes de gel HiG sont restées fermes. Les propriétés physicochimiques des alginates de sodium et de leurs gels ont été caractérisées extensivement. La viscosité intrinsèque de l'alginate IntG était 2,5 fois plus grande que celle de l'alginate HiG, ce qui suggère une masse molaire plus élevée. La spectroscopie de photoélectrons avec rayons X a indiqué que les deux alginates étaient similaires en termes de composition élémentaire, incluant la présence de faibles niveaux de contre-ions dans tous les gels. La mouillabilité des deux alginates et des gels était aussi identique, selon des mesures de l'angle de contact de l'eau sur des films secs. Les billes de Ba de l'alginate HiG résistaient beaucoup plus au gonflement et à la dégradation quand elles étaient immergées dans l'eau que les autres types de billes. Ces résultats suggèrent que les facteurs principaux qui contribuent à la biocompatibilité des gels d'alginate purifié sont le contenu de mannuronate/guluronate et/ou la viscosité intrinsèque.

Partie IV

La biocompatibilité et les caractéristiques physico-chimiques des microcapsules d'alginate-polycation

SOUS PRESSE : Tam SK, Bilodeau S, Dusseault J, Langlois G, Hallé JP, Yahia L. Biocompatibility and physicochemical characteristics of alginate-polycation microcapsules. Acta Biomaterialia 2010.

Il y a une nécessité de mieux comprendre la biocompatibilité des microcapsules d'alginate-polycation basé sur leurs caractéristiques physicochimiques. Des microcapsules composées d'alginate avec 44% (IntG) ou 71% (HiG) guluronate, gélifiées avec le calcium (Ca) ou le baryum (Ba), et entourées avec le poly-L-lysine (PLL) ou la poly-L-ornithine (PLO), suivi par une couche d'alginate IntG ont été comparées. Pour les microcapsules avec un cœur de gel IntG(Ca), l'utilisation de PLO au lieu de PLL a mené à moins d'adhésion de cellules immunitaires après deux jours dans les souris C57BL/6J. En plus, les microcapsules de PLO étaient caractérisées par une plus forte hydrophilicité et une résistance supérieure au gonflement et au dommage sous le stress osmotique. Pour les microcapsules avec une membrane de PLL, la substitution de cœur de gel IntG(Ca) par IntG(Ba) ou HiG(Ca) a mené à des réponses immunitaires plus fortes ($p < 0,05$). Ceci a été expliqué par une faible pénétration du PLL dans le gel, tel que démontré par des analyses de FTIR et par une rupture plus importantes des membranes pendant le gonflement osmotique. Les analyses de XPS ont démontré que toutes les microcapsules avaient la même quantité de polycation à leur surface. De plus, les revêtements d'alginate n'avaient pas des effets significatifs sur la biocompatibilité et les propriétés physicochimiques des microcapsules. Donc, les interactions alginate-polycation lors de la création de la membrane sont plus importantes pour la biocompatibilité que la quantité de polycation à la surface et le revêtement d'alginate.

Partie V

Induire la normoglycémie dans les souris diabétiques avec les îlots microencapsulés : Avec ou sans la membrane

PAS SOUMIS POUR PUBLICATION

La membrane polycationique est supposée résulter en des microcapsules d'alginate-polycation ayant une perméabilité contrôlée et une plus grande stabilité, mais sa biocompatibilité n'est pas optimale. Cette étude cherche à déterminer si une membrane légèrement immunogénique sert à diminuer ou à augmenter la capacité des microcapsules d'immunoprotéger. Nous avons évalué la capacité de 300 îlots de rats, encapsulés, soit dans les microcapsules alginate-PLO ou dans les billes d'alginate de baryum, de normaliser les taux de glucose sanguin des souris diabétiques immunocompétentes. Une des quatre souris qui a été transplantée avec des îlots dans les microcapsules PLO est devenue normoglycémique (pour 14 jours), tandis que les trois autres sont restées diabétiques. Toutes les souris qui ont reçu des îlots immobilisés dans des billes de gel sont devenues normoglycémiques pour des périodes entre 16 et 50+ jours. Une fois que l'hyperglycémie était rétablie, les échantillons étaient explantés et examinés sous microscope afin de comprendre pourquoi les greffes ont échouées. Dans le cas des microcapsules PLO, l'échec de la greffe semblait d'être à cause de la fragmentation et la dégradation des échantillons *in vivo*. Dans le cas des billes de gel, la cause de l'échec de la greffe était moins claire. Plusieurs des billes explantées étaient endommagées et/ou adhérées avec des cellules de l'hôte. Environ 10% des îlots pouvaient être récupérés, mais certains d'entre eux étaient en mauvais état ou dégradés. La durée de survie des greffes n'a pas corrélé avec la proportion de îlots récupérés ni avec la sévérité d'adhésion de cellules de l'hôte. En conclusion, la membrane PLO n'était clairement pas bénéfique pour la fonction et la survie des îlots *in vivo*, mais il reste incertain qu'une membrane d'un autre type est nécessaire pour l'immunoprotection.

CONCLUSIONS GÉNÉRALES

La biocompatibilité des microcapsules d'alginate-polycation n'a pas pu être optimisée dans notre laboratoire. Elles étaient régulièrement immunogéniques en termes de l'adhésion des cellules immunitaires à leur surface *in vivo*. Ces réactions inflammatoires étaient clairement induites par la présence du polycation qui forme la membrane de la microcapsule. De plus, un revêtement d'alginate, qui est ajouté dans le but d'améliorer la biocompatibilité des microcapsules en masquant les charges du polycation, n'a eu aucun effet significatif sur les propriétés physicochimiques, ni sur l'immunogénicité des microcapsules. Pourtant, la sévérité de la réponse immunitaire a varié dépendamment des interactions entre le polycation et le gel d'alginate, ce qui est relié à la composition chimique de la microcapsule. Cette observation démontre qu'il y toujours une possibilité d'améliorer la biocompatibilité des microcapsules en choisissant une combinaison de matériaux (l'alginate, le cation pour gélification, le polycation) qui optimise la diffusion et la liaison du polycation dans le gel d'alginate. Quand nous avons évalué l'efficacité des microcapsules à protéger les îlots transplantés, ce n'était pas la réponse immunitaire aux microcapsules qui était le plus problématique pour la survie de la greffe, mais plutôt la tendance de ces microcapsules de se dégrader *in vivo*.

Par contre, il a été démontré que l'alginate était parfaitement biocompatible dans des conditions spécifiques. Dans notre cas, ceci demandait un contenu intermédiaire de guluronate, une masse molaire plus grande, et un niveau de pureté adéquat. La biocompatibilité de ces gels, cependant, était contrariée par leur fragilité, ce qui présente un problème pour l'immobilisation et la protection des cellules transplantées. Néanmoins, la survie et le fonctionnement des îlots transplantés étaient améliorés avec l'utilisation des billes de gel d'alginate sans membrane.

RECOMMANDATIONS

Pour améliorer la biocompatibilité des microcapsules d'alginate-polycation, il est recommandé d'optimiser la diffusion et la liaison du polycation avec le gel d'alginate. Afin de trouver la meilleure combinaison de paramètres pour optimiser les interactions alginate-polycation (choix de matériaux, dynamiques de gélification, etc.), il est d'abord suggéré d'évaluer une gamme de formules en utilisant le FTIR pour surveiller les interactions

intermoléculaires. Après avoir passé cette étape de base, il est recommandé de vérifier les autres propriétés physicochimiques qui ont été corrélées avec la biocompatibilité, telles que l'hydrophilicité (angle de contact), la charge de surface (potentiel de zêta), et la morphologie de la surface (la microscopie à force atomique). Il est fortement suggéré de cesser d'essayer d'optimiser la biocompatibilité en manipulant le revêtement d'alginate.

Même si la biocompatibilité de la microcapsule est optimisée, ceci ne veut pas dire qu'une réponse immunitaire peut nécessairement être complètement éliminée. La chirurgie nécessaire à l'implantation et des défauts physiques sur des microcapsules individuelles sont deux facteurs qui sont essentiellement inévitables et qui risque de déclencher l'inflammation. Il est donc recommandé d'incorporer des anti-inflammatoires dans les microcapsules qui agissent à courte terme et localement.

Finalement, une fois que la biocompatibilité de la microcapsule d'alginate-polycation sera établie, il est nécessaire de vérifier l'effet des modifications sur la stabilité et la perméabilité des microcapsules, puisqu'une microcapsule biocompatible n'est pas utile si elle n'est pas appropriée pour l'immunoprotection des cellules transplantées.

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AO	Albino Oxford
AFM	Atomic force microscopy, a technique that is primarily used to map the topography and morphology of a solid surface at the nanoscale by measuring the force on a tiny probe scanning the surface
APA	Alginate-poly-L-lysine-alginate, a term used to describe the classic design of microcapsule for cell and tissue immunoprotection
at%	Relative atomic percentage
ATR-FTIR	Attenuated total reflectance Fourier transform infrared spectroscopy, a mode of FTIR spectroscopy in which a crystal of high refractive index is pressed against the sample in order to limit the analytic depth to < 5 μm
Ba ; Ba^{2+}	Barium ; Barium ion
BCA	Bicinchoninic acid, a reagent that is used to quantify proteins in solution
Ca ; Ca^{2+}	Calcium ; Calcium ion
CLSM	Confocal laser scanning microscopy, a technique that is used to provide high-resolution images of fluorescent samples at selected depths or focal planes with the aid of a focused laser beam
EU	Endotoxin units
FTIR	Fourier transform infrared spectroscopy, a technique that is used for the identification and semi-quantification of molecular structures or chemical groups within a sample, based on the adsorption of infrared radiation at frequencies characteristic of the molecular vibrations
G	Guluronate or Guluronic acid, one of the two main units of alginate
HEMA-MMA	Poly(hydroxyethyl methacrylate-co-methyl methacrylate)
HiG ; High G	Having a high guluronate content (> 60% G)

High M	Having a high mannuronate content (> 60% M)
ieq	Islet equivalents (generally normalized to 150 μm diameter)
Ig	Immunoglobulin
IL	Interleukin
IntG ; Int G	Having an intermediate guluronate content (40-60% G)
KRH	Krebs-Ringer-HEPES buffer solution
LAL	Limulus amoebocyte lysate, an extract of horseshoe crab blood cells that coagulates when in contact with endotoxins
M	Mannuronate or Mannuronic acid, one of the two main units of alginate
M/G content	Relative proportion of mannuronate to guluronate in an alginate sample
mM	Millimolar
mOsm	Milliosmole
Mw	Molecular weight
η	Viscosity
NMR	Nuclear magnetic resonance, a technique that is used to identify and quantify molecular structures within a sample, based on the absorption of electromagnetic radiation, at characteristic frequencies, by atomic nuclei placed in a strong magnetic field
PEG	Polyethylene glycol
PF	Peritoneal fluid
PLL	Poly-L-lysine
PLO	Poly-L-ornithine
PMCG	poly(methylene-co-guanidine)
RPMI	Roswell Park Memorial Institute medium for cell culture

SEM	Scanning electron microscopy, a technique that provides a high-resolution image of an electrically conductive sample by rastering a high-energy beam of electrons across the sample surface
STZ	Streptozotocin, a substance that is toxic to insulin-secreting beta cells
TNF	Tumor necrosis factor
ToF-SIMS	Time-of-flight secondary ion mass spectrometry, a technique that is used to detect and identify elements and molecules within the outer 1-2 monolayers of a sample surface, based on the measured mass of ions that are fragmented from the sample surface using an ion beam
TX	Transplantation
UHV	Ultra-high viscosity
w/v	Weight per volume
XPS	X-ray photoelectron spectroscopy, a technique that is used to identify and quantify elements and chemical bonds within the outer 10 nm of the sample surface, based on the characteristic energies of electrons that are ejected from the sample by x-ray radiation

LIST OF APPENDICES

INTRODUCTION

A NEED TO OPTIMIZE THE BIOCOMPATIBILITY OF IMMUNOPROTECTIVE MICROCAPSULES

The purpose

The purpose of microencapsulation is to physically protect transplanted therapeutic cells and tissues from graft rejection with the aid of a semipermeable membrane. This approach to graft immunoprotection has the advantage of not requiring general immunosuppression of the patient. Moreover, the use of microcapsules allows the option of using non-cadaveric sources of cells, including genetically engineered cells and xenografts, for cell therapy. For these two reasons alone, the use of microcapsules has the potential of making cell therapy more widely accessible than ever.

In practice, immunoprotective microcapsules can potentially be used to treat range of disorders by allowing the transplantation of cells that secrete a specific protein or hormone that the patient is lacking. One of the most popular applications for these microcapsules is to protect and transplant islets of Langerhans (i.e. clusters of insulin secreting cells that are found in the pancreas) in order to “cure” type 1 diabetes. Since encapsulated islet transplantation is also the main research axe of my co-director, Dr Hallé, this thesis focuses principally on the characterization, performance, and optimization of microcapsules designed for islet transplantation.

Researchers have proven on numerous occasions that encapsulated islets can successfully induce normoglycemia (normal blood sugar levels) in a range of animal models, and a handful of phase I/II clinical trials have begun. Proof-of-concept has been demonstrated, and hopes remain high for this approach to treat diabetes.

The problem

The greatest challenge that we are facing is that encapsulated islet graft survival is both limited and variable. That is, *in vivo* viability and function of encapsulated islets can still range from days to years. Many different biological factors have been associated with the failure of encapsulated islets, but also material factors. In particular, an inadequate biocompatibility of the microcapsules contributes to graft failure.

When a microcapsule is not sufficiently biocompatible, there is a non-specific foreign body reaction to the device. Activated immune cells adhere to the microcapsule surface and secrete cytokines and nitric oxide that can penetrate the microcapsule membrane and harm the islet cells within. As inflammation progresses, fibrotic tissue surrounds the microcapsules and can hinder the diffusion of oxygen and cell nutrients towards the enclosed islets. The diffusion of insulin and glucose is also hindered, which can lead to slower response times of the encapsulated islets. Increasing the lifetime and efficiency of encapsulated islets therefore requires that we minimize the immunogenicity of the microcapsules. Adequate biocompatibility of the microcapsule is also necessary in order to focus, without interference, on other aspects of the encapsulated islet system that must be optimized.

Although a couple of researchers have demonstrated their ability to produce alginate-polycation microcapsules that are impressively biocompatible, other labs have had difficulty reproducing such results. This underlines our lack of understanding about which factors are the most important for determining the biocompatibility of the microcapsules. This situation is intimately related to the fact that there currently exist no standards that can guide us in the fabrication of the microcapsules in order to achieve optimal biocompatibility and performance of the devices.

Our goal

The ultimate goal of all the researchers in this field is to optimize the performance of encapsulated islets to the point that the transplant can survive and properly function for the lifetime of the diabetic patient.

The goal of this research is to contribute to the optimization of one aspect of the encapsulated islet system : the biocompatibility of the microcapsule. The main question that we seek to answer is *why* one microcapsule is biocompatible while another is not, despite their similar designs. Our objective is thus to elucidate the correlations between the chemical make-up, physicochemical properties, and *in vivo* biocompatibility of the microcapsules.

Our hypothesis

This research is based on the hypothesis that the host response to microcapsule, including protein adsorption and cellular response, is governed by, and can therefore be controlled by, the physicochemical properties of the device and its material components.

Specific research aims

This experimental section of this thesis has been divided into five chapters, each representing a phase in my doctoral studies, and each driven by specific research aims :

1) To prove that immunoglobulins adsorb to the surface of alginate-polycation microcapsules, and to correlate this adsorption with the microcapsule chemistry
2) To test interlaboratory reproducibility in making biocompatible microcapsules, and to evaluate the suitability of our materials and fabrication protocols for subsequent studies
3) To determine which physicochemical properties of alginates affect the *in vivo* biocompatibility of their gels
4) To determine which physiochemical properties of alginate-polycation microcapsules are most important for determining their *in vivo* biocompatibility

5) To determine whether a modestly immunogenic membrane hinders or helps the ability of the microcapsule to immunoprotect islet xenografts in diabetic mice

CHAPTER 1

BACKGROUND AND CRITICAL REVIEW OF THE LITERATURE

1.1 Microencapsulation for immunoprotection

1.1.1 The benefits of immunoprotection by encapsulation

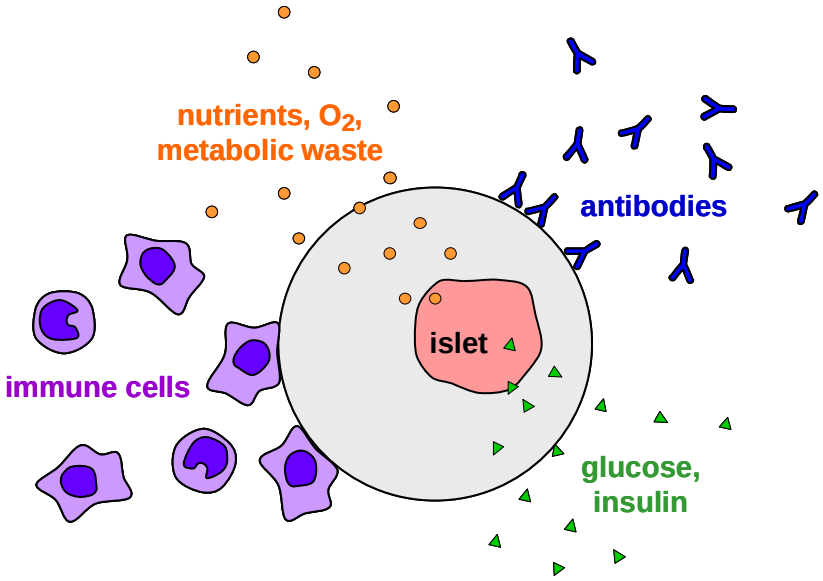

Figure 1.1 Schematic representation of immunoprotection by encapsulation using the example of an insulin-secreting islet enclosed within a microcapsule.

Immunoprotection refers to a strategy to protect cells and tissues from graft rejection by encapsulating them within a semi-permeable membrane. As a general rule, the encapsulated cells secrete some type of molecule (a hormone, enzyme, or another type of protein) and are transplanted into a patient that is unable to adequately produce this molecule. The semi-permeable membrane is intended to physically block out larger components of the host immune system (antibodies ~ 150 kDa and immune cells) while simultaneously allowing the free diffusion of smaller molecules (cell nutrients, oxygen, metabolic waste, and the therapeutic substance (e.g. insulin 5.8 kDa) into and out of the device. Ideally, the membrane is also capable of immuno*isolating* the therapeutic cells by preventing the host immune cells from both

detecting and reacting to the enclosed cells by blocking cell-to-cell contact [1, 2]. The concept of immunoprotection by encapsulation is illustrated in **Figure 1.1**, using the example of an islet (a cluster of insulin-secreting cells) enclosed within a microcapsule.

The main advantage of immunoprotection by encapsulation is that long-term general immunosuppression of the patient is not required in order to avoid graft rejection of the transplanted therapeutic cells. Since the development of the "Edmonton protocol" for isolating islets from a cadaveric donor pancreas, first published by Shapiro *et al* in 2000 [3, 4], immunosuppression has been used to successfully protect non-encapsulated islet transplants in hundreds of patients, with graft function sometimes extending as long as several years [5, 6]. However, because of the risks (vulnerability to infections, tumours and cancer) and undesirable side effects (mouth ulcers, diarrhea, acne) associated with immunosuppression [7], non-encapsulated islet transplantation is currently reserved for patients with severe hyperglycemia or who also require kidney transplants that demand immunosuppression.

Another benefit of using encapsulation devices is the possibility that the membrane can also potentially protect the host from the cells, a concept that was demonstrated in our lab [8]. This implies the option of transplanting non-cadaveric sources of cells in a safe manner, including animal cells (xenografts) [9, 10] and genetically engineered cells [11, 12]. This feature of encapsulation may help alleviate the current problem of insufficient sources of human islets available for widespread clinical transplantation [13].

Another potential advantage to encapsulated cell transplantation is the possibility of incorporating bioactive substances that enhance graft performance into the device. Such substances, for example, can improve oxygenation of the cells [14] or suppress acute inflammation upon transplantation of the grafts [15, 16]. Similarly, co-encapsulation with different cell types has also been explored as a strategy to improve the viability and function of the therapeutic cells [17, 18].

Finally, the encapsulation devices can also protect the transplanted cells from mechanical stress [19], thereby increasing their chance for proper function and survival.

1.1.2 The advantages of microcapsules

A range of devices for immunoprotection purposes have been developed, including intravascular devices, and extravascular macrocapsules (generally planar or tubular diffusion chambers), and extravascular microcapsules [20-22]. Intravascular devices have the advantage of placing the enclosed cells close to the bloodstream, but the surgery can be complicated and thrombosis (blood clotting) can block blood flow or necessitate the use of anti-coagulants [20, 22]. Extravascular macrocapsules, which are used to encapsulate a large number of cells or many islets at once, are easy to retrieve in the case of graft failure, but their geometry makes them generally unfavourable for oxygen and nutrient diffusion, as well as diffusion of the therapeutic agent [20, 22].

Extravascular microcapsules are generally < 1.5 mm in diameter. In the case of islet transplantation, each microcapsule is intended to enclose a single islet (human islets can measure 50 to 300 μm in diameter [23]). The advantages and disadvantages associated with microcapsules vs. macrocapsules have been compared in 'Tableau 1' of our book chapter (**APPENDIX II**). The greater surface-to-volume ratio is probably the greatest advantage of using microcapsules, since this geometry should theoretically allow an increased diffusion efficiency of the devices [22, 24]. In the case of islet transplantation, this can lead to a greater viability and quicker response time of the islet cells due to an increased diffusion of oxygen, nutrients, and glucose towards the enclosed islets, and of insulin out of the microcapsule [25]. Other important advantages of using microcapsules in place of macrodevices include the accessibility to a greater range of transplantation sites [26, 27] and the possibility of maintaining graft survival even if a small percentage of the microcapsules break.

Similar to microcapsules are also conformal coatings or nanoencapsulation, which involves directly coating a cellular aggregate (such as an islet) with a thin polymer layer (as thin as 10 μm), with idea of minimizing transplant volume and maximizing diffusion properties. This has been investigated by directly applying the first polycationic layer to the negatively charged islet surface [28, 29], by interfacial precipitation using poly(hydroxyethyl methacrylate-co-methyl methacrylate) HEMA-MMA [30], or a variety of techniques to graft polyethylene glycol (PEG) to the cell surface (i.e. PEGylation) [31-33], a polymer known to discourage protein and cell adhesion [34]. With the exception of PEGylation, however, conformal coating and

nanoencapsulation have not yet shown the same level of promise as microcapsules for cell immunoprotection.

1.1.3 Alginate-based microcapsules

The vast majority of microcapsules that have been developed for cell immunoprotection purposes have used a hydrogel as the main component [35]. Hydrogels have several favourable properties for encapsulation purposes (section 3.1 of our book chapter, **APPENDIX II**), the most important of these being their pliability, which minimally irritates surrounding biological tissues by friction, and their high water content, which minimizes the interfacial tension between the capsule surface and surrounding tissues/fluids, making protein and cell adhesion energetically unfavourable [36, 37]. A detailed description of hydrogels that have been used for cell encapsulation is presented in section 3.1 of our book chapter (**APPENDIX II**).

The most popular hydrogel in the cell encapsulation field remains alginate. Molecularly speaking, alginate is an unbranched copolymer with blocks of (1-4)-linked β-D-mannuronate (M) and α-G-guluronate (G) residues. These two residues are identical in atomic composition but take on different chair conformations. A schematic of a -GGMM- segment of alginate is illustrated in **Figure 5.1**. In addition to possessing the favourable qualities of other hydrogels, this natural polysaccharide has the added advantage of quickly gelling under conditions that are compatible with living cells [38]. To immobilize a cell within a microbead of alginate gel, one has only to suspend the cell in a viscous, aqueous solution of alginate and then immerse droplets of this suspension in a solution containing divalent cations (generally Ca^{2+} or Ba^{2+}).

Most research groups have opted to include some type of membrane in the design of their microcapsules in order to reduce the permeability and increase the stability of the devices. In most cases, these membranes are formed by polyelectrolyte complexation, which requires an electrostatic interaction between the negatively charged alginate gel and some type of polycation (i.e. a positively charged polymer). Membrane formation using this approach is simple to perform in the lab, as it generally requires successive incubations of the bead or capsule into polymer and buffer solutions. The general protocol for this technique, as well as alternative techniques for microcapsule fabrication, is described in detail in our book chapters in **APPENDIX II** and **APPENDIX III**.

Historically, Alginate-Poly(L-lysine)-Alginate (APA) microcapsules have been investigated more than any other microcapsule design. In 1980, Lim and Sun were the first to propose the APA microcapsule as a device for immunoprotecting islets in a paper that published in *Science* [39]. Since, APA microcapsules (or slight variations of) have continued to dominate the cell encapsulation field [40, 41], most likely because of the proven ability of these devices to function short-term *in vivo*, the apparent simplicity of the fabrication protocol, and the low cost of the materials required. Despite thirty years of research dedicated to developing the APA microcapsule, however, the design persistently shows limitations (including inconsistent biocompatibility [42]) that have made it difficult to advance towards regular clinical applications. For this reason, several researchers have decided to investigate alternative alginate-based microcapsule designs. These have recently been reviewed by others [41, 43] and are also listed in 'Tableau 2' of our book chapter (**APPENDIX II**). Overall, the best candidates for replacing the PLL as the polycation in for alginate-based microcapsules seem to be PLO [44, 45], PMCG [46, 47], and chitosan [48, 49].

Finally, a handful of research groups have opted to use alginate gel beads without adding a membrane [50-53]. Despite the risk of inadequate immunoprotection due to the high porosity of the alginate gel, the main motivation behind omitting the membrane is based on the challenge of overcoming the immunogenicity of the polycation (see **section 1.4.2**).

1.2 Recent progress of microencapsulated islet transplantation

The goal of encapsulated cell and tissue transplantation is to provide a specific molecule (hormone, enzyme, or other protein) to a patient that is unable to produce this molecule on their own. Encapsulated cell therapy has been investigated for the treatment of a variety of health problems, including hemophilia [54], cancer [55], renal failure [56], hyperparathyroidism [57], and dwarfism [58]. In our lab, we are investigating the encapsulation of the islets of Langerhans as a method to supply insulin to type 1 (insulin-dependent) diabetics. An overview of the history and recent progress of encapsulated islet transplantation is illustrated in **Figure 1.2** and are further discussed in this section.

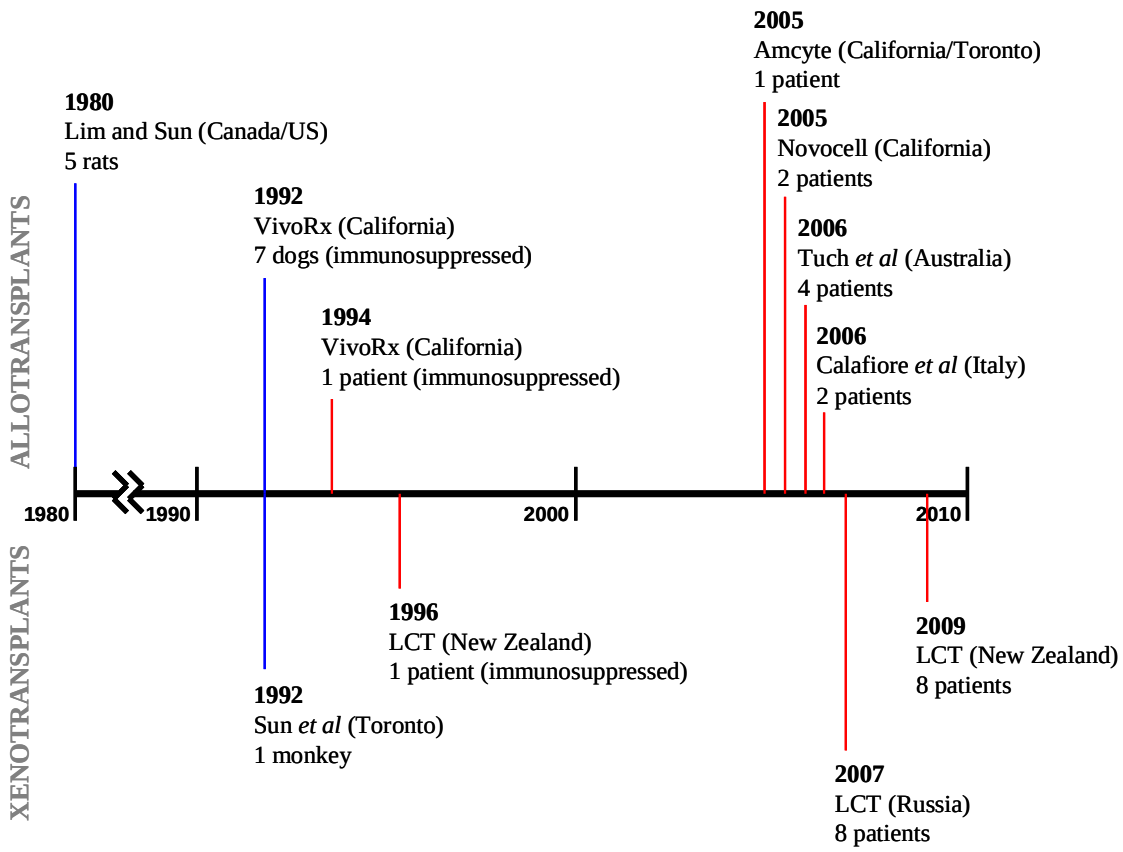

Figure 1.2 Timeline of key moments in allotransplantations (between same species) and xenotransplantations (between different species) of islet encapsulation. In this graph, xenotransplantation implies the transplantation of porcine islets. Animal model studies are represented by blue lines, while clinical trials are represented by red lines. LCT = Living Cell Technologies.

1.2.1 Key moments using animal models

1980: Lim and Sun (Canada/US) published the hallmark study for islet encapsulation, i.e. the first known publication to demonstrate the efficiency of encapsulated islets for treating diabetes *in vivo* [39]. These researchers implanted batches of 2000-3000 rat islets, encapsulated within alginate-PLL microcapsules, into five STZ-induced diabetic Wistar rats. In all five recipients, normoglycemia was induced and maintained for two to three weeks.

1992: This year marked the first experiments in large animal models. **Sun *et al* (Toronto)** demonstrated that porcine islets encapsulated within alginate-PLL could normalize the blood sugars of a spontaneously diabetic monkey for more than 150 days [59]. Four years later, they extended their study to nine diabetic monkeys. Without the use of immunosuppression, all nine

recipients became normoglycemic and then remained insulin independent for 120 to 804 days [60]. In the same year, **Soon-Shiong *et al* (VivoRx, California)** transplanted islet allografts into seven spontaneous diabetic dogs, also using alginate-PLL microcapsules, but in combination with a low dose of immunosuppressants [61, 62]. They reported successful reversal of diabetes in all seven dogs, which remained insulin-free for a period of 63 to 172 days.

1992 to present: Several studies use large animal models to demonstrate the efficiency of encapsulated islet transplantation. Here we name a few. Lanza *et al* reversed diabetes in dogs for 60 to >175 days in diabetic dogs using islet allografts encapsulated in uncoated alginate beads in combination with low doses of immunosuppressants [63]. Kendall *et al* announced that long-term normoglycemia (nine months) was induced in a diabetic baboon using porcine islets enclosed within alginate-PLO microcapsules [64]. Kin *et al* used a microcapsule composed of agarose and polystyrene sulfonic acid mixed gel to immunoprotect pig islets when transplanted in diabetic dogs, achieving moderate success [65]. Living Cell Technologies transplanted neonatal porcine islets immobilized in alginate–PLO microcapsules into eight diabetic monkeys, which resulted in lower insulin requirements and, in one monkey, insulin independence [66]. Novocell tested PEG-coated islets in combination with low doses of immunosuppressants in two sets of experiments involving diabetic baboons, with about half of the recipients achieving insulin independence several months post-transplantation [67]. Wang *et al* allotransplanted islets encapsulated within multi-layered alginate-based microcapsules into diabetic dogs without the use of immunosuppression, and all nine dogs were normoglycemic and insulin-free for 64 to 214 days [68]

1.2.2 Clinical trials of allotransplantation (human islets)

1994: Soon-Shiong *et al* (VivoRx, California) were granted approval for Phase I/II trials of encapsulated islets using patients that are candidates for whole organ pancreas transplantations [69]. The first patient received two intraperitoneal injections of 10 000 then 5 900 human ieq/kg enclosed in alginate-PLL microcapsules, and was insulin independent by nine months post-transplantation. According to a follow-up report two years later, the patient continued to show a reduction in exogeneous insulin [69]. However, it has been pointed out that this patient was already under immunosuppression due to a kidney graft, so the effectiveness of the

microcapsule remains questionable [70]. Results concerning other patients have been difficult to find, likely due to legal problems associated with the company in the late 90's [71, 72].

Feb 2005: As part of a phase I/II clinical trial **AmCyte (now ReNeuron Group, California)** transplanted islets encapsulated within alginate-PLL microcapsules into the abdominal cavity of a man at the **Toronto General Hospital** with no long-term immunosuppression. [73-75]. No follow-up reports have been found.

Nov 2005: Novocell (now ViaCyte, California) started Phase I/II clinical trials of the safety and efficacy of PEG-encapsulated primary islet allografts implanted subcutaneously in type I diabetic patients [76]. Preliminary evidence of safety and function in two patients was demonstrated. At 12 months post-transplantation, there were no significant side effects and evidence of islet function [77, 78]. However, in **May 2010**, ViaCyte announced that they have discontinued their work on PEG-encapsulated islets in order to focus on stem cell derived treatments for diabetes (Pro-Islet^{TM}) [79].

Feb 2006: Tuch *et al* (Prince of Wales Hospital, Australia) started pilot clinical trials for the transplantation of human islets encapsulated within in barium alginate beads in the absence of anti-rejection drugs [80, 81]. In a 2009 publication, they describe that over a period of 19 months, four patients received one to four intraperitoneal infusions of (on average) 178 200 encapsulated ieq [82]. Any effect of the transplant on blood glucose or insulin requirements didn't last more than a day. In one patient, they examined the transplants by laparoscopy 16 months after the first infusion and noted that the microcapsules were attached to internal organs, surrounded by fibrous tissue, and the islets were necrotic. A few months later, the same researchers published a study that implied a need to go back to small animal models to understand what went wrong [83].

2006: Calafiore *et al* (University of Perugia, Italy) received approval for a phase 1 pilot clinical trial of microencapsulated human islet allografts into ten non-immunosuppressed patients with type 1 diabetes [84]. A total of 400 000 or 600 000 ieq enclosed within alginate-PLO microcapsules were transplanted into two patients by injection in the abdomen. After several weeks, the patient show improved blood glucose levels and less need for insulin [85]. In a 2010 publication, Calafiore reported that two more patients have since been transplanted, and they also showed a reduction in insulin requirements [70].

1.2.3 Clinical trials of xenotransplantation (porcine islets)

Clinical trials of encapsulate xenotransplantation has been dominated by one New Zealand-based company called **Living Cell Technologies (LCT)**. This company's currently leading product, described under the brand name DIABECELL®, consists of neo-natal porcine islets encapsulated within alginate-polyornithine microcapsules [86]. LCT uses an ultra-pure alginate that was developed by Calafiore *et al* (University of Perugia) and holds an exclusive license and rights to the Perugia alginate [87].

1996: LCT (New Zealand) initiated the first Phase I/II clinical trials of encapsulated animal islets (neo-natal porcine islets) in Type 1 diabetic patients that have kidney transplants (thus are already immunosuppressed). A follow-up report in 2007 describes that a total of 1 305 000 ieq enclosed in alginate-PLL microcapsules, and then re-encapsulated within larger microcapsules, were transplanted intraperitoneally into one patient [88]. At 12 weeks post-transplantation, insulin requirements were reduced, but by 49 weeks, the patient was back to original insulin dosages. At 9.5 years post-transplantation, the microcapsules were embedded in the omentum, opaque and rigid, but some of the islets appeared to have survived but were not well-functioning [88].

2000: LCT explains that trials with encapsulated porcine islets have begun in Type 1 diabetic patients *without* immunosuppression. However, due to worldwide concerns on xenotransplantation, the New Zealand Ministry of Health placed a hold on further clinical trials until new viral tests, etc. can be developed [89].

2007: LCT circumvents approval issues by starting a Phase I/IIa clinical trial for DIABECELL® in **Moscow, Russia**. Up to ten patients are to receive implants of varying dose strength, and some patients will receive multiple doses [90]. By June 2009, eight patients have been transplanted, and two were still off insulin injections, while all patients showed better blood glucose control an reduced insulin dosages [91].

Jul 2009: LCT started a Phase I/II Clinical trial in **New Zealand** that investigates the safety and effectiveness of DIABECELL® in non-immunosuppressed patients with type I diabetes [92]. Three study groups are to receive dosages of 10, 15 and 20 thousand ieq/kg injected into the abdominal cavity via laparoscopy. The first implant was in Oct 2009. In **Apr 2010**, study

received a major grant from the JDRF to support the project [93]. On **4 Aug 2010**, the company announced that the Minister of Health has approved an addition to the New Zealand Phase II clinical trial of DIABECELL® to include four more patients. To date, eight patients have already received the transplants. So far, none show adverse side effect and all have experienced less frequent hypoglycemia [94].

Apart from LCT, other companies are trying to enter the marked of encapsulated porcine islet transplantation. For instance, **MicroIslet (California)** plans to pilot xenotransplantation human clinical studies employing the MicroIslet-P^{TM} in a yet to be determined foreign country. This company employs an alginate-based capsules for the transplantation of porcine islets into the abdominal cavity [95, 96].

1.2.4 Main obstacles to regular clinical applications

Despite the promise expressed in the recent progress of pilot clinical trials of encapsulated islet transplantation, we are still a number of years away from regular clinical applications. The main obstacles that are being faced are :

***Lack of human donor organs*:** As much as allotransplantations are easier to deal with from an immunological point of view than xenotransplantations, suitable human pancreases and islets are not easy to come by (not all donated organs are suitable for transplantation) [97, 98].

***Safety and regulatory approval*:** As a strategy to overcome the obstacle of limited human islets available, xenotransplantation has become an interesting alternative, particularly the use of porcine islets. However, the risks of transmitting animal viruses and/or inducing a strong immune response have made it difficult to get approval for xenotransplantations [99, 100]. No clinical trials involving xenotransplantation have been approved by Health Canada to date [101]. Researchers have tried to overcome approval issues by setting up clinical trials in foreign countries that impose less strict regulations. The situation is similar for the use of genetically engineered cells that secrete insulin.

***Limited graft survival*:** Of course, if the graft does not work efficiently, hopes for clinical applications will remain dim. There are a number of factors that contribute to death or dysfunction of encapsulated cells. These have been reviewed in a number of recent publications [22, 41, 102-107] and won't be explained in detail here. However, it is important to understand

that, from a biological point of view, the life of an encapsulated islet is very stressful. If one can imagine, the islet must first endure the stress of being isolated from its native pancreas [108]. After being isolated, the islet must then be able to survive the encapsulation procedure [109]. Once inside the microcapsule and transplanted, the islet must try to survive inside this artificial environment. This encapsulated islet cannot count on blood vessels to bring it necessary nutrients and oxygen since the microcapsule prevents revascularization of the tissues. Instead, the islet relies solely on diffusion for nutrient and oxygen transport [110, 111]. If this diffusion is not efficient, islet cells (particularly those in the middle of the cluster) risk dying from hypoxia [112, 113]. In addition to all these factors involving the islet, it is believed that an inadequate biocompatibility of the microcapsules contributes to graft failure. This topic is discussed in more detail in **section 1.3.4**.

***Uncertainty of long-term performance*:** Associated with the fact that we are still struggling to maintain long-term survival of the transplanted islets, there is an uncertainty about how the transplant will perform, or should perform, over the course of several years. This uncertainty also applies to the performance of the microcapsules themselves. In particular, are the capsules expected to eventually biodegrade, thus requiring regular injections of fresh encapsulated islets, or should we be aiming to develop a 'permanent' capsule that will remain stable for the lifetime of the patient ?

1.3 Microcapsule biocompatibility : Biological considerations

What do we mean when we say that a microcapsule is "biocompatible" ? In the case of encapsulated cell transplantation, a "biocompatible" system is interpreted as inducing minimal host cell adhesion, cellular overgrowth or fibrotic overgrowth. There is accumulated evidence that the host response to the microcapsules is a non-specific foreign body response (as opposed to an adaptive immune response). This response, which is similar for all biomaterial types, is briefly reviewed here.

1.3.1 Relevant aspects of the host response to implants

James Anderson elegantly described the host response to implants in his article "*Biological Responses to Biomaterials*", published in the Annual Review of Materials Research [114]. Extracts of this paper that are considered to be relevant to understanding the host response to microcapsules have been summarized here.

Anderson [114] has described the host response to implanted medical devices as a sequence of eight events. These are :

(1) ***Injury*****:** This refers to injury to vascularised connective tissue involved with the transplantation procedure. The inflammatory response is initiated by injury.

(2) ***Blood-material interactions*****:** Immediately following injury, fluid, proteins, and blood cells flow towards to the injured tissue. This is accompanied by changes in blood composition and a range of cellular events. Chemicals are released from plasma, cells, and injured tissue that mediate, and therefore characterize, the inflammatory response. Classes of chemical mediators include complement components, oxygen-derived free radicals, cytokines, and growth factors.

(3) ***Provisional matrix formation*****:** Development of a provisional matrix at the implant site occurs within minutes to hours following implantation. This matrix consists of cross-linked fibrin and various inflammatory products. Components within or released from this matrix initiate processes such as inflammatory cell and fibroblast recruitment. The fibrin network also provides a substrate for cell adhesion and migration.

(4) ***Acute inflammation*****:** Acute inflammation lasts from minutes to days, depending on the extent of injury. It is characterized by the exudation of fluid and plasma proteins and the emigration of leukocytes (predominantly short-lived neutrophils) towards the implantation site. The major role of neutrophils is to phagocytose microorganisms and foreign materials. Recognition of the implant by the neutrophils and other phagocytes is facilitated when the implant surface is coated by opsonins (see **section 1.3.2**). Although biomaterials are not generally phagocytosed because they are much larger than cells, certain events in phagocytosis may occur, including frustrated phagocytosis. This

process does not involve engulfment of the biomaterial but does cause the extracellular release of leukocyte products in an attempt to degrade the biomaterial.

(5) ***Chronic inflammation*****:** Persistent inflammatory stimuli lead to chronic inflammation. The chemical and physical properties of the biomaterial, as well as motion of the implant, may lead to chronic inflammation. This phase is characterized by the presence of macrophages and monocytes (which differentiate into macrophages), as well as plasma cells and lymphocytes. Macrophages, however, are the predominant cell type during chronic inflammation and these can survive several weeks. Macrophages may be activated upon adherence to the biomaterial surface. Like neutrophils, macrophages are phagocytes, and when activated, will attempt phagocytosis of the biomaterial. Macrophages are also principally responsible for normal wound healing in the foreign body reaction. By processing and presenting antigens to immunocompetent cells, macrophages also play a role in the development of immune reactions.

(6) ***Granulation tissue*****:** Granulation tissue is associated with healing inflammation. Depending on the extent of injury, granulation tissue may be seen as early as three to five days following implantation of a biomaterial. Characteristic histological features of granulation tissue include the formation of new small blood vessels from pre-existing ones (i.e. neovascularization) and the proliferation of fibroblasts, which are active in synthesizing collagen and proteoglycans. Collagen, especially type I collagen, forms the fibrous capsule.

(7) ***Foreign body reaction*****:** The foreign body reaction is composed of foreign body giant cells, macrophages, fibroblasts, and capillaries. Foreign body giant cells are formed by the fusion of monocytes/macrophages in an attempt to phagocytose larger foreign bodies. The specific composition of the foreign body reaction, however, is determined by the form and surface topography of the biomaterial. On relatively flat and smooth surfaces, this can consist of one or two layers of macrophages, while on rough surfaces, foreign body reaction may consist of foreign body giant cells as well as macrophages. High surface-to-volume implants also lead to higher ratios of macrophages and foreign body giant cells.

(8) ***Fibrosis/Fibrous capsule development*:** The end-stage healing response to biomaterials is generally fibrosis or fibrous encapsulation. Essentially, connective tissue (collagen) surrounds the implant and its interfacial foreign body reaction, thus isolating the implant from the local tissue environment.

The predominant cell type that is present during inflammation varies with the age of the injury. The size, shape, topography and chemical and physical properties of the biomaterial may be responsible for variations in the intensity and time duration of the inflammatory and wound healing processes. Thus intensity and/or time duration of inflammatory reaction may characterize the "biocompatibility" of a biomaterial. Implant biocompatibility with tissue has usually been described in terms of the acute and chronic inflammatory responses, and of the fibrous capsule formation that is observed at various post-implantation time points. Evaluation of implant biocompatibility commonly involves histological evaluation of tissue adjacent to implanted materials as a function of implant time.

1.3.2 Protein adsorption to biomaterials

As mentioned in the previous section, blood-material interactions occur immediately after injury. Amongst these interactions is the flow of proteins towards the site of injury. It is now generally accepted that protein adsorption is the host tissue's first interaction with the biomaterial [115]. Within one second upon implantation, proteins can already be observed on biomaterial surfaces, and within minutes a monolayer of protein adsorbs to most surfaces [116, 117]. Proteins are present in all biological fluids (including blood and peritoneal fluid), and are a primary source of information for biological recognition [37]. Simply put, the cellular response to implanted biomaterials is actually determined by the nature of the adsorbed protein layer rather than to the biomaterial itself [118].

In turn, the type of proteins that adsorb to a biomaterial, as well as the manner in which they adsorb (quantity, conformation) is thought to be influenced by the surface properties of the biomaterial. Thermodynamically speaking, the major interactions which drive the interfacial activity and adsorption of proteins are the electrostatic and hydrophobic interactions [37]. Accordingly, neutral, highly hydrophilic polymers are known to have minimal or weak interactions with most aqueous proteins [119]. In addition to surface charge and wettability,

topography and chemistry are also material properties that govern protein adsorption [120]. This observation, however, does not necessarily imply that all neutral, hydrophilic polymers will be biocompatible when implanted, or that protein-attracting surfaces will necessarily be immunogenic, as some proteins can be beneficial for biocompatibility when adsorbed to the surface [121].

Certain protein types, when adsorbed to a biomaterial surface, may be more potent than others in terms of their role in facilitating an unwanted inflammatory response. In particular, opsonizing proteins, or opsonins, adsorb readily to foreign surfaces so that phagocytic cells (macrophages, neutrophils) having cell receptors for these proteins, can recognize, adhere to, and attempt to phagocytose the foreign body. The two major opsonins are the complement factor C3b that participates in complement activation and the antibody IgG that plays a role in the immune response [114, 120, 122, 123]. Other proteins that can mediate cell adhesion include fibronectin and vitronectin [120].

1.3.3 Observed responses to microcapsules

In general, problems with microcapsule biocompatibility have been described in terms of 'cellular overgrowth', 'fibrotic overgrowth', or 'fibrosis' that is observed to surround the microcapsules after a certain transplantation periods *in vivo*. In many cases, similar inflammatory responses to both empty and cell-containing microcapsules imply that it is the microcapsule, rather than recognition of the enclosed islets, that induces the inflammatory response [124-126]. The most common immune and inflammatory responses that have been described against microcapsules designed for cell transplantation are described here :

***Protein adsorption*:** As early as 1992, Sawhney and Hubbell [127] observed protein adhesion and complement binding to different versions of APA microcapsules. De Vos *et al* [128, 129] measured the nitrogen levels on explanted APA microcapsules to demonstrate that rat proteins adsorb to their microcapsules when implanted into the peritoneal cavity. In our own lab, Dusseault *et al* [130] used an elution technique combined with mass spectrometry to identify a few dozen kinds of mouse serum/plasma proteins are capable of adsorbing to APA microcapsules *in vitro*. Xie *et al* recently demonstrated that bovine serum fibrinogen and immunoglobulin (IgG) bind to alginate-chitosan microcapsules *in vitro* [131, 132].

***Infiltration of immune cells to the implantation site*:** In our own lab, Robitaille *et al* [133] demonstrated that, within the first few hours after implanting APA microcapsules in the peritoneal cavity of rats, there is increase in free-floating neutrophils towards the implantation site. By 48 hours, macrophages, lymphocytes, and eosinophils take over.

***Cellular adhesion, overgrowth, and fibrosis*:** As implied in **section 1.3.1**, the specific composition of the cellular overgrowth or foreign body reaction surrounding microcapsules appears to vary with time. The majority of the studies mentioned here employed standard histological or immunohistochemical staining in order to determine the composition of the overgrowth. De Vos *et al* [128] noted that at 24 hours post-transplantation of APA microcapsules, the overgrowth appears to be mainly macrophages and some granulocytes, with small numbers of other cell types (mainly erythrocytes associated with trauma, but some basophiles). By five days post-transplantation, the overgrowth was still dominated by macrophages and the appearance of some fibroblasts and multinucleated giant cells [128]. By one week post-transplantation, macrophage population slightly decreased and fibroblasts became more important (about 30% of the cellular composition) [128]. King *et al* [134] observed that, in their case, cellular overgrowth on APA microcapsules became significant only at one week post-transplantation, and this reaction did not increase in severity by day 28. Fritschy *et al* [125] noted, at two weeks post-transplantation, the presence of macrophages, fibroblasts, fibrocytes, foreign body giant cells, granulocytes, and collagen deposition, but not B lymphocytes, T lymphocytes, dendritic cells, nor natural killer (NK) cells. The lack of B- and T-lymphocytes suggest a non-specific foreign body response to the microcapsules as opposed to an adaptive immune response [135]. Observations by Clayton *et al* [40] of monocytes, macrophages, fibroblasts, and collagen fibres, but not T cells nor B lymphocytes, at three weeks post-transplantation confirmed the results of Fritschy *et al* [125]. Also supporting these observations, Gotfredsen *et al* [126] saw, at various post-transplantation periods of two weeks and later, no evidence of lymphocytic invasion, but rather an overgrown layer of histiocytes and numerous layers of fibroblasts. By two months post-transplantation, de Vos *et al* [136] observed that macrophages had totally disappeared from the overgrowth, as they only found several layers of fibroblasts and connective tissue.

***Activation of immune cells*:** In 1993, Pueyo *et al* [137] demonstrated that APA microcapsules are capable of stimulating IL-1β release and intracellular IL-1β and IL-1α production by human macrophages *in vitro*. Similarly, Orive *et al* demonstrated that APA microcapsules can activate the proliferation of murine splenocytes, as well as stimulate the release of TNF-α from mouse peritoneal macrophages *in vitro* [138]. Moreover, King *et al* [134] demonstrated activation of macrophages by APA microcapsules *in vivo* by collecting peritoneal exudates of mice that received microcapsule implantations and then measuring the expression of IL-1β and TNF-α using real time PCR. We later confirmed the ability of implanted APA microcapsules to stimulate the expression of TNF-α, IL-1β and TGF-β_1 *in vivo* [133].

***Antibody response*:** After injecting microcapsules into Balb/c mice, serum samples from the recipients have been positive for immunoglobulins that have been developed against the material components of APA microcapsules [139, 140].

A schematic that visually summarizes the inflammatory response to alginate-polycation microcapsules, as based on the evidence just described, is presented in **Figure 1.3** below.

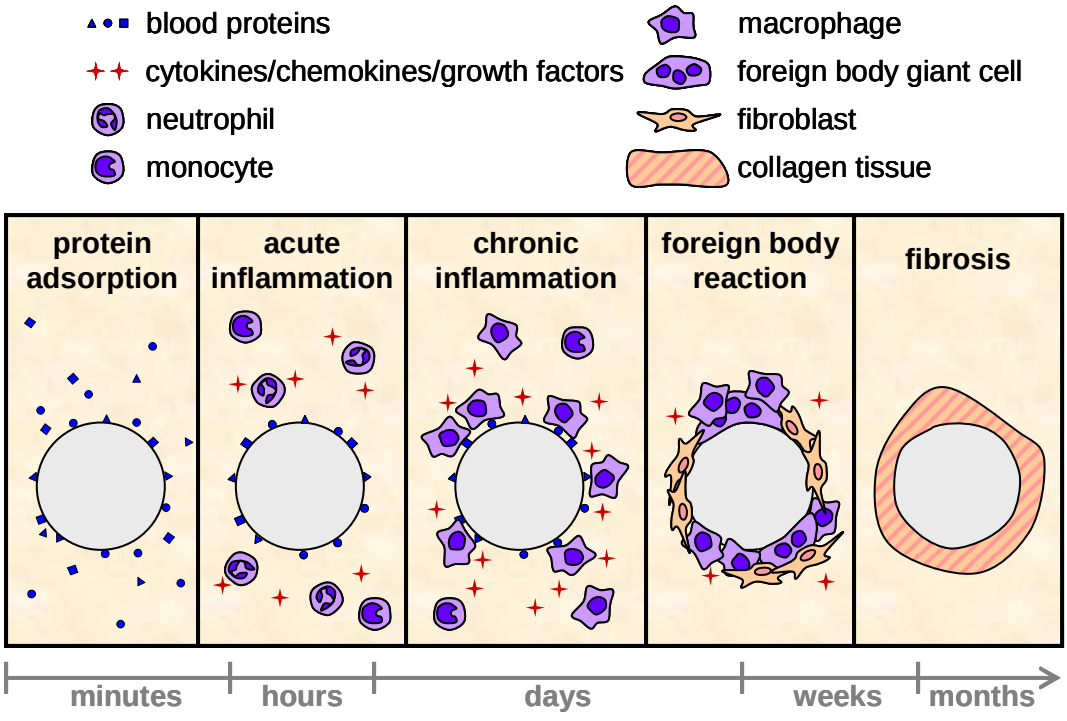

Figure 1.3 Schematic describing the probable time-course of the host response to alginate-polycation microcapsules upon implantation.

1.3.4 Contribution of non-biocompatibility to graft failure

There has been evidence that an inflammatory response to the microcapsules is hazardous for the function and survival of the enclosed islets, and thereby contributes to graft failure. Indeed, in the early 90's, graft failure was almost synonymous with "cellular overgrowth" or "fibrotic overgrowth" that develops around the alginate-polycation microcapsule within a few days of their implantation *in vivo* [124, 141, 142]. Graft failure has also been seen to be associated with lower recovery rates and greater proportion of capsules with pericapsular infiltrate [125]. The main concern for graft survival is that the fibrotic tissue can block or hinder the free diffusion of oxygen and nutrients across the capsule membrane, resulting in cell dysfunction or death, particularly hypoxia-induced islet necrosis [104, 143]. Similarly, hindered diffusion of insulin and glucose can slow the response time of the transplanted islets, making them less efficient. In addition to creating a physical barrier, cellular overgrowth may form a metabolic barrier to nutrient diffusion since cells within the overgrowth compete with the enclosed islets for glucose at the implantation site [134, 144]. More recently, there are additional concerns that activated macrophages and other immune cells are stimulated by the microcapsules to secrete a range of products that are small enough to penetrate the capsule membrane and damage the cells within, including the cytokines IL-1β (17.5 kDa), TNF-α (25-51 kDa) [145, 146], as well as nitric oxide [147-149].

Also, the non-biocompatibility of the microcapsule hinders progress of the cell encapsulation approach by interfering with the process of understanding other aspects of graft failure.

1.4 Microcapsule biocompatibility : Material considerations

The best approach towards optimizing the biocompatibility of microcapsules intended for cell encapsulation is to consider what we already know. Here we review studies that have investigated the biocompatibility of alginates and alginate-based microcapsules and critically discuss what the study conclusions suggest as far as recommendations for producing a biocompatible microcapsule.

1.4.1 Alginate considerations

There currently exists an international standard for the characterization and testing of alginates as starting materials intended for use in biomedical and tissue-engineering applications [150]. While this standard provides an excellent overview of recommended techniques for alginate characterization, it does not specifically concern alginates intended for cell encapsulation, nor does it explicitly state what combination of properties leads to the most biocompatible alginate. In this section, we present the current status of research in relation to selecting the best alginate for biocompatibility, although, as you will see, which alginate to choose ultimately depends on whether the alginate is soluble, gelled, or used as a microcapsule component.

Purity

Alginates that are used for cell encapsulation are derived from marine algae, and can therefore contain many kinds of natural contaminants, particularly proteins, endotoxins, and polyphenolic compounds [151, 152]. Logically, purifying the alginates to rid of such contaminants is the first step to improving their biocompatibility.

In 1992, the Würzburg group [153] demonstrated that commercial alginates contained fractions of impurities that showed mitogenic activity. Upon removing these fractions using free-flow electrophoresis, the purified alginates did not induce mitogenic activity *in vitro*. When barium alginate beads were implanted intraperitoneally in rodents for three weeks, purified samples showed no fibrosis, in contrast to non-purified samples. Shortly after, they developed a chemical extraction method for purification that resulted in alginates that showed no mitogenic activity *in vitro* and induced significantly less fibrosis *in vivo* than non-purified alginates [154, 155]. Also in the 90's, de Vos *et al* [136] demonstrated that, by purifying their alginates in the lab, they could dramatically reduce the extent of cellular overgrowth on APA microcapsules implanted in the peritoneal cavity of AO rats. More recently, Leinfelder *et al* [156] developed a highly sensitive cell assay based on the apoptosis of Jurkat cells to validate their alginate purification methods. As well, the Spanish group has demonstrated that the use of pure alginates vs. non-pure alginates leads to less serum antibody response in mice implanted with APA microcapsules [139], as well as less secretion of TNF-α from murine macrophages and reduced

proliferation of splenocytes *in vitro* [138]. Mallet and Korbutt [157] also demonstrated that using purified alginate increases graft survival and function of islets encapsulated in simple alginate microcapsules, as well as dramatically reduced capsular overgrowth.

We have seen similar benefits of purifying alginates in our own lab, including the production of gel beads that induce significantly less mRNA expression of TNF-α and IL-1β from a rat macrophage cell line *in vitro* [158]. More recently, we demonstrated that, while purifying commercial alginates significantly reduces the extent of cell adhesion, thickness of fibrotic layer, and amount collagen deposition surrounding alginate gel beads *in vivo*, the severity of foreign body response varied with the efficacy of purification protocol applied [159]. In particular, we provided evidence that proteins in the commercial alginate were particularly problematic for biocompatibility, as removal of these proteins by dialysing the alginates against saline correlated with less host cell adhesion to gel beads implanted in C57BL/6 mice [160].

Relative proportion of mannuronate to guluronate (M/G content)

Researchers agree that, due to the conformational differences between the M and G residues, the alginate's physical properties (including its gels) is strongly dependent on the composition and distribution of each of these residues along the polymeric chain. In contrast, the effect of the alginate's M/G content on its biocompatibility is a subject of continual debate. To complicate this affair, the effect of M/G content on biocompatibility seems to depend on the whether the alginate is in solution form, gelled, or crosslinking with a polycation.

In the early 1990's, the Norwegian group demonstrated that, when in solution form, high M alginates are more potent stimulators than high G alginates of monocytes to produce cytokines such as TNF-α, IL-6 and IL-1 [161-163]. To emphasize their point, they later implanted APA microcapsules into rats, and observed that the microcapsules based on high M alginates induced an antibody response while the high G microcapsules did not [140]. It has been suggested that the immunogenicity of the high M microcapsules is associated with the fact that the mannuronic-rich fragments, which do not take part in the gelling process, can leach out the capsules and trigger an immune response [164]. In the same era, Clayton *et al* [40] observed the opposite result. That is, when implanted in rats, APA microcapsules coated with high M alginate provoked a weaker immune response than those coated high G alginate (both

microcapsule types had a high M alginate gel core). However, in this study, they did not mention purifying their alginates, nor did they test the biocompatibility of their alginates in the absence of PLL, so one can not definitely conclude that their high M alginate was actually more biocompatible.

In response to the claimed immunogenicity of high M alginates by the Norwegian group, the German group published a study to demonstrate that all alginates can be rendered non-mitogenic and biocompatible if they are properly purified, including those with a high M content [154, 155]. A study from the Groningen group suggested the same point of view, having demonstrated that (in the absence of a polycation), purified alginate with a high G content is just as biocompatible as an alginate with intermediate G content, as evaluated by the extent of cellular overgrowth on gel beads *in vivo* [165].

The Boston group showed that (uncoated) barium alginate beads of high G alginate induced slightly more fibrotic overgrowth when implanted in mice than high M gel beads (which were biocompatible) [52]. In a more recent study of theirs, however, they compared the *in vivo* biocompatibility of two high M alginate and one high G alginate in Lewis rats, and while all beads showed minimal overgrowth, only one of the two high M alginates performed better than the high G alginate, indicating that some factor other than M/G content influenced the biocompatibility of their alginate gels [53].

When a polycationic membrane is added to form alginate-polycation microcapsules, the story becomes even more complex. Some have claimed that using high M alginate results in APA microcapsules of greater biocompatibility [140, 165], others have claimed that APA microcapsules based on high G alginate are more biocompatible [40], and others have observed no significant difference between the two in terms of inducing fibrotic overgrowth [52]. This situation implies that the alginate is not alone in influencing the biocompatibility of the complete alginate-polycation microcapsule (see **section 1.4.2** for more details on this topic).

Molecular weight (Mw) and solution viscosity

These two properties of alginate are intimately related, since the viscosity of an alginate solution depends on (among other factors such as concentration), the average molecular weight of the sample [166].

In 1993, Otterlei *et al* [167] reported that TNF-α production of human monocytes *in vitro* by alginate solutions depended strongly on the molecular weights of poly M and high M alginate, with maximal TNF-α production occurring at Mw's above 50,000 and 200,000, respectively. The implications of these results for the biocompatibility of microcapsules, however, is unclear since the alginate is normally crosslinked or complexed when it is incorporated into the microcapsule.

More recently, Schneider *et al* [168] reported that gel beads and multilayer microcapsules made from alginates with a lower Mw average alginate elicited a much stronger fibrotic response compared to samples based on higher Mw average alginate when implanted in rats. However, this study has (with reason) been criticized by Wandrey for its scientific value [169], including the authors' incorrect calculations of molar mass and their neglect to mention that the alginates were purified.

That said, the German group has been strongly promoting the use of ultra-high viscosity (UHV) alginates [14, 170, 171], stating that both viscosity and biocompatibility increase with molecular mass.

Other considerations

Although not an intrinsic property of the alginate per se, how the alginate is crosslinked to form a gel may have an influence on the gel biocompatibility. For instance, Duvivier-Kali *et al* [52] observed that, for gel beads of high M alginate, barium cross-linked alginate induced slightly less fibrosis *in vivo* than calcium cross-linked alginates.

1.4.2 Membrane considerations

Many of the published works investigating the relationship between the membrane and the biocompatibility of alginate-based microcapsules have been based on simple comparisons of different microcapsule formulations. Here, we review and discuss a number of such studies.

Let's, for the moment, ignore the benefits of adding a membrane for optimizing the permeability and stability of the microcapsule, and concentrate on what influence this membrane has on biocompatibility. Unfortunately, more than one study has provided evidence that the microcapsule membrane is immunogenic. This is demonstrated by observations that the

in vivo and *in vitro* biocompatibility of alginate gel beads is significantly worsened once it is coated with a polycation to form a membrane [52, 134, 158, 172, 173]. In contrast, not a single study that we know of has suggested that adding a membrane can actually enhance the biocompatibility of the microcapsule.

Despite the results of the above-mentioned studies, a few groups have managed to develop microcapsules (with membranes) that are not significantly immunogenic. These include the Groningen group, who uses APA microcapsules [136, 174], and the Perugia group, who prefers alginate-PLO microcapsules [44, 175]. The success of these groups demonstrates the feasibility of developing biocompatible alginate-polycation microcapsules with the appropriate combination of materials and techniques.

If one should decide to add a membrane to form the microcapsule, it is advised to take care that there are no unbound, positively charged functional groups of the polycation exposed. This precaution is supported by a study by Strand *et al* [176], which demonstrated that unbound PLL is capable of inducing massive cell death (necrosis) and stimulating monocytes to produce TNF *in vitro*, and moreover, that this effect can be reduced by combining the polycation with alginate. In accordance with this view, the most common strategy to improving the biocompatibility of the microcapsule membrane is to add a final coating of dilute polyanion (usually alginate), with the intention of binding any uncomplexed portions of the polycation which, as we just stated, is considered to be immunogenic [104]. As far as we know, only one group has clearly observed that adding this alginate coating had an effect of reducing (but not eliminating) the immunogenicity of the PLL membrane of alginate-based microcapsules [172]. This study, and at least one other, suggested that the extent of the alginate coating's effect depends on the chemical composition of the alginate [172, 177]. Strangely enough, there is a notable lack of published studies that have demonstrated the efficiency of the alginate coating for enhancing biocompatibility by directly comparing the performance of alginate-polycation microcapsules before and after the final alginate coating.

Of course, the choice of polycation is also expected to influence the overall biocompatibility of the microcapsule membrane. A few groups have directly compared the biocompatibility of alginate-based microcapsules made with different polycations, including Ponce *et al* [129], who concluded that PLL is a better choice than each PDL and PLO, as well as

Calafiore *et al* [44], who observed that PLO microcapsules resulted in the attraction of fewer host cells than PLL microcapsules. These results, as you may note, are contradictory, which suggests that the type of polycation is not the most important determining factor for the biocompatibility of the membrane.

Indeed, it has been recently stressed that the properties of the alginate that make up the core of the microcapsules (i.e. the gel matrix onto which the polycation must bind) may have an important effect on the biocompatibility of the complete microcapsule [104]. De Vos *et al* [165] clearly demonstrated that, in their laboratory, using alginates with an intermediate G content results in biocompatible APA microcapsules whereas using alginate with a high G content does not. These results support previous observations by Clayton *et al* [40], yet contradict the results of Kulseng *et al* [140], who concluded that APA microcapsules based on high G alginate are the less immunogenic. To add to the debate, Duvivier-Kali *et al* [52] observed no clear correlations between the type of alginate employed (high G vs. high M) and the extent of fibrosis induced by APA microcapsules.

1.5 Physicochemical analyses to understand microcapsule biocompatibility

As discussed in the previous section, when investigating the biocompatibility of alginate-based microcapsules, relying on simple comparisons of different designs leads to contradictory results. This is undoubtedly related to the complexity of the microcapsule structure and properties, despite the apparent simplicity of the fabrication protocol. In order to better understand the microcapsules in relation to their performance and biocompatibility, a number of researchers have investigated the physicochemical properties of the devices using a range of techniques. Not only can this approach help us to understand why one microcapsule formulation may be more biocompatible than another, but the knowledge acquired will hopefully help us to "fine-tune" the more successful microcapsule designs as a step towards the standardization and regular clinical application of the devices. The physicochemical properties of alginate-based microcapsules that have already been explored with the intention of explaining biocompatibility are summarized in **Table 1.1**. These are then explained in more detail in the subsequent sections.

Table 1.1 Published studies that explore the physicochemical properties of alginate-based microcapsules with the intention of understanding biocompatibility

Microcapsule property	**Technique for physicochemical analysis**	**Correlation with microcapsule biocompatibility?**
Size	Light microscopy [53, 178-180]	Evidence that smaller is more biocompatible [53, 178-180]
Surface morphology & topology	SEM [183] AFM [14, 184-187] TEM [185] Scanning acoustic microscopy [188] Optical interferometer [132]	Evidence that smoother is more biocompatible [183, 185, 132]
Intermolecular interactions & polycation binding	FTIR [189, 194, 195] Labelling techniques [190, 191] CLSM [192] Magnetic resonance microscopy [193]	Single study suggests that greater uptake of PLL is less biocompatible [194]; Lack of studies directly testing correlation with biocompatibility
Surface chemical composition	XPS [131, 174, 195, 196] ToF-SIMS [195]	Single group suggests that more PLL at surface is less biocompatible [174, 196]; Lack of studies directly testing correlation with biocompatibility
Surface charge	Zeta potential [131, 132, 198] Surface functional groups [199]	Evidence that more positive charge is less biocompatible [131, 132, 198, 199]
Elasticity or pliability	Compression tests [47, 200-205] Microcapillary vacuum [206] AFM [187]	Lack of studies directly testing correlation with biocompatibility
Membrane degradation & leaching	Labelling techniques [190, 191] ATR-FTIR with SEM [207-209]	Lack of studies directly testing correlation with biocompatibility
Surface hydrophilicity	Contact angle [152, 131]	Single study suggests no correlation [131]

1.5.1 Size

In our lab, we have shown that smaller APA microcapsules (326 ± 16 μm diameter) show a better *in vivo* biocompatibility than larger microcapsules (1247 ± 120 μm) of the same formulation [178]. To explain these results, it was proposed that the smaller microcapsules are less prone to breakage and defects, and are smoother than their larger counterparts, as well as induce less stress on surrounding tissues. Previous to our own study, Lum *et al* [179] had also suggested that smaller APA microcapsules (250-350 μm diameter) are more biocompatible than

standard-sized microcapsules. More recently, Sakai *et al* [180] also saw a correlation between smaller size and a reduced immune response towards agarose gel microcapsules ranging in diameter (90-925 μm). As well, Omer *et al* [53] recently noticed that, when enclosing islets, smaller barium alginate beads (500-700 μm diameter) were more stable and substantially more biocompatible *in vivo* than larger beads (800-1100 μm). Such similar trends despite the use of different microcapsule chemistries suggest that size may indeed have a large impact on microcapsule biocompatibility.

1.5.2 Surface morphology and topography (smoothness)

As previously mentioned, the topography of the implant can influence the composition of the foreign body reaction (**section 1.3.1**), and as a general rule, smooth surfaces have been associated with greater tissue biocompatibility [181]. Indeed, in 1991, Lanza *et al* [182] demonstrated that, for tubular diffusion chambers designed for islet encapsulation, the use of a smooth skin instead of a rough one resulted in significantly less fibrotic response when transplanted in rats. Since this study, there has been great interest in characterizing the surface morphology and topography of microcapsules designed for cell encapsulation in relation to their biocompatibility. Most of these studies have been carried out using atomic force microscopy (AFM), which allows one to examine samples while they are immersed in a liquid (see **APPENDIX IV** for more details on AFM), although other techniques have also been applied.

In 1998, Chen *et al* [183] were among the first to have successfully correlated microcapsule surface smoothness with an improved *in vivo* biocompatibility. In their case, they evaluated the surface smoothness of APA microcapsules coated with PEG derivatives using scanning electron microscopy (SEM).

In the same year, Xu *et al* [184] were, to our knowledge, the first to demonstrate the use of AFM to characterize the surface topography and quantify roughness of alginate-based microcapsules. While they demonstrated an effect of the cationic solution for gelation on these surface properties, no direct correlation with biocompatibility was investigated. A few years later, Zimmermann *et al* [14] used AFM to demonstrate that the smooth surface of their UHV alginate gels (films) could successfully prevent cell adhesion and migration *in vitro*. They also

exploited this technique to monitor changes in morphology on the gel bead surfaces due to mechanical shearing forces and/or deposition of fibrils after their implantation *in vivo*.

By 2003, Bünger *et al* [185] used AFM, in combination with transmission electron microscopy (TEM), to clearly demonstrate that switching the type of polymer coating on alginate-PLL microcapsules (alginate vs. polyacrylic acid vs. heparin) had an impact on each the surface roughness and the biocompatibility of the samples. That is, the microcapsules coated with polyacrylic acid were both the smoothest and the most biocompatible *in vivo*. During the same period, both Zimmermann *et al* [186] used AFM to validate the surface smoothness of their newly developed alginate gel with enhanced gel stability, while Lekka *et al* [187] used AFM to demonstrate the influence of chemical composition on surface morphology (among other properties). Although both of the latter studies saw the value in verifying surface morphology, they did not attempt to directly correlate this property with the biocompatibility of their microcapsules.

In recent years, other interesting methods have been proposed to examine the surface morphology and topography of hydrated alginate-based films and microcapsules. One of these was investigated by Klemenz *et al* [188], who used high frequency scanning acoustic microscopy to produce 3D images and topography profiles of the microcapsule surface with a lateral resolution of 1.5 μm. The implications of this technique for elaborating correlations with biocompatibility have yet to be demonstrated. As another example, Xie *et al* [132] used SEM in combination with a non-contact, three-dimensional optical interferometer to quantify surface roughness of ACA membranes. In their case, they correlated increased roughness (which was affected by each the alginate Mw and the chitosan concentration) with an increased adsorption of bovine plasma fibrinogen *in vitro* as well enhanced changes in the adsorbed protein conformation.

1.5.3 Intermolecular interactions and polycation binding

As previously mentioned (**section 1.4.2**), adding a polycationic membrane presents a risk in terms of microcapsule biocompatibility, since there is evidence that the unbound polycation (PLL) is immunogenic [176]. It naturally follows that researchers were curious to understand how the polycation binds to the alginate, and vice versa, since this may provide answers to

certain issues in membrane biocompatibility, but also explain other microcapsule features such as membrane stability and permeability.

Having already understood the implications that such information could have for the cell encapsulation field, Dupuy *et al* [189], in 1994, used Fourier transform infrared spectroscopy (FTIR) to demonstrate the force of interactions between alginate and PLL within films. Their results suggested that high M alginate is more efficient for binding PLL than high G alginate. Indeed, this can be explained by the fact that the MG blocks in alginate are more flexible and are not involved in gelation by crosslinking with divalent cations as are the GG blocks [38]. Shortly later, Thu *et al* published a sophisticated study [190, 191] in which they used a range of labelling techniques to investigate the interactions between alginate and PLL during microcapsule formation. Not only did they confirm that PLL binds more rapidly to alginate gels of high M, but they also demonstrated that increasing the alginate concentration at the surface of the gel beads (to produce 'inhomogeneous gel beads') can enhance PLL binding, and that PLL binding depends as well on the choice of crosslinking ion in the alginate gel. In the same study, they also investigated the binding of the alginate coating to the alginate-PLL microcapsule, and demonstrated that more of this alginate binds to high G microcapsules, and that binding was enhanced by reducing the molecular weight of the alginate, although the M/G content of the coating had no effect on its binding efficiency. A few years later, Strand *et al* [192] used confocal laser scanning microscopy (CLSM) to visually and quantitatively study the distribution of polymers and cross-linking ions in APA microcapsules, and demonstrated that PLL is capable of diffusing into the microcapsule during storage in an ionic solution, as well as confirmed that the M/G content of the alginate coating had no effect on the its binding properties to the microcapsule. Most recently, Constantinidis *et al* [193] have demonstrated the use of ^{1}H magnetic resonance microscopy to visualize and quantify the PLL, as well as monitor temporal changes in the alginate gel *in vitro*, without requiring labelling of the polymers.

While the studies described above provided important information about alginate-PLL interactions that can be useful for interpreting and explaining microcapsule biocompatibility, Van Hoogmoed *et al* [194] were the first to directly correlate such evidence with the *in vivo* biocompatibility of APA microcapsules. They used attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) to compare the properties of APA microcapsules

based on high G alginate (which are immunogenic) with microcapsules based on Int G alginate (which are biocompatible). The spectral peak areas allowed them to estimate that the membranes of the high G microcapsules contained about 20% more PLL than the membranes of the Int G microcapsules. They also suggested that variable degrees of binding existed between the alginate and PLL, as interpreted by the conformation of the PLL within the microcapsule membrane. The results of my Master's research confirmed, using ATR-FTIR, that alginate-PLL binding is not always optimal (i.e. associated with α-helical conformations of the polycation) within our own APA microcapsules [195].

1.5.4 Surface chemical composition

Intuitively, we understand that the surface chemical composition of a biomaterial influences its biocompatibility. In the case of alginate-based microcapsules, however, the chemical composition of their surface was not directly measured until issues with the immunogenicity of the membrane started to emerge.

In 2002, de Vos *et al* [174, 196] were, as far as we know, the first to directly analyse the chemical composition of the APA microcapsule surface, and they did this using x-ray photoelectron spectroscopy (XPS). Their results offered an explanation as to why their microcapsules based on High G alginate were more immunogenic than those based on Int G alginate. Specifically, they measured less negatively charged binding sites available for binding PLL on the surface of the High G alginate gel cores, and yet, the also detected higher quantities of PLL at the High G microcapsule surface. These results implied an increased risk of greater quantities of unbound PLL at the surface of the more immunogenic microcapsules. Simultaneously, they realised that since the PLL was present at the microcapsule surfaces, the outer alginate coatings did not exist as a distinct layer. A few years later, as part as my Master's work [195], we confirmed the exposure of PLL at the surface of our own APA microcapsules using XPS in combination with time-of-flight secondary ion mass spectrometry (ToF-SIMS), which is even more surface sensitive (1-2 monolayers) than XPS. At the same time, using ToF-SIMS imaging, we proved that this exposure was not due to defects in the microcapsule membrane, which was homogeneous in chemical composition. Using confocal laser scanning microscopy (CLSM), Strand *et al* [192] have previously demonstrated that, indeed, the alginate coating has rather a tendency to diffuse into the microcapsule membrane rather than bind to the

surface as a distinct layer. Most recently, the use of XPS to quantify the amount of polycation at the surface of alginate-based microcapsules has been expanded to the investigation of alginate-chitosan microcapsules [131], although in their case the quantity of chitosan at the surface did not correlate with the extent of fibrinogen and IgG adsorption *in vitro*.

1.5.5 Surface charge

Most proteins bear a net negative charge, and thus anionic surfaces generally tend to adsorb less protein than cationic ones [37]. Similarly, macrophages and other cells also have a negative surface charge [197] and can therefore be expected to be attracted by positively charged surfaces. As previously discussed in **section 1.3**, protein adsorption and cell adhesion can define the immune response to implants. Moreover, the point of alginate coating is to neutralize the positive charges of the polycation, and it was already proven that this polycation is present at the microcapsule surface. Whether the positive charges are efficiently balanced by the alginate coating is another question. Therefore, there is an interest in knowing the surface charge of the microcapsules in order to explain their biocompatibility. However, these studies have only recently been published because of the apparent technical difficulties involved with measuring such a property.

In 2007, De Vos *et al* [198] successfully used the streaming potential technique to measure the zeta potentials of APA microcapsules of different composition. This measurement is directly related to the average surface charge density of several microcapsules in a buffer solution. They observed that adding a PLL membrane (with alginate coating) to alginate gel beads consistently resulted in an increase in net positive charge. More interesting, the microcapsules that were associated with the greatest increase in net positive charge upon addition of the membrane were also the more immunogenic *in vivo*. This result supports the view that the positively charged PLL plays an important role in inducing an immune response to APA microcapsules. Also in line with this view are the results of Bakeine *et al* [199], who compared the *in vivo* biocompatibility of various synthetic latex microcapsules ranging in surface charge as well as APA microcapsules. They concluded that those microcapsules with a positively charged functional group at their surface (including the APA microcapsules) induced a significantly greater development of fibrosis. Most recently, Xie *et al* also measured zeta potentials to characterize the surface charge of alginate-chitosan microcapsules [131, 132].

Interestingly, they saw a net negative charge on their microcapsules, rather than positive. Still, they found that the capsules with the “least negative charge” corresponded with a greater adsorption of fibrinogen and immunoglobulin G *in vitro*.

1.5.6 Elasticity or pliability

One of the main reasons that alginate gels are used for microencapsulation purposes is because of their pliability, which should minimize injury to surrounding tissues due to frictional or mechanical forces [36].

Traditionally, bulk elastic properties of alginate-based microcapsules have been measured by applying a controlled compressive force to a group of capsules (essentially ‘squishing’ the capsules) and relating this force to the changing diameter of the microcapsules as they flatten and burst [47, 200-202]. Other more sophisticated methods for evaluating the pliability and elasticity of individual microcapsules have also been developed, including micromanipulation (involving a tiny probe that applies force) [203-205] and the use of a microcapillary to apply negative pressure to the membrane [206].

In principal, the more the microcapsule compresses (flattens) before bursting, the more elastic or pliable it is. The evidence shows that adding a polycationic membrane leads to a more rigid microcapsule that ‘squishes’ to a lesser extent when a compressive force is applied, although the specific material properties and fabrication protocol will affect the extent of this effect [47, 200-202, 207, 208]. While perhaps improving microcapsule strength (in terms of more force required to deform the sample), this feature may simultaneously present a problem for biocompatibility since the pliability of the hydrogel is compromised. However, such implications have not been directly explored by direct comparisons between elasticity and biocompatibility.

The local elastic properties of alginate-based microcapsules (as opposed to their bulk elastic properties) have been examined closely by one group. Specifically, Lekka *et al* [187] expanded the use of AFM beyond surface topography measurements in order to measure local elastic properties of their alginate-polycation microcapsules, as well as correlate these results with bulk mechanical characteristics of the beads. Although they demonstrated the effect of

microcapsule composition on the local Young's moduli, the implications for alginate biocompatibility were only implied, rather than tested directly.

1.5.7 Membrane degradation and leaching

Using labelling techniques, Thu *et al* [190, 191] demonstrated that PLL is capable of leaching out of the microcapsule when stored in an ionic solution. Given the apparent immunogenicity of unbound PLL [176], this observation has an important implication for the biocompatibility of the microcapsules in an *in vivo* environment.

A decade later, and in the same line of thought, Thanos *et al* [207-209] started using ATR-FTIR and SEM to monitor the surface degradation of alginate-PLO microcapsules *in vivo* over time. Degradation was characterized by a relative increase in PLO content in the microcapsule membrane (ATR-FTIR) in correlation with surface pitting and erosion (visible by SEM). When comparing a range of commercially available alginates, they saw no clear influence of the alginate M/G content on the results, but associated alginates of higher molecular weight with greater degradation [207, 209]. However, when they compared a commercial alginate with an ultra-pure alginate developed at the University of Perugia, the latter performed significantly better in terms of resisting degradation in an *in vivo* environment [208]. The implications for microcapsule biocompatibility were not directly measured, but it stands to reason that degradation of the membrane leads to an increased risk of exposing the potentially immunogenic polycation to the host tissues.

1.5.8 Surface hydrophilicity

It is often emphasized that one of the reasons that alginates are used for cell encapsulation is because they, like all hydrogels, are highly hydrophilic and therefore expected to discourage protein adsorption [36, 37]. Despite this popular point of view, few have investigated this property directly for alginate-based microcapsules. Based on my Master's work [152], we examined the effect of alginate purification on, among other properties, its hydrophilicity, as interpreted by the contact angle of water droplets on dry solution films. It turned out that hydrophilicity was a good predictor for alginate purity, and therefore in an indirect manner, alginate biocompatibility. More recently, at least one other group has decided to adapt the same technique in order to evaluate the immunological potential of their

microcapsules. Specifically, Xie *et al* [131] used the contact angle technique to measure the wettabilities of membranes representing alginate-chitosan-alginate microcapsules. In their case, they demonstrated that their samples were hydrophilic but failed to make a correlation between hydrophilicity and the adsorption of bovine fibrinogen and IgG on their microcapsules *in vitro*.

1.6 References

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CHAPTER 2

RESEARCH OBJECTIVE AND GENERAL APPROACH

2.1 Research objective

The objective of this research is to elucidate the relationships between the chemical makeup, the physicochemical properties, and the biological response to alginate-based microcapsules intended for islet transplantation. Our intention is to provide guidelines for optimizing the biocompatibility of the microcapsules, and thus contribute to the success of encapsulated islet transplantation.

2.2 General approach

The experimental research that was carried out can be divided into five phases. Each phase was directed by specific research aims, described here. The results of each phase are presented as separate chapters in this thesis.

2.2.1 Phase I : Microcapsule fabrication and protein adsorption

De Vos *et al* have developed alginate-poly-L-lysine-alginate (APA) microcapsules that are arguably the only ones worldwide that display a consistent biocompatibility, characterized by a high retrieval rate and minimal cellular overgrowth after long implantation periods in rodents. Interested in learning their "tricks of the trade", I started my doctoral studies with a six-month internship in D^{r} de Vos' lab at the University Medical Centre Groningen (the Netherlands).

Aim 1. *To learn, firsthand, how the Groningen group makes their microcapsules:* This also included a crash course in basic biochemistry (don't forget I'm an engineering student!). Since this was a learning objective, rather than a scientific study, per se, there are no results to present in this thesis.

Aim 2*. To prove that immunoglobulins adsorb to the surface of APA microcapsules, and to correlate this adsorption with the microcapsule chemistry:* We chose immunoglobulin adsorption as a research topic because protein adsorption is considered to be the first and governing phase of the biological response to implanted biomaterials. Moreover, the Groningen lab had already proved that proteins adsorb to their microcapsules upon their implantation in rats. The technique that we applied was relatively simple and easily accessible for any lab. Basically, APA microcapsules of different designs were incubated *in vitro* with human serum or peritoneal fluid, and then stained with fluorescently-marked antibodies to the immunoglobulins.

The results of this collaborative study have been published in the Journal of Biomedical Materials Research Part A, and are presented in **CHAPTER 3** of this thesis.

2.2.2 Phase II : Interlaboratory comparisons of alginates and microcapsules

Upon returning to Montreal, we wanted to see if we could produce biocompatible microcapsules if we imitated the Groningen protocol for capsule fabrication. This would have been very helpful for us, not only to understand what fabrication parameters may affect microcapsule biocompatibility, but if we had succeeded, to also have an appropriate negative control for subsequent studies.

Aim 1. *To verify the quality of our alginate in comparison to the alginate used by the Groningen group:* We purchased the same brand (although different batch number) of alginate that the Groningen group uses, but followed different protocols to purify our alginates and to make our gel beads. Despite these differences, we wanted to determine whether our alginates could perform just as well as theirs, particularly since we preferred to use our own alginate for subsequent studies rather than relying on them for our materials for microcapsule fabrication. Comparing the alginates consisted of analysing their chemical composition and purity, and testing the *in vivo* biocompatibility of their gels.

Aim 2. *To reproduce Groningen style microcapsules in our lab and compare their biocompatibility with that of our own microcapsules:* Using the first-hand experience that I gained in making microcapsules in the Groningen lab, we were curious to know whether we

were capable of making similarly biocompatible microcapsules using our own materials and lab equipment. Not only was this essentially a test of lab-to-lab reproducibility, but by comparing these microcapsules with our own, we expected to gather some important preliminary data on what fabrication parameters are important for microcapsule biocompatibility.

The results for these studies were not submitted for publication, but are presented in **CHAPTER 4** of this thesis.

2.2.3 Phase III : Characterization and biocompatibility of alginates

The first logical step towards understanding the biocompatibility of alginate-polycation microcapsules is to examine the characteristics and biocompatibility of the base material. Although alginate is generally considered to be non-immunogenic, several years of research have shown that this is not always the case. There is a continuing debate in the field about which combination of alginate properties can provide optimal biocompatibility for cell encapsulation purposes.

Aim. *To determine which properties of an alginate influence its biocompatibility:* We chose to compare two commercially available alginates that are commonly employed for cell encapsulation. We quickly established that the *in vivo* biocompatibility of these alginates differed significantly, and we wanted to explain why this was so. To do so, we thoroughly characterized the alginates and their gels in terms of chemistry, purity, solution viscosity, and hydrophilicity.

The results of this study should soon be published in the Journal of Biomedical Materials Research A (resubmitted after major revision), and are presented in **CHAPTER 5** of this thesis.

2.2.4 Phase IV : Microcapsule characterization and biocompatibility

Here, the term ‘microcapsule’ refers to a bead of alginate gel having a polycationic membrane. With the exception of recent publications, the majority of studies that have investigated microcapsule biocompatibility have focussed primarily on the chemical makeup of the

microcapsules, without further analysis of their physicochemical properties. After establishing that our alginate was biocompatible, we were in a good position to focus on the role of the polycationic membrane, including polycation-alginate interactions, on both the physicochemical properties and the biocompatibility of the microcapsules.

Aim. *To explain how microcapsule biocompatibility is influenced by their chemistry, in terms of their physicochemical properties:* As others, we investigated the effect of the type of alginate, gel, and polycation on microcapsule biocompatibility. However, we also included analyses of the elemental surface composition, molecular interactions within the membrane, wettability of the polyelectrolyte complexes, and stability of the polycationic membrane. We used these results to explain, in greater depth, how the different microcapsule designs can lead to their ranging *in vivo* biocompatibilities.

The results of this have been submitted for publication in Acta Biomaterialia, and are presented in **CHAPTER 6** of this thesis.

2.2.5 Phase V : Inducing normoglycemia using encapsulated islets

The ultimate application of our microcapsules is to immunoprotect transplanted islets *in vivo* so that they may efficiently normalize the blood sugar levels of a diabetic recipient. Most researchers believe that using a polycationic membrane is essential for achieving proper immunoprotection, despite that there is evidence that this membrane is responsible for microcapsule immunogenicity. Others have been trying to prove that microbeads without a membrane, which are more biocompatible, can still immunoprotect. We wanted to determine which of these two situations applied in our case.

Aim 1. *To determine whether a moderate immune response to the microcapsules is enough to hinder their ability to immunoprotect the islets within:* Using our most biocompatible microcapsules (which still induce a moderate immune response), we encapsulated rat islets and transplanted them into immunocompetent diabetic mice. Graft success was measured by the ability of the encapsulated islets to normalize the blood glucose levels of the recipients. Upon graft failure, we examined the state of each the microcapsules and islets under the microscope.

Aim 2. *To determine whether a membrane is even necessary for the immunoprotection of transplanted islets:* We compared the ability of rat islets enclosed within gel beads (without a membrane) with islets enclosed within microcapsules (with a membrane) to reverse diabetes in mice. Normally, the gel beads should not induce a significant immune response, but may be too porous to effectively block out components of the host immune system.

The results of this study were not submitted for publication, but are nevertheless presented in **CHAPTER 7** of this thesis.

CHAPTER 3

ARTICLE 1 : ADSORPTION OF HUMAN IMMUNOGLOBULIN TO IMPLANTABLE ALGINATE-POLY-L-LYSINE MICROCAPSULES: EFFECT OF MICROCAPSULE COMPOSITION

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3.1 Abstract

Alginate-poly-L-lysine-alginate (APA) microcapsules continue to be the most widely studied device for the immuno-protection of transplanted therapeutic cells. Producing APA microcapsules having a reproducible and high level of biocompatibility requires an understanding of the mechanisms of the immune response towards the implants. Here, we investigate the adsorption of immunoglobulins (IgG, IgM, and IgA) onto the surface of APA microcapsules *in vitro* after their exposure to human serum and peritoneal fluid. Immunoglobulins (Ig) are considered to be opsonizing proteins, thus they tend to mediate inflammation when adsorbed to foreign surfaces. Ig adsorption was monitored using direct immunofluorescence. The amount of Ig adsorbed to the microcapsule surface was not significantly influenced by the guluronic acid content nor the purity level of the alginate, although microcapsules of intermediate-G purified alginate corresponded with the lowest

adsorption levels. Ig adsorption was negligible when the poly-L-lysine membrane was omitted, suggesting that positive charges at the microcapsule surface are responsible for binding Ig.

3.2 Keywords

protein adsorption; immunofluorescence; biocompatibility; alginate; microcapsules

3.3 Introduction

Alginate-based microcapsules are commonly used for the immuno-protection of transplanted therapeutic cells. The biocompatibility of the encapsulated cell system, including the microcapsule itself, continues to be a subject of concern [1, 2]. In the 1990s, the development of fibrotic tissue around the implanted microcapsules was considered to be one of the major obstacles hampering the progress of encapsulated cell transplantation [3]; fibrosis arising from the normal wound-healing process was stated to hinder the diffusion of cell nutrients and therapeutic products across the capsule membrane. Moreover, fibrosis was frequently associated with graft failure. More recently, it was demonstrated that, by using optimal biomaterials and fabrication methods, it is feasible to produce alginate-poly-L-lysine-alginate (APA) microcapsules that induce minimal fibrosis *in vivo*, even 2 years after implantation in rats [4]. Despite this encouraging step forward, the ultimate goal of these research efforts, i.e. permanent survival of the encapsulated cellular grafts, was unfortunately not achieved as graft survival varied between 4 and 6 months.

During recent years it has become more accepted that the immune response against the encapsulated cell system is far more complicated than initially assumed. The complexity of this response partially explains the wide range of biocompatibility that the microcapsule system has displayed in the literature. The fact is activation of the immune system starts with the mandatory surgery to implant the microcapsules, that is before the implant is even introduced into the body. More precisely, injury to vascularized connective tissue caused by the incision induces an immediate inflammatory response associated with the influx of inflammatory cells and plasma proteins, as well as the release of bioactive factors such as cytokines and nitric oxide [5, 6].An important and likely event that can decide the ultimate biocompatibility of the microcapsule is the adsorption of opsonins onto its surface immediately upon its implantation. In general, the surface adsorption of opsonins is necessary for immune cells to recognize a pathogen or

biomaterial as being ‘‘foreign’’ and to subsequently attack it. To date, no one has verified experimentally whether this adsorption occurs in the case of microcapsules designed for cellular therapy. The immunoglobulins IgM, IgA, and especially IgG have the ability to opsonize foreign bodies, thus their capacity to adsorb to microcapsules is of interest when investigating their biocompatibility. IgG and IgM are particularly effective for complement activation, while IgG and IgA can mediate phagocytosis, though IgA is generally restricted to mucosal secretions [7-11].

In the present study, we investigate the adsorption of immunoglobulin (Ig) to the surface of APA microcapsules i*n vitro*. This was done for microcapsules of different compositions.

3.4 Materials and Methods

3.4.1 Study Design

The adsorption of human immunoglobulins IgG/IgM/IgA to the surface of APA microcapsules *in vitro* was monitored using direct immunofluorescence. Ig adsorption was achieved by incubating microcapsules in serum and/or peritoneal fluid (PF). These fluids were selected in order to simulate the *in vivo* situation where microcapsules come into contact with both blood and PF during implantation into the peritoneal cavity, i.e. the conventional transplantation site. Subsequent to incubation, the protein-covered microcapsules were rinsed then immersed in a solution containing a fluorescently labeled antibody to human immunoglobulin (Ig). The extent of Ig adsorption was measured in terms of fluorescence intensity. Ig adsorption levels to APA microcapsules composed of alginates varying in chemical composition (i.e. high vs. intermediate guluronic acid content) and in purity level were compared because these properties of alginate have a significant influence on the *in vivo* bioperformance of the microcapsules. Furthermore, to investigate the relative influence of the alginate matrix and the poly-L-lysine (PLL) membrane on Ig adsorption, the adsorption to complete APA microcapsules and to calcium alginate beads (having no PLL membrane) was compared.

3.4.2 Materials

Intermediate-G sodium alginate (Keltone® LVCR, International Specialty Products Corp, UK) having 40% guluronic acid (as specified by the manufacturer) and high-G sodium alginate (Manugel® DMB, International Specialty Products Corp, UK) having 50% guluronic acid (as specified by the manufacturer) were used for microcapsule fabrication. The alginates were purified using previously described methods [12]. The alginates were dissolved in a 220 mOsm Ca^{2+}–free Krebs-Ringer-Hepes (KRH) solution consisting of 90.0 m*M* NaCl, 4.7 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, and 25.0 m*M* Hepes. Alginates were dissolved at a concentration of 3.4% w/v for the unpurified intermediate-G alginate, 3.5% w/v for the purified intermediate-G alginate and 1.9% w/v for each of the high-G alginates. These concentrations provided alginate solutions of appropriate viscosity for microcapsule fabrication. All alginate solutions were sterilized by filtration (0.22 μm). PLL hydrochloride (Sigma-Aldrich, USA) having a molecular weight of 22,200 (as specified by the manufacturer) was used to form the microcapsule membrane. For protein adsorption studies *in vitro*, human serum isolated from whole blood was donated by healthy volunteers. Human PF was obtained from a male donor within 12 h of his decease. For the experiments, only the PF supernatent was used. To detect the immunoglobulin adsorbed to the microcapsule surface, a fluorescein isothiocyanate (FITC)-conjugated polyvalent antibody to human IgA/IgG/IgM developed in rabbit (Sigma-Aldrich, USA) was used.

3.4.3 Microcapsule Fabrication

Microcapsules were produced using an air-driven droplet generator following previously described methods [13]. Alginate solution was extruded from a 23G needle using a syringe and a co-axial air stream to produce droplets. The alginate droplets were immersed in a 100 m*M* $CaCl_2$ solution and allowed to gel for 5 min after extrusion of the last droplet (the extrusion process lasted 2–4 min). Gelled calcium alginate beads measured 650–675 μm in diameter. To form the microcapsule membrane, the calcium alginate beads were rinsed and then immersed for 10 min in a PLL solution that consisted of PLL dissolved 0.1% w/v in 310 mOsm Ca^{2+}-free KRH (135.0 m*M* NaCl, 4.7 m*M* KCl, 25.0 m*M* Hepes, 1.2 m*M* KH_2PO_4, and 1.2 m*M* $MgSO_4$). The microcapsules were rinsed again and then immersed in a 10× diluted solution of alginate

(i.e. 0.19%, 0.34% or 0.35% w/v in Ca^{2+}-free KRH) for 5 min, with the aim of binding any unbound PLL at the surface. In all cases, the same type of alginate was used for both the microcapsule gel core and the coating step. After final rinsing, the microcapsules were stored in KRH until analysis. Final APA microcapsules measured 600–750 μm in diameter. All solutions used for microcapsule fabrication were sterilized by filtration (0.22 μm).

3.4.4 Protein adsorption to microcapsules

Samples of 30 microcapsules were transferred to a polypropylene test tube. The supernatant (i.e., KRH) was removed by aspiration. Serum was diluted 1:1 in KRH and 300 μL of the diluted serum was added to each test tube. Samples were incubated in a warm water bath at 37°C with gentle agitation for 1 h. Afterwards, the serum was removed and the microcapsules were rinsed five times with KRH. For certain experiments (see the results section), the serum was replaced with human PF or a 1:1 mixture of serum and PF.

3.4.5 Measurement of Ig adsorption

The amounts of Ig that adsorbed to the microcapsule surfaces were measured using direct immunofluorescence. Seventy-five microliters of dilute FITC-conjugated rabbit antibody to human IgG/IgM/IgA (diluted 1:600 in KRH) was added to each sample of microcapsules. Immediately afterwards, the samples were kept in the darkness at room temperature for 1 h. The microcapsules were then rinsed five times with KRH and subsequently transferred to a 96-well plate (Corning® flat bottom). The fluorescence intensity emitted from the samples was quantitatively measured using a Bio-Tek FL600™ fluorescence microplate reader (Bio-Tek Instruments, USA). An excitation wavelength of 485 nm and emission wavelength of 530 nm was used in order to detect the FITC label. A top-reading measurement was taken at a sensitivity setting of 200. Readings were always taken in triplicate. The labeling efficiency of the antibody was also confirmed by fluorescence microscopy using an Olympus IMT-2 inverted microscope (Olympus, Japan).

3.4.6 Statistical Analysis

Measured values were compared using the Wilcoxon Signed Ranks Test with the aid of SPSS® statistical analysis software (SPSS, USA). A difference for which $p < 0.05$ was

considered to be statistically significant. In general, quantitative results (represented as bar graphs) are expressed as the mean fluorescence intensity over an *n* value of 6 ± the standard error of the mean (SEM).

3.5 Results

Direct immunofluorescence was applied in order to quantify the extent of immunoglobulin (Ig) adsorption to microcapsules preincubated in either serum or PF. A microscopic examination of APA microcapsules prepared of purified intermediate-G alginate and preincubated in serum (**Figure 3.1**), confirmed that the fluorescence staining was specific for the protein-covered microcapsule surface. These results not only confirm that Ig adsorbs to APA microcapsules, but also demonstrate the adequacy of our experimental approach.

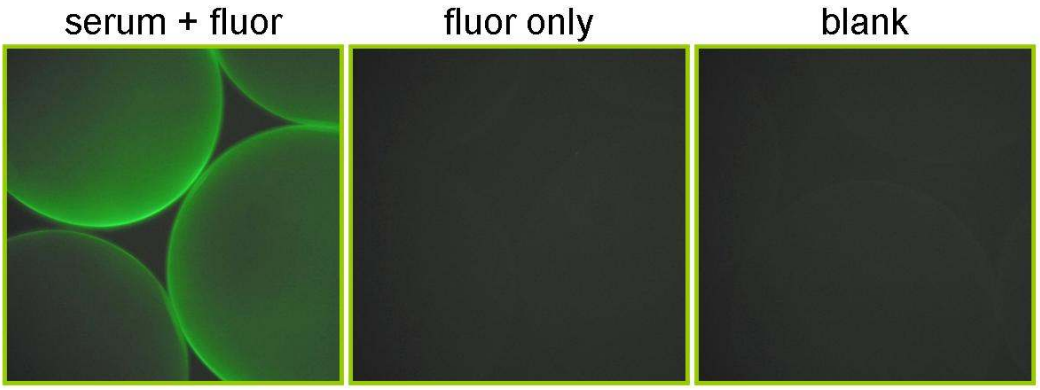

Figure 3.1 Fluorescent signal emitted from alginate-poly-L-lysine-alginate (APA) microcapsules upon excitation by a light having a wavelength (λ) of 485 nm, as observed by microscopy. Microcapsules were either pre-incubated in serum then incubated with a FITC-labeled antibody to human immunoglobulins IgG/IgM/IgA (‘‘serum + fluor’’), incubated with only the FITC-labeled antibody (‘‘fluor only’’), or incubated with neither (‘‘blank’’).

In the case of *in vivo* studies, the microcapsules are in contact with both blood and PF upon their implantation in the peritoneal cavity (i.e., the conventional transplantation site). Therefore, we quantified and compared the adsorption of Ig onto APA microcapsules after incubation in serum, PF, and a 1:1 mixture of PF and serum. As shown in **Figure 3.2**, the Ig adsorbed in significantly greater amounts ($p < 0.05$) to the microcapsules when they were incubated in serum (8.4 ± 1.8 arbitrary fluorescent units, AFU) compared to those that were

incubated in either PF (2.9 ± 0.9 AFU) or the serum/PF mix (3.4±0.7 AFU). Since incubation in serum was associated with the highest adsorption levels, we used serum as the Ig source for subsequent studies.

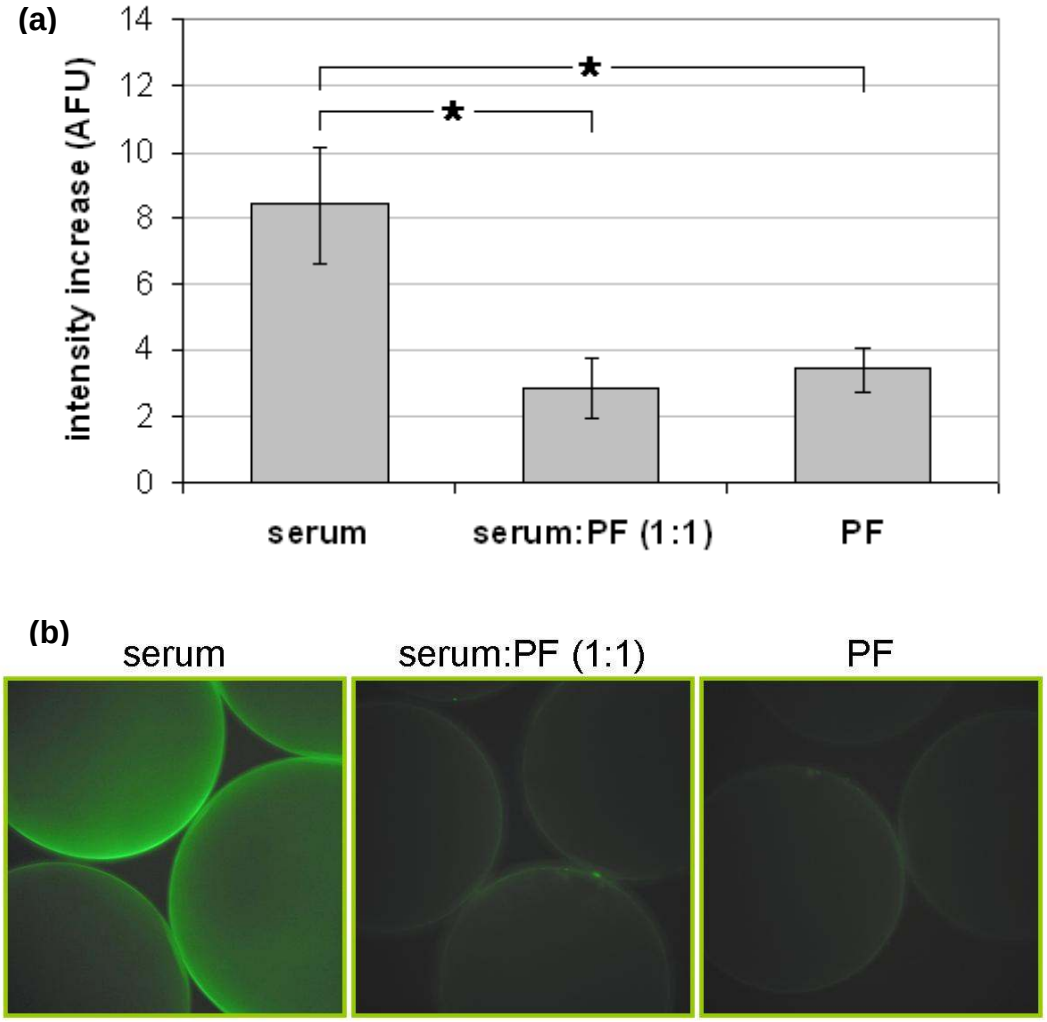

Figure 3.2 Extent of immunoglobulin (Ig) adsorption to APA microcapsules that were incubated in serum, peritoneal fluid (PF) or a 1:1 mixture of serum and PF. Ig adsorption is proportional to the intensity of fluorescence emitted from the samples upon excitation. (a) Quantification of Ig adsorption, which is represented by an ‘‘intensity increase’’ (i.e. the average increase in fluorescence intensity vs. microcapsules that were incubated with only the FITC-labelled antibody) ± standard error of the mean, SEM (n = 6). AFU = arbitrary fluorescent units, * $p < 0.05$. (b) Microscope observation of the fluorescent light emitted by the microcapsules.

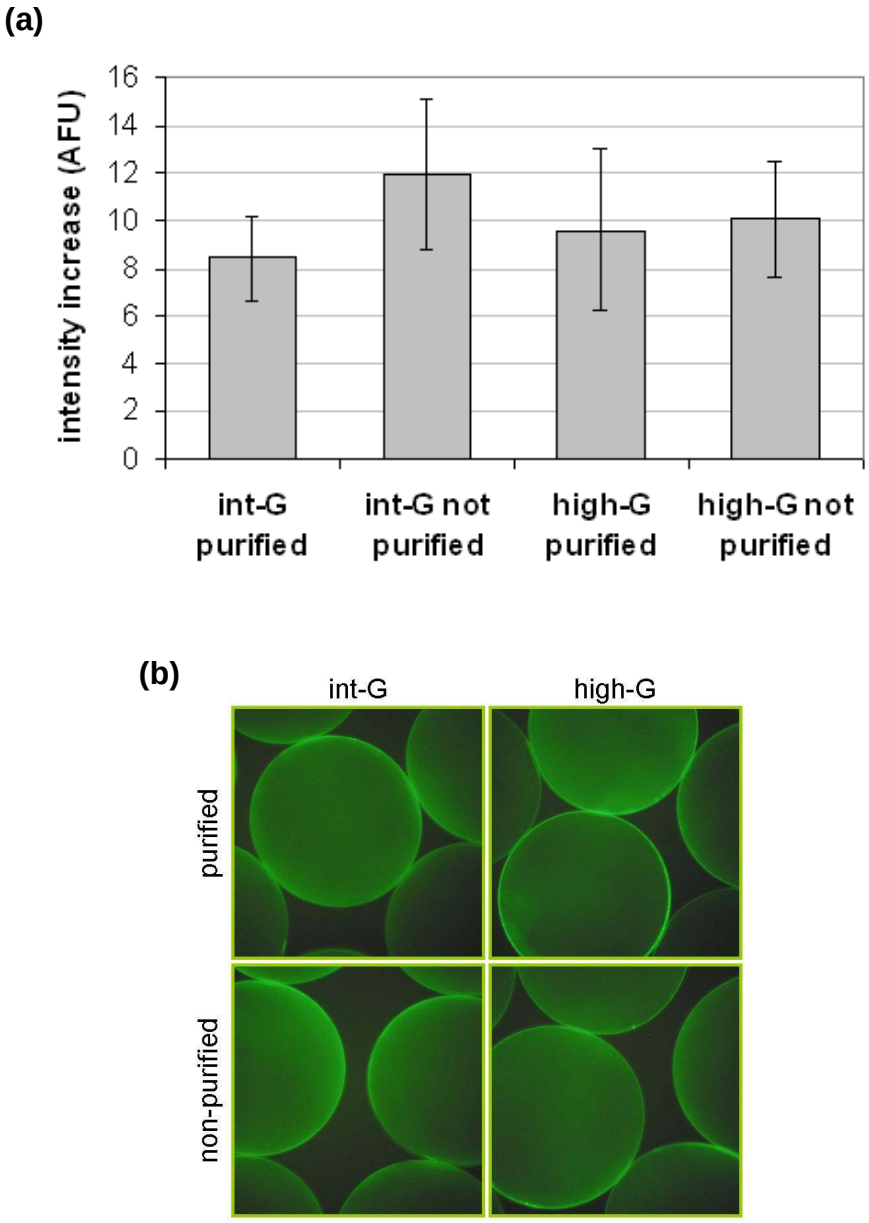

Figure 3.3 Extent of Ig adsorption to APA microcapsules that were incubated in serum. The microcapsules were prepared of alginates varying in chemical composition (intermediate-G vs. high-G) and in purity (purified vs. nonpurified). Ig adsorption is proportional to the intensity of fluorescence emitted from the samples. (a) Quantification of Ig adsorption, which is represented by an ‘‘intensity increase’’ (i.e. the average increase in fluorescence intensity vs. microcapsules that were incubated with only the FITC-labeled antibody) ± SEM (*n* = 6). AFU = arbitrary fluorescent units. (b) Microscope observation of the fluorescent light emitted by the microcapsules.

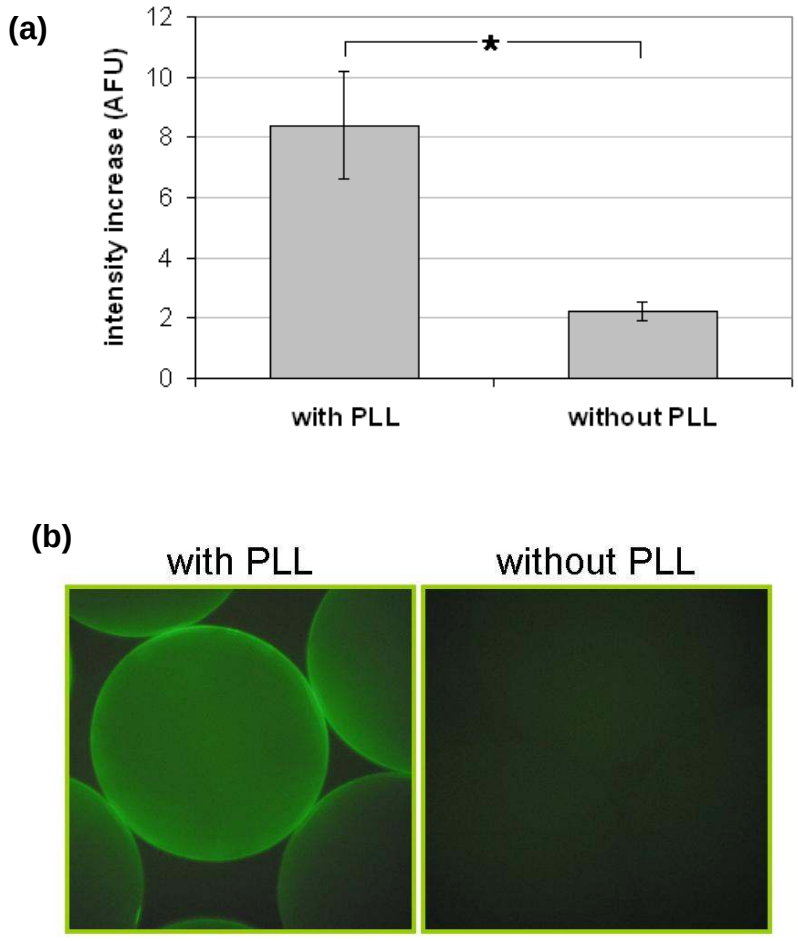

Figure 3.4 Extent of Ig adsorption to APA microcapsules (‘‘with PLL’’) or to calcium alginate beads (‘‘without PLL’’) that were incubated in serum. The samples were prepared of purified intermediate-G alginate. Ig adsorption is proportional to the intensity of fluorescence emitted from the samples. (a) Quantification of Ig adsorption, which is represented by an ‘‘intensity increase’’ (i.e. the average increase in fluorescence intensity vs. samples that were incubated with only the FITC-labeled antibody) ± SEM (n = 6). AFU = arbitrary fluorescent units, * $p < 0.05$. (b) Microscope observation of the fluorescent light emitted by the samples.

To investigate the influence of the microcapsule characteristics on the extent of Ig adsorption, we quantified and compared the adsorption onto APA microcapsules prepared of purified versus nonpurified alginates, and of intermediate-G versus high-G alginates. There were no statistically significant differences between the amounts of Ig adsorbed onto each of the samples tested (**Figure 3.3**).

Next, we investigated whether it is the PLL-based membrane or the alginate matrix that is responsible for the observed adsorption of Ig to the capsules. To do this, we compared the extent of Ig adsorption onto calcium alginate beads to the adsorption onto APA microcapsules after incubating each in serum. As shown in **Figure 3.4 (a)**, in the case of samples prepared of purified intermediate-G alginate, the measured fluorescence intensity was fourfold higher when PLL was included in the membrane. The experiments were repeated for samples prepared of high-G alginates and nonpurified alginates; the results (not shown) consistently confirmed that the PLL membrane is a principal mediator of Ig adsorption to APA microcapsules.

3.6 Discussion

Generally speaking, following the implantation of a medical device such as microcapsules, the typical sequence of events is as follows: Immediately after injury, there is acute inflammation that, if persists, leads to chronic inflammation, granulation tissue, foreign body reaction, and finally fibrosis [5, 6, 14-17]. Acute inflammation, the first phase upon injury, is characterized by the exudation of fluid and plasma proteins, as well as the attraction of leukocytes to the site of injury. Subsequently, neutrophils and macrophages become activated. In general, these immune cells will recognize the implant as being ‘‘foreign’’ only if it is coated by opsonins, i.e. serum proteins for which these cells have specific membrane receptors [17-19]. Several immunoglobulins, particularly IgG but also IgA and IgM, are considered to be opsonins. In the case of cell-containing microcapsules, opsonization of the implant during acute inflammation plausibly leads to events that can be disastrous for graft viability, particularly the further release of cytotoxic cytokines associated with chronic inflammation and frustrated phagocytosis, as well as the formation of fibrotic tissue during the final stage of the healing process.

It is well recognized that the biological response to APA microcapsules is significantly influenced by the chemical properties and the quality of the alginate. We have observed that microcapsules prepared of purified intermediate-G alginate remain free of any significant foreign body response for prolonged periods of time after implantation, while microcapsules prepared of high-G alginate are consistently associated with low recovery rates and extensive overgrowth [20, 21]. It is also well known that microcapsules composed of alginates that are not properly purified induce a severe inflammatory response [12, 22-24]. Yet, the mechanisms that

explain the influence of the alginate properties on the overall biocompatibility of the microcapsule are still not well understood. Therefore, we investigated the extent of immunoglobulin (Ig) adsorption onto the surface of APA microcapsules composed of alginates varying in chemistry and purity level as these microcapsules consistently demonstrate various degrees of biocompatibility when implanted. This approach was based on the hypothesis that opsonization by Ig is the principal event that mediates or leads to the immune response to implanted microcapsules. However, it was observed that altering the alginate properties had no statistically significant effect on the quantity of Ig that adsorbed to the corresponding APA microcapsules. Taking into account our previously observed *in vivo* response to microcapsules of the same chemical composition, these results suggest that Ig adsorption plays a negligible or a minor role in mediating an immune response to the implanted microcapsules. At the same time, it is worth noting that the microcapsules prepared of purified intermediate- G alginate adsorbed the lowest amount of Ig. This finding corroborates our *in vivo* observation that microcapsules prepared of this particular alginate provoke a minor inflammatory response compared to microcapsules prepared of high-G or impure alginates [20, 21]. Thus, it remains plausible that Ig adsorption has a certain degree of influence on the inflammatory response to implanted APA microcapsules.

We observed that the extent of Ig adsorption to the microcapsules was significantly lower in the absence of the PLL-based membrane. Since we have recently confirmed, using zeta potential measurements, that adding the PLL membrane creates a net charge at the surface of APA microcapsules [25], the results of this study indicate that the positive charges of the polycation are attracting the immunoglobulin. This is in alignment with the general observation that, as opposed to highly charged surfaces, neutral (and hydrophilic) polymers have minimal or weak interactions with most aqueous proteins [26]. The biological implications of these results can be interpreted in two ways. On one hand, if it is to be assumed that Ig adsorption inevitably leads to inflammation, then the observation that the PLL attracts Ig to the microcapsule surface supports the numerous published studies which imply that exposed PLL is mainly responsible for the inflammatory response to APA microcapsules [15, 21, 27, 28]. On the other hand, these results do not necessarily reflect our own *in vivo* observations. We have seen that, in the case of APA microcapsules prepared of purified intermediate-G alginate, complete microcapsules that include the PLL membrane are just as biocompatible as the corresponding alginate gel beads.

From this point of view, our results suggest that Ig adsorption to the microcapsule surface does not always lead to a severe inflammatory response or fibrosis.

In this study, we observed that Ig adsorption to the microcapsules was greater from serum than from PF or from a serum/PF mix. This is explained by the differences in protein concentrations between the two fluids. PF is a transudate of plasma, thus they share similar protein compositions. However, due to the fact that the peritoneal membrane limits the diffusion of larger molecules (>20 kDa), PF contains similar concentrations of smaller solutes, lower concentrations of high molecular-weight molecules, and an overall protein concentration of ~50% that of plasma protein concentrations [29-32]. The molecular weight of human immunoglobulin ranges from about 150 to 900 kDa [11], thus their concentration is expected to be lower in PF than in serum. In turn, protein adsorption patterns from mixed solutions are influenced by (among other factors) the relative bulk concentrations and molecular weights of each protein type. For instance, smaller proteins at higher concentrations tend to adsorb before larger proteins [26, 33].

The results of this study provide indications that Ig adsorption may influence the inflammatory response to APA microcapsules, yet it is clear that the combined effect of IgG, IgM, and IgA adsorption is not the only determining factor for microcapsule biocompatibility. While IgG is known to be a highly effective opsonizer, there are a number of other serum proteins that have also been seen to influence implant biocompatibility, for example the complement factor C3b, whose potential role in these experiments must not be ignored [17]. A growing number of researchers recognize that proteins of all types are very important to biomaterials science because of their tendency to deposit on surfaces as a tightly bound adsorbate, and more importantly due to the strong influence that these deposits have on subsequent cellular interactions with an implant surface [34]. Proteins have been measured on biomaterial surfaces within 1 s of implantation, thus protein adsorption occurs well before cells arrive at the surface [35]. On another note, in the particular case of microcapsules for cell therapy, protein adsorption should be considered not only in terms of its influence on the cellular response to the implants, but also in its influence on the capsule bioperformance by its potential to alter the selective permeability of the microcapsule's immuno-protective membrane. While this aspect has not been directly studied in the case of alginate microcapsules, protein

adsorption has been seen to reduce the hydraulic permeability and solute sieving of polysulphone membranes [36, 37].

3.7 Conclusions

It was clearly proven that human immunoglobulin (IgG/IgM/IgA) adsorbs to the surface of APA microcapsules *in vitro*. This was demonstrated using the relatively simple method of direct immunofluorescence. The extent of immunoglobulin (Ig) adsorption is not significantly influenced by the chemical composition and purity of the alginate used for microcapsule fabrication, yet it is highly dependent on the presence of the polylysine membrane, indicating that the positive charges of the polycation are mainly responsible for binding the Ig. Since IgG, IgM, and IgA are known to be opsonizing proteins that lead to complement activation upon their adsorption to foreign surfaces, these findings are useful for explaining the mechanisms of the immune response to APA microcapsules.

3.8 References

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CHAPTER 4

AN INTERLABORATORY COMPARISON OF ALGINATES AND APA MICROCAPSULES (GRONINGEN VS. MONTRÉAL)

NOT SUBMITTED FOR PUBLICATION

4.1 Abstract

The Groningen lab is known for producing biocompatible alginate-poly(L-lysine)-alginate (APA) microcapsules. We attempted to reproduce their results by following their protocol for microcapsule fabrication but using the materials and the equipment available in the Montréal lab. Alginate that we purchased and purified ourselves was slightly contaminated, but similar in composition than the alginate provided by the Groningen lab. Nevertheless, both alginates were biocompatible *in vivo*. Both alginates also produced APA microcapsules of similar immunogenicity. Using the Montréal alginate, we could make perfectly biocompatible gel beads regardless of the protocol for bead fabrication employed. However, our version of the Groningen protocol for APA microcapsule fabrication resulted in microcapsules that were more immunogenic ($p < 0.01$) than those fabricated using the Montréal protocol. This discrepancy was attributed to deviations that were taken from the original Groningen protocols, including the method for droplet generation and choice of animal model. The results of this study demonstrate the difficulty of achieving lab-to-lab reproducibility. Moreover, Montréal protocols for alginate purification, bead fabrication and APA microcapsule fabrication may be considered adequate for subsequent implantation studies.

4.2 Introduction

The Groningen lab is known for producing alginate-poly(L-lysine)-alginate (APA) microcapsules that are biocompatible *in vivo*. Since the late 1990's, they have been demonstrating that when they implant their microcapsules into the peritoneal cavity of Albino Oxford (AO) rats, only minimal cell overgrowth and fibrosis is induced, even after several

months [1, 2]. Although other research groups have also reported adequate biocompatibility of their APA microcapsules under specific conditions [3-5], these studies tend to lack follow-up, which puts into question the reproducibility of their results. Other groups have published the fact that the APA microcapsules that they produced in their labs (sometimes as controls) induced some type of an immune response *in vivo* [6-8].

After having studied the Groningen protocol for APA microcapsule fabrication first-hand, we (the Montréal group) attempted to reproduce their results using their protocol but our own lab equipment and materials. First, we tested whether the alginate that we purchased and purified ourselves performed as well as the Groningen group's alginate. Next, we wanted to know if we were capable of producing biocompatible APA microcapsules if we followed (as closely as possible) their protocol for microcapsule fabrication. If we were to succeed, we would not only be able to move forward with the study of other aspects of microcapsule bioperformance using immunocompetent mice, but we would also have a very useful control for our studies on capsule biocompatibility.

4.3 Materials and Methods

4.3.1 Materials

IntG$_{Gron}$: Keltone® LVCR sodium alginate, lot #015821A, was purchased from ISP Corp (UK). This alginate was purified in the Groningen lab according to their own protocol, described in ref [1].

IntG$_{Mtl}$: Keltone® LVCR sodium alginate, lot #721322, was provided by ISP Corp (New Jersey, USA). We purified the alginate in Montréal according to our own protocol, described in refs [9, 10].

PLL : Poly(L-lysine) hydrobromide was purchased from Sigma (St Louis, USA) and had a molecular weight of 15,000 - 30,000 by viscosity (as specified by the manufacturer).

All solutions used in this study were made in Montréal. Their formulae are described in **APPENDIX I**. Solutions were sterilized by filtration (0.22 μm) before their use. Labware and other laboratory equipment were sterilized whenever possible.

4.3.2 Protein and Endotoxin measurements

For the alginates purified in Montréal, the protein and endotoxin content was measured using the microBCA and LAL assays, respectively. Details on these techniques for evaluating alginate contamination are described in **section 5.4.2** [11].

4.3.3 Proton nuclear magnetic resonance (^{1}H-NMR)

The composition and sequential structure of the alginates were determined using ^{1}H-NMR. The protocol details for this technique are described in **section 5.4.5** [11].

4.3.4 Contact angle technique

The wettabilities of the samples were estimated by measuring the static contact angles of 1 μL water droplets on dry films. Alginate films were cast from aqueous solutions directly onto glass microscope slides. Details for film fabrication and the contact angle technique are described in **section 5.4.9**.

4.3.5 Gel bead and APA microcapsule fabrication

Gel beads and APA microcapsules were fabricated using either the Montréal protocols ("Montréal") or modified versions of the Groningen protocols ("Groningen$_{mod}$"). Details for the Montréal protocol for bead and microcapsule fabrication are described in **section 5.4.3** and **section 6.4.2**, respectively. The original Groningen protocols ("Groningen$_{orig}$") are described in **section 3.4.3** and have been published in [12]. In this study, slight modifications from the original protocols were necessary in order to adapt to the lab equipment and materials available for sample fabrication. The principal steps for the each of the protocols for bead and microcapsule fabrication are compared in Error! Reference source not found. and Error! Reference source not found..

Table 4.1 Comparison of fabrication protocols for gel beads

Step	**Groningen$_{orig}$**	**Groningen$_{mod}$**	**Montréal**
Alginate solution	IntG$_{Gron}$ 2.7% w/v in 220 mOsm Ca^{2+}-free KRH	IntG$_{Mtl}$ 1.75% w/v in 220 mOsm Ca^{2+}-free KRH	IntG$_{Mtl}$ 1.63% w/v in 154 mM saline IntG$_{Gron}$ 2.80% w/v In 154 mM saline
Droplet production	Co-axial air-jet with 23G needle	Electrostatic pulse with 25G needle	Electrostatic pulse with 25G needle
Gelation	8+ min in 10 mM $BaCl_2$(Gron)	8+ min in 10 mM $BaCl_2$(Gron)	30 min In 10 mM $BaCl_2$(Mtl)
Rinse	1×1 min in 310 mOsm Ca^{2+}-free KRH 3×1 min in 25 mM KRH	3×5 min in Ringer	3×5 min in Ringer

Table 4.2 Comparison of fabrication protocols for APA microcapsules

Step	**Groningen$_{orig}$**	**Groningen$_{mod}$**	**Montreal**
Alginate solution	IntG$_{Gron}$ 2.7% w/v in 220 mOsm Ca^{2+}-free KRH	IntG$_{Mtl}$ 1.75% w/v in 220 mOsm Ca^{2+}-free KRH	IntG$_{Mtl}$ 1.63% w/v in 154 mM saline IntG$_{Gron}$ 2.80% w/v in 154 mM saline
Droplet production	Co-axial air-jet with 23G needle	Electrostatic pulse with 25G needle	Electrostatic pulse with 25G needle
Gelation	8+ min in 100 mM $CaCl_2$	8+ min in 100 mM $CaCl_2$	30 min In 100 mM Ca Lactate
Rinse	1 min in Spoelhepes	2 min in Spoelhepes	n/a
PLL coating	10 min in 0.1% w/v PLL in 310 mOsm Ca^{2+}-free KRH	5 min in 0.05% w/v PLL in 310 mOsm Ca^{2+}-free KRH	5 min in 0.05% w/v PLL In 154 mM saline
Rinse	3×1 min in 310 mOsm Ca^{2+}-free KRH	3×3 min in 310 mOsm Ca^{2+}-free KRH	5 min in 154 mM saline
Alginate coating	5 min in alg diluted 1:10 in 310 mOsm Ca^{2+}-free KRH	5 min in alg diluted 1:10 in 310 mOsm Ca^{2+}-free KRH	5 min in alg diluted 1:10 in 154 mM saline
Rinse	1×1 min in 310 mOsm Ca^{2+}-free KRH 3×1 min in 25 mM KRH	1×2 min in 310 mOsm Ca^{2+}-free KRH 3×2 min in 25 mM KRH	3×5 min in 154 mM saline

4.3.6 In vivo biocompatibility

The protocol for evaluating the *in vivo* biocompatibility of the microcapsules is described in detail in **section 6.4.3**. Briefly, 500 beads or microcapsules were handpicked and then implanted into the peritoneal cavity of male C57BL/6J mice. After two days, the samples were explanted and individual beads/capsules were classified into one of four categories according to how much of their surface was covered with immune cells. This classification system allowed a calculation of a weighted 'cell adhesion score' that ranges from 0 (no cell adhesion) to 10 (complete coverage by cells and fibrosis).

4.3.7 Statistical Analysis

Samples were compared using the unpaired Student *t*-test. Differences for which $p < 0.05$ were considered to be statistically significant.

4.4 Results

4.4.1 Further characteristics of the alginates

Alginate characteristics that were either measured directly or extracted from the literature are compared in **Table 4.3**. Our alginate ($IntG_{Mtl}$) and the alginate provided by the Groningen group ($IntG_{Gron}$) contained similar proportions of guluronate (G) and mannuronate (M) both before and after their purification. The alginate that was purified in Montreal had negligible amounts of proteins and slight quantities of endotoxins [11]. In comparison, we did not directly measure the contamination levels of the Groningen-purified alginate, although a published study indicated a minimal amount of endotoxins (< 0.01 EU/mL) without specifying the concentration of the solution tested [12]. However, we previously demonstrated that the contact angle of water on alginate films is a good indicator of their purity level [13]. Using this approach, we observed that (after purification) the $IntG_{Mtl}$ alginate had a slightly greater contact angle than the $IntG_{Gron}$ alginate, which suggests that the Groningen group's alginate was slightly more pure.

Table 4.3 Further characteristics of the alginates

	$IntG_{Gron}$	**$IntG_{Mtl}$**
Composition	Crude : 43%G : 57%M (n = 1) Pure : 40%G : 60%M (ref [1])	Crude : 36%G : 54%M (n = 2) Pure : 44:%G : 56%M (n = 3)
Contaminants (after purif)	Proteins : no data Endotoxins : < 0.01 EU/mL (ref [12])	Proteins : 3.7 ± 0.4 μg/g (n = 3) Endotoxins : 231 ± 127 EU/g (n = 4)
Contact angle	Crude : 68.5 ± 2.0° (n = 4) Pure : 33.1 ± 2.1° (n = 4)	Crude : 50.0 ± 8.1° (n = 2) Pure : 39.5 ± 4.1° (n = 5)

4.4.2 Characteristics of the beads and microcapsules

As detailed in Error! Reference source not found., we had to slightly modify the Groningen protocol for bead fabrication before we could implant the beads. We initially tried to use the rinsing solutions described in the original protocol, but when we placed the barium gel beads in contact with 310 mOsm Ca^{2+}-free KRH, some sort of precipitation developed on their surfaces (**Figure 4.1**). We determined that this was some sort of reaction between the 10 mM $BaCl_2$(Gron) and the 310 mOsm Ca^{2+}-free KRH because when we mixed these two solutions together (in the absence of beads), the same type of precipitation was observed on the bottom of the petri dish (**Figure 4.1**). Because of this inconvenience, we simply substituted the KRH with Ringer's solution for rinsing the beads. The resulting gel beads looked just fine, as did the Montréal-style gel beads.

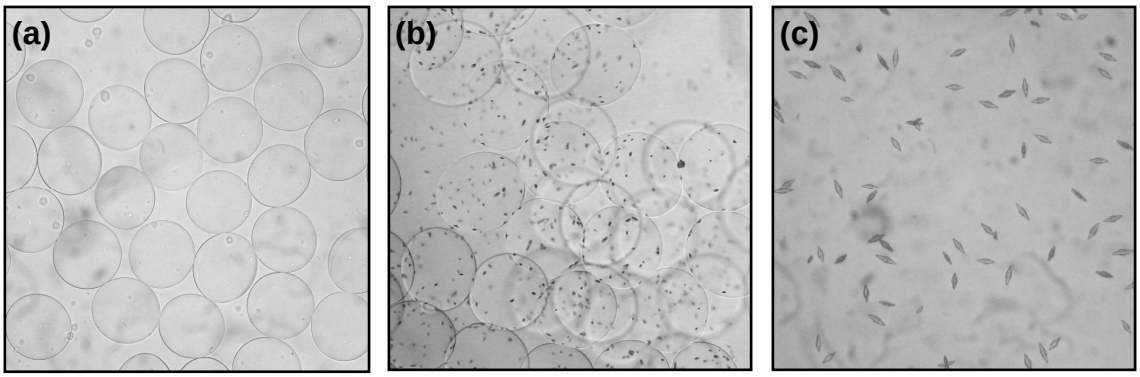

Figure 4.1 View through optical microscope of (a) barium alginate gel beads in 10 mM $BaCl_2$(Gron), (b) the same gel beads after transferring them into 310 mOsm Ca^{2+}-free KRH, and (c) a mixture of 10 mM $BaCl_2$(Gron) and 310 mOsm Ca^{2+}-free KRH solutions.

Similarly, slight deviations from the original Groningen protocol for APA microcapsule fabrication were necessary in order to produce an implantable sample (Error! Reference source not found.). When we attempted to follow the original coating protocols for membrane formation on our smaller gel beads, the membrane was much too thick (**Figure 4.2**). Therefore, we reduced the concentration and incubation time for the PLL coating step. The resulting APA microcapsules looked similar to the capsules made using the Montréal protocol for fabrication. However, these microcapsules swelled less during membrane formation (154% diameter increase) than did the microcapsules from the Montréal protocol (200% diameter increase).

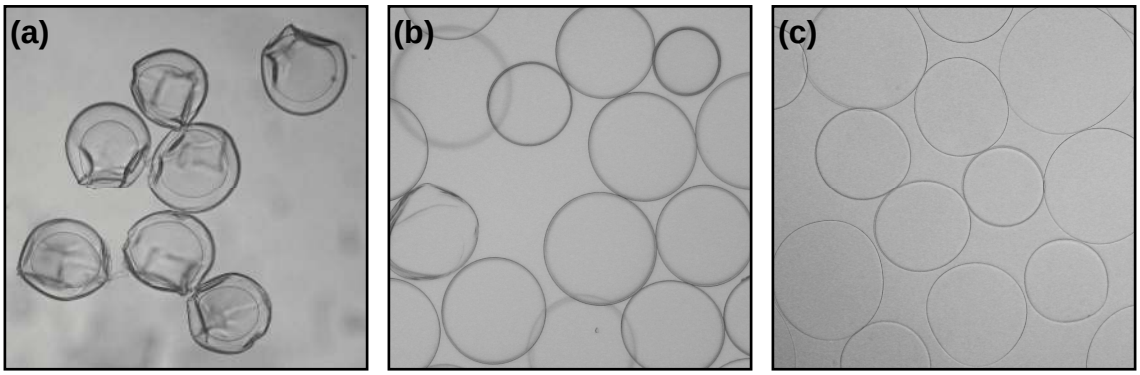

Figure 4.2 View through optical microscope of APA microcapsules when following (a) the $\text{Groningen}_{\text{orig}}$ protocol (excluding the use of co-axial air-jet) (b) the $\text{Groningen}_{\text{mod}}$ protocol, and (c) the Montréal protocol.

4.4.3 In vivo biocompatibility

The *in vivo* biocompatibilities of the samples were evaluated in terms of the extent of immune cell adhesion after two days in the peritoneal cavity of C57BL/6J mice. In all cases, the APA microcapsules showed a moderate immune response that corresponded to cell adhesion scores between 4.0 and 7.0. When the PLL membrane was omitted, the gel beads were very biocompatible, as cell adhesion scores were consistently below 1.5.

The biocompatibility of the $\text{IntG}_{\text{Gron}}$ and the IntG_{Mtl} alginates were compared by implanting barium alginate gel beads that were fabricated using the Montréal protocol. The Groningen alginate showed signs of being slightly immunogenic while the Montréal alginate was perfectly biocompatible (**Figure 4.3**). However, the difference between their cell adhesion

scores was not statistically significant. When these alginates were used to make APA microcapsules (Montréal protocol), their *in vivo* performance was almost identical.

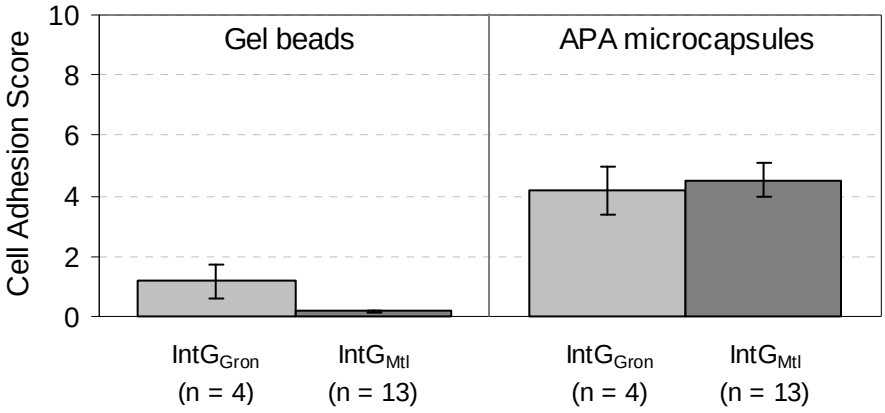

Figure 4.3 Effect of alginate source (IntG$_{Gron}$ vs IntG$_{Mtl}$) on the *in vivo* biocompatibility of gel beads and APA microcapsules. Samples were made using the Montreal fabrication protocol. Bars represent the mean cell adhesion score ± SEM.

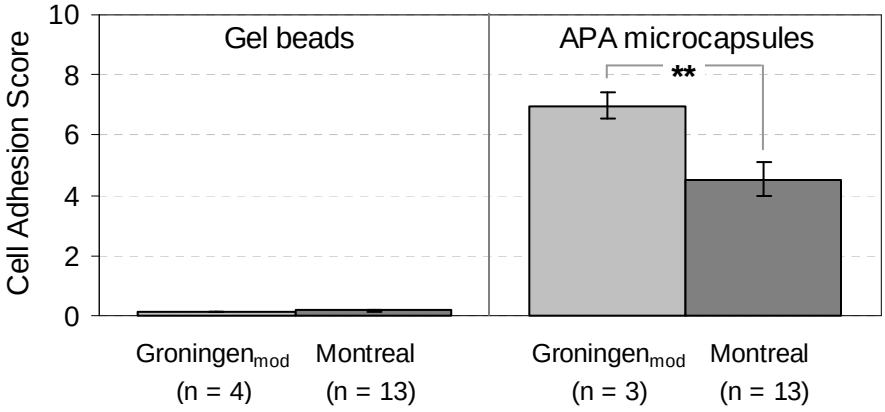

Figure 4.4 Effect of fabrication protocol (Groningen$_{mod}$ vs Montreal) on the *in vivo* biocompatibility of gel beads and APA microcapsules. Samples were made using the IntG$_{Mtl}$ alginate. Bars represent the mean cell adhesion score ± SEM. ** $p < 0.01$.

The Groningen$_{mod}$ and Montréal fabrication protocols were compared using the same alginate (IntG$_{Mtl}$). Both protocols for gel bead fabrication resulted in perfectly biocompatible beads (**Figure 4.4**). The different protocols for APA microcapsule fabrication, however, gave significantly different results ($p < 0.01$). That is, the Montréal protocol for fabrication resulted in APA microcapsules that were more biocompatible.

4.5 Discussion

Despite originating from different lots, each the $IntG_{Gron}$ and the $IntG_{Mtl}$ alginates were purchased from the same company and under the same brand name (Keltone® LVCR). Therefore, they were expected to have similar characteristics before being processed in the lab. Once purchased, however, the alginates were subjected to two different protocols for purification. While the final purity of the alginates could not be directly compared (we did not analyse the Groningen alginate for contaminants), both types of alginate proved to be biocompatible, with no significant difference in their performance *in vivo*. This result demonstrates that both purification protocols are suitable for treating alginates to be used in *in vivo* studies.

That said, caution must be made when generalizing the effectiveness of the purification protocols applied. We have previously shown that, under certain conditions, the Groningen protocol for purification was not effective for removing all contaminants [10] and resulted in non-biocompatible alginates [14]. It is likely that, in these earlier studies, the Groningen protocol for purification was rendered ineffective because, first of all, it was adapted to the Montreal lab, and second of all, it was applied to purify an alginate having a higher guluronate content (Protanal® LF10/60). In contrast, the "Groningen alginate" used in this study was actually purified in the Groningen lab and it was applied to an alginate having an intermediate guluronate content.

Once the PLL membrane was added to the alginate beads to form complete APA microcapsules, the type of alginate ($IntG_{Gron}$ vs $IntG_{Mtl}$) had absolutely no effect on biocompatibility. This could be expected, as we have shown that the main factor governing the immune response to APA microcapsules is the interaction between the polycation and alginate gel (**CHAPTER 6**). Since the alginates had similar characteristics to begin with, and the same PLL as well as the same fabrication protocol were employed, the microcapsules also had similar biocompatibilities.

Gel beads of $IntG_{Mtl}$ alginate were consistently biocompatible, regardless of fabrication protocol applied to make the gel beads. Therefore, both the $Groningen_{mod}$ and the Montréal protocols for bead fabrication are appropriate for implantation studies. More importantly, this confirms that the Montréal alginate is adequately biocompatible.

When we used a modified version of the Groningen protocol ($Groningen_{mod}$) for APA microcapsule fabrication, the resulting samples were significantly more immunogenic than Montréal-style APA microcapsules ($p < 0.01$). This was despite the fact that we used the same alginate ($IntG_{Mtl}$) in both cases. This result was unexpected since de Vos *et al* have proven that their protocol leads to very biocompatible APA microcapsules [1, 2]. It can be presumed that the modifications that we made to the Groningen protocol when we adapted it to the Montreal lab were responsible for inducing an immunogenicity of the microcapsules.

The most important discrepancy between the original version and our modified version of the Groningen protocol for APA microcapsule fabrication is the method for droplet generation. The Groningen group uses a co-axial airjet system that results in droplets with a diameter of approximately 600 μm. In contrast, the Montréal lab is equipped with a pulsed electrostatic system that generates droplets that are closer to 300 μm in diameter. In collaboration with Stöver *et al* of M^cMaster University, we are currently collecting data that demonstrates that PLL penetrates deeper into smaller alginate gel beads compared to larger gel beads. This is exactly the effect that we observed when trying to apply the Groningen procedure for PLL coating to our smaller gel beads (**Figure 4.2**). In order to reduce the membrane thickness, we were obliged to use a 0.05% w/v solution of PLL in place of a 0.1% w/v PLL solution, as the original Groningen protocol requires. The collapse of smaller capsules with the addition of PLL was also seen by Strand *et al* [15], although they managed to counter this effect by rinsing the beads with 0.3 M mannitol solution instead of saline before the exposure to PLL. Since we also provided evidence that the ability of the PLL to diffuse into and bind to the alginate gel bead is a crucial factor in determining APA microcapsule biocompatibility (**CHAPTER 6**), this deviation from the original Groningen protocol for microcapsule fabrication could very well have induced the immunogenicity that we observed in this study.

Another possible explanation for our inability to reproduce the Groningen results for APA microcapsule biocompatibility lies in the choice of animal model. In Groningen, AO rats were used as implant recipients, whereas in this study we used C57BL/6J mice. Studies have shown that the choice of animal model can significantly influence the severity of immune response to alginate-based microcapsules [8, 16], and C57BL/6J mice may be particularly sensitive to this effect [17]. Since we did not test the microcapsules that we fabricated in AO

rats, we cannot exclude the possibility that the C57BL/6J mice are simply more sensitive as a species. Although, it should be pointed out that De Vos *et al* have tried implanting their APA microcapsules in pre-diabetic BB rats (which are reportedly highly responsive to these capsule types [8]) and still saw less than 10% overgrowth at 1 month [1].

This study serves as a good example of how difficult it is to reproduce *in vivo* results for APA microcapsules between labs. We failed to reproduce the results achieved by the Groningen group despite having had firsthand experience in fabricating their microcapsules in their lab. One can imagine that reproducing results is even more difficult when relying solely on the published protocol for microcapsule fabrication. This situation is one of the greatest obstacles to the standardization of alginate microcapsules for implantation and to the understanding of their biocompatibility.

Overall, since both the $IntG_{Gron}$ and the $IntG_{Mtl}$ alginates performed similarly, and the Montréal protocol for APA microcapsule fabrication resulted in a better biocompatibility than the $Groningen_{mod}$ protocol, we feel that it is appropriate to use our own materials and protocols for alginate purification and microcapsule fabrication for subsequent studies.

4.6 Conclusions

The alginate purchased and purified by the Montréal group had similar characteristics to the alginate provided by the Groningen group. Moreover, our alginate was proven to be just as biocompatible, if not a slight more, than the Groningen alginate. Using our alginate, we showed that both the Groningen and Montréal protocols for fabrication were adequate for producing biocompatible gel beads. However, when we attempted to follow the Groningen protocol for APA microcapsule fabrication, the resulting microcapsules were even more immunogenic than APA microcapsules that were produced using our usual protocol for fabrication. Since the Groningen protocol was expected to result in biocompatible APA microcapsules, it was assumed that the parameters that were necessarily modified when adapting the protocol to the Montréal lab were responsible for inducing immunogenicity. While we did not succeed in producing biocompatible APA microcapsules by following the Groningen protocol (which is a good example of how difficult it is to reproduce implantation results between labs), we showed

that the Montréal protocols for alginate purification, bead fabrication, and microcapsule fabrication are adequate for subsequent implantation studies.

4.7 References

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CHAPTER 5

ARTICLE 2 : FACTORS INFLUENCING ALGINATE GEL BIOCOMPATIBILITY

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5.1 Abstract

Alginate remains the most popular polymer used for cell encapsulation, yet its biocompatibility is inconsistent. Two commercially available alginates were compared, one with 71% guluronate (HiG), and the other with 44% (IntG). Both alginates were purified, and their purities were verified. After two days in the peritoneal cavity of C57BL/6J mice, barium (Ba)-gel and calcium (Ca)-gel beads of IntG alginate were clean, while host cells were adhered to beads of HiG alginate. IntG gel beads, however, showed fragmentation *in vivo* while HiG gel beads stayed firm. The physicochemical properties of the sodium alginates and their gels were thoroughly characterized. The intrinsic viscosity of IntG alginate was 2.5-fold higher than that of HiG alginate, suggesting a greater molecular mass. X-ray photoelectron spectroscopy indicated that both alginates were similar in elemental composition, including low levels of counterions in all gels. The wettabilities of the alginates and gels were also identical, as measured by contact angles of water on dry films. Ba-gel beads of HiG alginate resisted swelling and degradation when immersed in water, much more than the other gel beads. These

results suggest that the main factors contributing to the biocompatibility of gels of purified alginate are the mannuronate/guluronate content and/or intrinsic viscosity.

5.2 Keywords

alginate; hydrogel; biocompatibility; microcapsules; cell encapsulation

5.3 Introduction

Alginate remains the most popular polymer used for cell encapsulation [1-3]. This natural polysaccharide is a preferred choice for this application because of its low toxicity and its ability to gel in conditions that are compatible with living cells [4]. Molecularly speaking, alginate is an unbranched copolymer with blocks of (1-4)-linked β-D-mannuronate (M) and α-G-guluronate (G) residues. A schematic of a -GGMM- segment of alginate is illustrated in **Figure 5.1**. For alginates derived from algae, the distribution of the M and G residues along the alginate chains, as well as their molecular mass, generally varies with the algal source and processing treatment of the biopolymer [5].

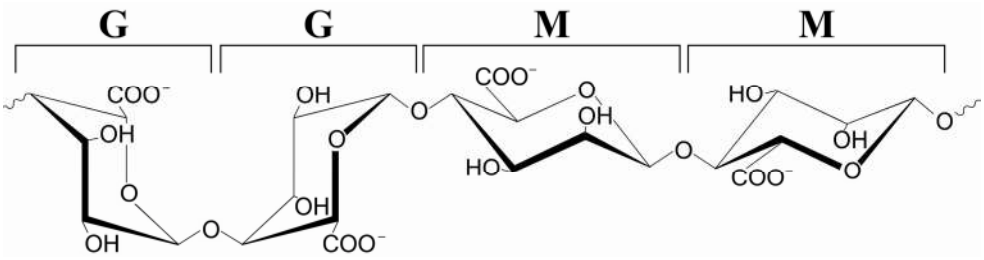


Figure 5.1 Molecular structure of alginate. **M** = mannuronate residue ; **G** = guluronate residue.

Because of this variability, the biocompatibility of alginates, particularly commercially available alginates, is not guaranteed [6]. First and foremost, before an alginate can be used for any medical applications, it must be properly purified in order to minimize the risk of an immune response stimulated by contaminants, such as proteins or endotoxins, that are often contained in crude alginates. The importance of alginate purity for bioperformance has been clearly emphasized in the literature [7-11]. Beyond purity level, published studies have suggested an effect of alginate's molecular weight [12, 13], solution viscosity [14, 15], or mannuronic (M) and guluronic (G) acid content [16-19] on the biocompatibility of alginate-based microcapsules or microbeads for cell therapy. However, the conclusions of such studies

are not always in agreement. Moreover, very few published works have focussed on the biocompatibility of properly purified alginates in the absence of a polycation, despite that uncoated alginate microbeads are becoming increasingly popular in the cell encapsulation field [18, 20, 21]. Indeed, there is still a need for a better understanding of the factors influencing alginate biocompatibility.

In this study, we compared two commercially available sodium alginates, one having a high guluronate (G) content, and the other having an intermediate G content. The alginates were purified in-house, and purity levels were verified in order to exclude the effect of contaminants on the results. For the analyses, the alginates were either in the form of an aqueous solution or crosslinked with Ba^{2+} or Ca^{2+} ions to form a gel. The samples in this study did not incorporate any polycations that could potentially induce an immune response or otherwise interfere with the interpretation of results. Both Ba^{2+}- and Ca^{2+}-crosslinked alginates were evaluated because barium alginate gel beads (without a polycationic membrane) have become a feasible option for immunoisolated transplantation [18, 20] and calcium alginate gels are most frequently employed to form the inner core of immunoprotecting microcapsules (having a polycationic membrane) [1]. This study aims to correlate an observed variation in biocompatibility with the physical and chemical details of the alginates and their gels, as this type of information would directly contribute to the rational selection and standardization of alginates for implantation purposes [22]. As mentioned above, others have observed (often contradictory) effects of each the M/G distribution and the solution viscosity on the biocompatibility of alginates [14-19], therefore these two properties were also evaluated in this study. In addition, the elemental compositions and molecular details of the alginates and their gels were assessed so as to monitor the possible role of counterion-alginate interactions on gel biocompatibility. Finally, the wettabilities of the alginates and their gels, as well as the swelling/degradation behaviour of the gel beads in water, were evaluated since alginate biocompatibility is often attributed to its properties as a hydrophilic and water-absorbing hydrogel [2] and because bead stability is an important feature for long-term bioperformance.

5.4 Materials and Methods

5.4.1 Materials

Sodium alginate Protanal® LF10/60 (Batch no. S13636) was provided by FMC BioPolymer (Drammen, Norway). Keltone® LVCR (CAS no. 9005-38-3, Lot no. 721322) was provided by ISP Corporation (NJ, USA). These alginates are denoted "HiG" and "IntG", respectively. Sterile water for irrigation, USP, was purchased from Baxter (Toronto, ON, Canada). All solutions were sterilized by filtration (0.22 μm) before their use. Reusable labware was specially cleaned and then sterilized by either Pasteur oven (200°C for 2h), autoclave or ethylene oxide gas before each use ; disposable plastic materials were considered as sterile and pyrogen-free.

5.4.2 Alginate purification

Sodium alginates were purified as previously described [9, 23]. Briefly, sodium alginate powder was extracted three times with chloroform then dissolved in sterile water (1.5% w/v for HiG ; 1.8% w/v for IntG). The solution was charcoal treated, filtered (0.22 μm), and then converted into gel beads using 50 mM $BaCl_2$. The gel beads were extracted over several days using 1M acetic acid, 500 mM sodium citrate, 50% ethanol, and then 70% ethanol. The beads were rinsed (water, 20 mM $BaCl_2$) and then dissolved in 250 mM EDTA. The resulting alginate solution was filtered (0.22 μm) and then dialysed against 150 mM saline and sterile water, successively (50,000 MWCO). Finally, the alginate was precipitated from the solution using 100% ethanol, freeze-dried, and stored at 4°C until its use.

Alginate purity was evaluated in terms of endotoxin and protein content, as well as the presence of foreign elements. For these tests, alginates were first dissolved in sterile water then filtered 0.2 μm. A commercially available Limulus amebocyte lysate (LAL) assay (E-toxate™ Kit, Sigma-Aldrich #ETO300, St Louis, MO, USA) was used to estimate the endotoxin content of alginates dissolved 1% w/v and diluted sequentially from 1:2 to 1:8000. The sensitivity limit of the LAL assay ranged between 0.03 - 0.125 EU/mL. E.coli 0.55:B5 lipopolysaccharide (LPS) included in the kit was used as a standard. A commercially available bicinchoninic acid (BCA) protein assay (Pierce Micro BCA™ Protein Assay Kit, Thermo Fisher Scientific, Rockford, IL,

USA) was used to quantify proteins in 1% w/v alginate solutions. The solutions were diluted 1:10 in sterile water in order to meet the kit's linear range of protein concentrations (0.5 - 20 μg/mL). Bovine serum albumin (BSA) included in the kit was used for the standard curve. Each measurement represents the mean of duplicate readings. Dry films of alginate solutions (2% w/v) were analyzed by x-ray photoelectron spectroscopy (XPS) in order to detect foreign elements. Details for the XPS technique are described elsewhere in *Materials and Methods*. For both HiG and IntG alginates, at least two purification batches were tested using each assay (n = 2 to 4).

5.4.3 Alginate gelation

Alginates were dissolved at a concentration of 2.0% w/v (HiG) or 1.63% w/v (IntG) in 154 mM saline (12.5 mM HEPES, pH 7.4) and then filtered 0.22 μm. These concentrations were selected to target a suitable viscosity (220 ± 20 cps) for droplet production. For gel beads, the alginate solution was first converted into tiny droplets using a pulsed electrostatic droplet generator equipped with a 25G blunt tip needle. For gel films, 200 μL of alginate solution was first spread uniformly over a glass microscope slide pre-coated with poly-L-lysine. Gelation consisted of immersing the alginate solution (droplets or films) for 30 minutes in a cross-linking solution, followed by rinsing the gel three times with Ringer's solution (154 mM NaCl, 5.6 mM KCl, 1.7 mM $CaCl_2$, 5 mM HEPES, pH 7.4). Either 10 mM $BaCl_2$ (16 mM HEPES, pH 7.4) or 100 mM Ca lactate was used as the cross-linking solution.

5.4.4 In vivo biocompatibility studies

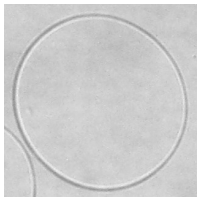
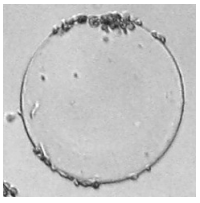
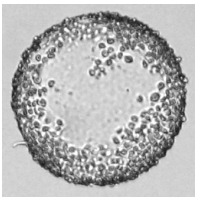
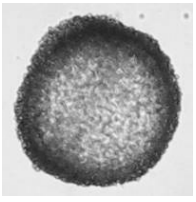
Each sample consisted of 500 gel beads suspended in ~ 0.5 mL of Ringer's solution. Samples were injected via a 22G catheter into the peritoneal cavity of male C57BL/6J mice aged 8 to 12 weeks (Jackson Laboratory, Bar Harbor, Maine, USA). Two days later, the beads were explanted. To do this, the mice were euthanized by cervical dislocation and a small ventral incision was made. The peritoneal cavity was flushed several times using ~ 30 mL of Ringer's solution. After flushing, the cavity was opened and inspected for beads adhered to the organs or peritoneal wall. The explanted beads were separated from free-floating cells by rinsing with ~ 20 mL of Ringer's solution over a 41 μm nylon mesh. The explanted samples were examined

under optical microscope. Each bead retrieved was classified into one of four categories, according to the extent of host cell adhesion to its surface, as described in **Table 5.1**.

For each sample, the cell adhesion score was calculated according to **Eq. 5.1** where *A*, *B*, *C*, and *D* are the number of beads retrieved that fall into categories A, B, C, or D, respectively, and *tot* is the total number of beads that were retrieved for that sample. The retrieval rate for a given sample was calculated according to **Eq. 5.2**.

Protocols for these *in vivo* studies were approved by the animal care ethics committee of the Maisonneuve-Rosemont Hospital Research Centre and have been stated to conform to the ethical guidelines of the Canadian Council for Animal Care. These guidelines were respected throughout this study.

Table 5.1 Classification of alginate gel beads retrieved after two days in the peritoneal cavity of C57BL/6J mice, according to the extent of host cell adhesion to the bead surface. This classification system is used to calculate the cell adhesion score (**Eq. 5.1**) and the retrieval rate (**Eq. 5.2**) of the explanted beads.

Category	**A**	**B**	**C**	**D**
Portion of bead surface covered by host cells	none 	< 50% 	> 50% 	100% 
Score	**0**	**3.3**	**6.6**	**10**

Eq. 5.1 $$cell\quad adhesion\quad score = \left(0 \times \frac{A}{tot}\right) + \left(3.3 \times \frac{B}{tot}\right) + \left(6.6 \times \frac{C}{tot}\right) + \left(10 \times \frac{D}{tot}\right)$$

Eq. 5.2 $$retrieval\quad rate = \frac{tot}{500} \times 100\%$$

5.4.5 Proton Nuclear Magnetic Resonance (^{1}H NMR)

NMR was used to determine the composition and sequential structure of the alginates, closely following the protocol described in the ASTM International Standard F 2259-03 [24], based on the work by Grasdalen *et al* [25-27]. Briefly, alginate powder was dissolved 0.1% w/v

in sterile water and filtered 0.22 μm. The alginate was acid hydrolyzed by lowering the solution pH to 5.6 (using HCl) and putting the sample in a water bath at 100°C for 1 h. The solution pH was then adjusted to 3.8 and put back in the water bath at 100°C for 30 min. After bringing the solution pH back to 7-8 using NaOH, the sample was freeze-dried. Next, 11 ± 1 mg of each sample was dissolved in 99.9% D_2O and freeze-dried again. Less than 24h before NMR analysis, the alginates were dissolved in 1 mL D_2O and mixed with 29 μL of 0.3 M TTHA (pH* 5-5.5). For spectral acquisition, sample temperature was maintained at 80°C. Spectra were acquired using a Bruker Avance 500 MHz NMR spectrometer (Bruker Biospin). Spectra were acquired without pre-saturation and with a 2 s relaxation delay. For each spectrum, 64 scans were made over a range of 0 to 7 ppm. Spectra were processed using Spinworks software (www.umanitoba.ca). The assignments and intensities of the ^1H NMR signals were interpreted as described in the ASTM Standard [24].

5.4.6 Viscometry

Alginates were dissolved ≤ 2.0% w/v in 154 mM saline (12.5 mM HEPES, pH 7.4) then filtered 0.22 μm, followed by sequential dilutions with saline down to 0.1% w/v. The viscosities of alginate solutions, η, were measured using a Brookefield synchro-lectric rotational viscometer (LV Model, Brookefield Engineering Laboratories, Stoughton, MA, USA) equipped with a no. 18 spindle and connected to a heating bath / circulator (Model D8-G, Haake Mess-Technik GmbH u. Co., Germany). During viscosity measurements, the solution temperature was maintained at 25.0 ± 0.1°C. Viscometer dial readings were allowed to stabilize (generally > 5 min of rotation) before they were recorded. Readings were taken at rotational speeds ranging from 0.3 to 60 revolutions per minute (rpm). One viscosity measurement represents the average of four dial readings (two speeds, two readings per speed). Three measurements were taken for each sample (n = 3). At least two purification batches were tested for each alginate type.

The intrinsic viscosity [η] of each alginate type was estimated by plotting each the reduced viscosity, $\eta_{red} = \frac{(\eta/\eta_0 - 1)}{c}$, and the inherent viscosity, $\eta_{inh} = \frac{\ln(\eta/\eta_0)}{c}$, as a function of solution concentration, c, and then extrapolating these plots to zero concentration [28]. These plots were constructed using the measured viscosities of the alginate solutions, η, for a range of

concentrations, as well as the measured viscosity of the solvent (154 mM saline), η_0. Reduced viscosity η_{red} is generally related to intrinsic viscosity $[\eta]$ by a power series (**Eq. 5.3**) [29], where k_1, k_2, k_3 … are dimensionless constants. Truncating **Eq. 5.3** to the second term gives the Huggins equation (**Eq. 5.4**) [30] that is commonly used to describe viscosity data at low polymer concentrations. Similarly, the inherent viscosity η_{inh} is related to the intrinsic viscosity $[\eta]$ by the Kraemer equation (**Eq. 5.5**) [31]. **Eq. 5.4** and **Eq. 5.5** dictate that, for dilute polymer solutions, plots of η_{red} vs. c and η_{inh} vs. c are expected to be linear. Moreover, these plots should normally converge to almost the same value for $[\eta]$ when extrapolated to inifinite dilution ($c = 0$).

Eq. 5.3 $$\eta_{red} = [\eta] + k_1[\eta]^2 c + k_2[\eta]^3 c^2 + k_3[n]^4 c^3 + \ldots$$

Eq. 5.4 $$\eta_{red} = [\eta] + k_H[\eta]^2 c$$

Eq. 5.5 $$\eta_{inh} = [\eta] + k_K[\eta]^2 c$$

5.4.7 X-Ray Photoelectron Spectroscopy (XPS)

XPS was used to quantitatively determine the elemental composition of the alginates and their gel beads (with the exception of hydrogen, H, which is not detectable by XPS). Because XPS is carried out in an ultra-high vacuum environment, it was necessary to dehydrate the samples before their analysis. For the alginates, sodium alginate powder was dissolved 2.0 % w/v in sterile water then filtered 0.22 μm. The solutions were then subjected to ultrasonic stirring for 30 min (Branson 5210 Ultrasonic Cleaner, Branson Ultrasonic, Danbury, CT, USA) in order to homogenize the solution. The solution was gently pipetted onto a 1 cm × 1 cm square of silicon wafer. For the Ba- and Ca- gels, ~ 1 mL of gel beads was quickly rinsed with 10 mL of sterile water. Excess water was removed and the beads were transferred onto a square of silicon wafer. All samples were then allowed to dry ≥ 24h under a sterile flowhood and ≥ 24h in a vacuum before being analyzed by XPS.

XPS spectra were acquired and processed using an Escalab MKII Surface Analysis System equipped with an Avantage data system (Thermo VG Scientific, West Sussex, UK). An unmonochromated Mg Kα source anode operated at 216 W (18 mA, 12 kV) was used to generate x-rays. Survey spectra were recorded for a single scan of 0-1200 eV binding energy

range, at a pass energy of 100 eV. Charge shift corrections of the spectra were made by setting the C_{1s} peak to 285.0 eV. Peaks integrations were calculated with subtraction of a Shirley background. Each sample type was analyzed at least twice by XPS ($n \geq 2$), and at least two purification batches were analyzed for each alginate type.

5.4.8 Micro-Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

MicroATR-FTIR was used to qualitatively examine the molecular groups found in the alginates and their gel beads. FTIR spectra were acquired using a Varian 7000 FT-IR Research Spectrometer equipped with a UMA 600 microscope and germanium microATR crystal (Digilab®, Randolph, MA, USA). Because water produces a strong absorption band in the spectral region of interest, the alginates and gel beads were prepared and dehydrated onto squares of silicon wafer as described for XPS measurements. Using the microscope view as a guide, the microATR crystal was gently pressed against the alginate film or against a single gel bead for analysis. Sample spectra were recorded for a range of 4000 to 700 cm^{-1} at a resolution of 4 cm^{-1}, with 128 scans co-added per spectrum. Background spectra consisted of the bare crystal under the same experimental conditions. Each sample type was examined twice by microATR-FTIR ($n = 2$), and at least two purification batches were analyzed for each alginate type.

5.4.9 Contact angle technique

Sample films were cast on microscope slides pre-coated with poly-L-lysine (PLL). The PLL enhanced adhesion of the cast films to the slides yet had no impact on the contact angle measurements (results not published). To coat the slides, they were first cleaned for 10 min in 1 M HCl at 100°C then rinsed extensively with distilled water. The slides were then immersed for 10 min in a solution containing 20 mg PLL-HBr (Sigma-Aldrich, Product no. P7890, St-Louis, MO, USA), 296 mL of distilled water, and 4 mL of 1 M Tris, and then allowed to dry under a sterile flowhood. For the sodium alginate films, alginates were dissolved 2.0% w/v in sterile water and then filtered 0.22 μm. 200 μL of each solution was gently spread over a slide surface. For the gels, alginate was dissolved in 154 mM saline at 2.0% w/v (HiG) or 1.63% w/v (IntG).

The solution was filtered 0.22 μm and then 200 μL was spread over a glass slide. The alginate-covered slide was then placed into a large Petri dish and the appropriate solutions (30 mL at a time) were poured over the film in order to form a gel, as described in the section *Alginate gelation*. To remove excess salts that can interfere with the results, the gel films were rinsed once for 60 ± 15 sec using 30 mL of sterile water. All films were allowed to dry overnight under a sterile flowhood before contact angle analysis.

Contact angle measurements were made using a VCA Optima Contact Angle Surface Analysis System (AST Products, Inc., Billerica, MA, USA). One (1) μL of sterile water was dispensed from a mechanically controlled 100 μL syringe fitted with a 22G blunt tip needle. The sample was raised to touch the suspended water drop. Within 2 sec of contact with the water drop, a photo was taken. The contact angle of the droplet to the surface was measured using the provided software. Each measurement represents the average of 2 to 4 spots on the same film. At least three measurements were made for each sample type ($n \geq 3$), and at least two purification batches were tested per alginate type.

5.4.10 Swelling and degradation

For each sample, 30 ± 5 gel beads (suspended in Ringer's solution) were transferred to a 24-well polystyrene plate. Their diameters were measured using the gradient scale mounted in the eyepiece of an optical microscope. Using a pipette, as much of the Ringer's solution as possible was carefully removed from the well containing the gel beads and then 1 mL of sterile water was added. After 5-10 min within adding the water, the diameters were measured again. Beads that were not clearly visible using the microscope, and thus their diameters were not measureable, were considered to have been degraded. This water rinse and examination by microscope was repeated twice more. For each sample type, swelling / degradation tests were made for three different batches of beads ($n = 3$), and at least two purification batches were tested per alginate type.

5.4.11 Statistical analysis

In cases where $n \geq 3$, samples were compared using the unpaired Student *t*-test. Differences for which $p < 0.05$ were considered to be statistically significant.

5.5 Results

5.5.1 Alginate composition and monomer sequence

The composition and monomer sequence of each alginate type used in this study was measured using Proton Nuclear Magnetic Resonance (^{1}H NMR). The results for the purified alginates are shown in **Table 5.2**. The HiG alginate was composed of 71% guluronate (G) and 29% mannuronate (M), while the IntG alginate was composed of 44% G and 56% M. On average, blocks of consecutive guluronate monomers were both more frequent and longer in length for the HiG alginate than for the IntG alginate, as interpreted by the values for F_{GG}, F_{GGG}, and $N_{G>1}$.

Table 5.2 Composition and monomer sequence of purified sodium alginates, as determined by ^{1}H NMR. Values represent the mean ± SEM for at least two purification batches (n = 3). F_x = frequency of monad /diad /triad x ; $N_{G>1}$ = average number of consecutive G monomers in G-blocks.

	F_G	F_M	F_{GG}	F_{MM}	F_{GM} F_{MG}	F_{GGG}	F_{GGM} F_{MGG}	F_{MGM}	$N_{G>1}$
HiG	0.71 ± .01	0.29 ± .01	0.60 ± .00	0.18 ± .02	0.11 ± .01	0.56 ± .00	0.04 ± .01	0.07 ± .00	16.8 ± 1.7
IntG	0.44 ± .01	0.56 ± .01	0.27 ± .01	0.39 ± .01	0.17 ± .00	0.23 ± .00	0.05 ± .00	0.12 ± .00	7.1 ± 0.1

5.5.2 Alginate purity

The effectiveness of the alginate purification process is demonstrated by the results presented in **Table 5.3**. Before their purification, the commercial alginates contained high levels of endotoxins (> 12 000 EU/g) and a significant amount of proteins (~ 9 mg/g). XPS analyses were not performed on the non-purified alginates, although previously published results indicate that the HiG alginate contained traces of sulphur, phosphorus, and nitrogen [32]. After purification, endotoxin levels were reduced 50- to 4000-fold and protein levels were significantly lowered ($p < 0.05$). XPS analyses confirmed that the purified alginates contained no detectable traces of foreign elements (i.e. elements other than C, O, and Na) that should be of concern for immunogenicity. Chlorine, detected in amounts of < 1 at%, was presumably derived

from the NaCl used in the alginate purification process. For a single measurement, silicon (Si) was detected, indicating that the silicon wafer substrate was not completely covered by the alginate film for this particular sample. Statistically, the purified alginates did not differ significantly in purity level.

Table 5.3 Measured levels of endotoxin, proteins, and foreign elements in sodium alginates before and after their purification, as measured by LAL assay, microBCA assay, and XPS. Alginates were dissolved in sterile water (1% or 2% w/v). Values represent the mean ± SEM (n = 3 for proteins and for foreign elements ; n = 2 (before purification) or n = 4 (after purification) for endotoxins). EU = endotoxin units ; at% = relative atomic percentage.

		Proteins	Endotoxins	Foreign Elements
HiG	**Before purification**	8.6 ± 0.6 mg/g	37000 ± 13000 EU/g	n/a
	After purification	4.3 ± 0.4 mg/g	9 ± 3 EU/g	Cl: 0.38 ± 0.28 at%
IntG	**Before purification**	9.3 ± 0.7 mg/g	12250 ± 250 EU/g	n/a
	After purification	3.7 ± 0.4 mg/g	231 ± 127 EU/g	Cl: 0.68 ± 0.40 at% Si: 0.64 ± 0.64 at%

5.5.3 Alginate viscosity

Solutions of IntG alginate were noticeably more viscous than solutions of HiG alginate. Their reduced viscosities η_{red} and inherent viscosities η_{inh} were graphed as a function of solution concentration, c, for concentrations of ≤ 0.01 g/mL (**Figure 5.S1**). The plots of η_{inh} vs. c were fairly linear and could therefore be fitted using Kraemer's equation (**Eq. 5.5**). However, the plots for η_{red} vs. c were more accurately described by a 2nd order polynomial rather than by a linear fit. Therefore, **Eq. 5.3** truncated to the 3rd term was used instead of the Huggins equation (**Eq. 5.4**) to fit this data. The intercepts of these plots at c = 0 were interpreted as the intrinsic viscosities $[\eta]$ of the alginates. The values of the intercepts are listed in **Table 5.4**, along with the coefficients of determination (R^2 values) for the polynomial / linear regressions applied. For the same alginate, the values for $[\eta]$, as estimated by the plot intercepts at c = 0, were in close

agreement with each other. The average value for [η] was 213 mL/g for the HiG alginate and 523 mL/g for the IntG alginate.

Table 5.4 Intrinsic viscosities [η] of purified sodium alginates as calculated using extrapolations of the reduced viscosity (η_{red}) and the inherent viscosity (η_{inh}) to zero solute concentration (c = 0).

	$[\eta] = (\eta_{red})_{c=0}$		$[\eta] = (\eta_{inh})_{c=0}$		Mean [η]
	Polynomial (R^2)	**Intercept (c = 0)**	**Linear (R^2)**	**Intercept (c = 0)**	
HiG	> 0.99	203 mL/g	0.60	223 mL/g	213 mL/g
IntG	0.99	546 mL/g	0.90	500 mL/g	523 mL/g

5.5.4 Alginate gel biocompatibility

After two days in the peritoneal cavity of C57BL/6J mice, gel beads of IntG alginate were consistently almost free of host cell adhesion. This is reflected in a cell adhesion score of < 1.00 for all mice tested (**Figure 5.2(a)**). In contrast, gel beads of HiG alginate beads were consistently covered with host cells, with cell adhesion scores ranging from 3.20 to 7.70. This result was independent of the type of gelling cation (Ca^{2+} or Ba^{2+}) employed. The difference in biocompatibility between the IntG and the HiG alginate was statistically significant for both Ba-gels ($p < 0.001$) and Ca-gels ($p < 0.05$).

The retrieval rates of the gel beads were variable, ranging from 20 to 90% for individual samples (**Figure 5.3**). Factors that appeared to lower retrieval rates included the poor visibility of beads adhered to the recipient organs, the degradation of the beads *in vivo*, and on the rare occasion, leaky catheters or sticking to tubes. The mean retrieval rate for HiG Ba-gel beads (38%) was significantly lower ($p < 0.05$) than for the other three bead types (57% - 62%). In this case, sample loss was mainly due to the poor biocompatibility of the HiG beads, which generally leads to their adhesion to the peritoneal wall or organs. Furthermore, it was observed that explanted beads of IntG alginate tended to be damaged or fragmented (**Figure 5.2(c)**), while beads of HiG alginate were more often intact (**Figure 5.2(b)**). Despite the apparent fragility of the IntG samples, these biocompatible beads were, on average, recovered in higher proportions than the HiG gel beads.

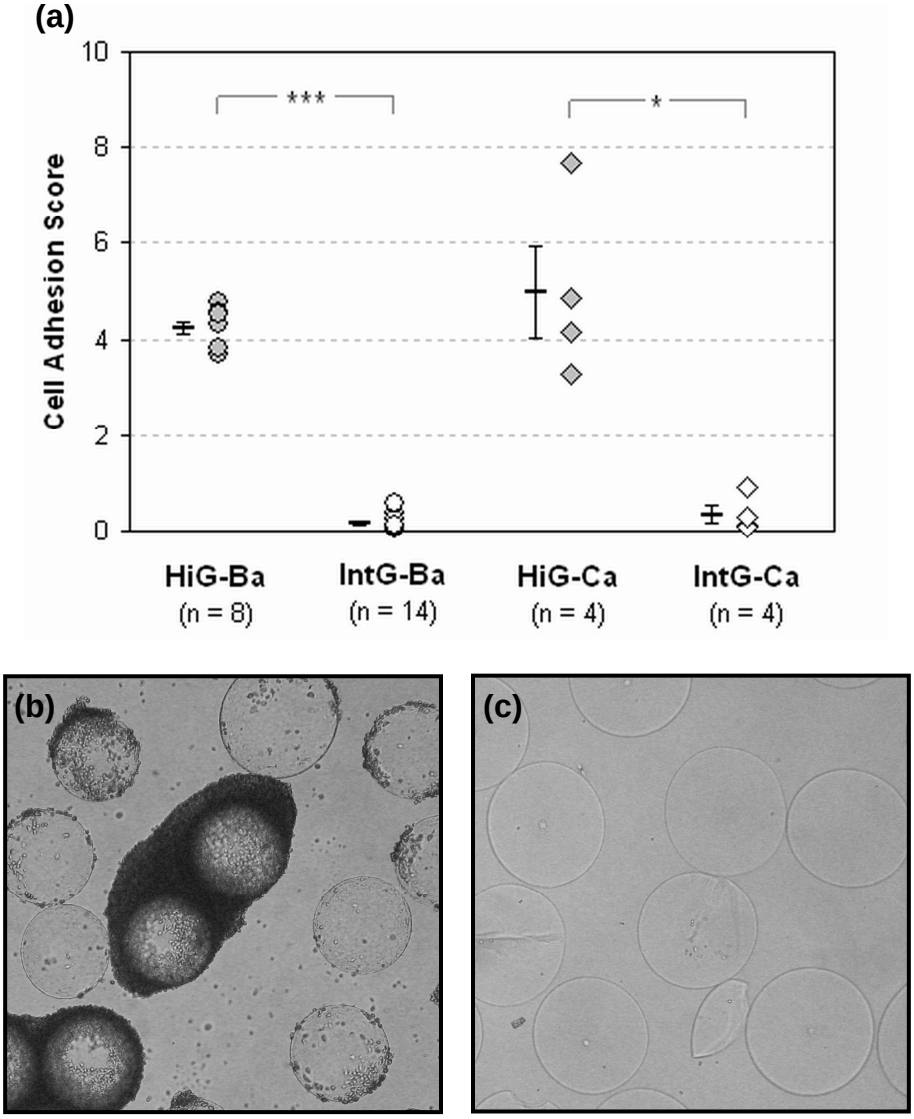


Figure 5.2 Extent of host cell adhesion to Ba- and Ca-gel beads of purified alginate retrieved after 2 days in the peritoneal cavity of C57BL/6J mice. **(a)** Cell adhesion is represented by a score, on a scale of 0 (no cell adhesion) to 10 (complete coverage by host cells), as described in **Table 5.1**. Each point represents an individual mouse. Bars represent the mean cell adhesion score ± SEM. *** $p < 0.001$, * $p < 0.05$. Typical samples of **(b)** HiG Ba-gel beads and **(c)** IntG Ba-gel beads upon explantation, as viewed through optical microscope.

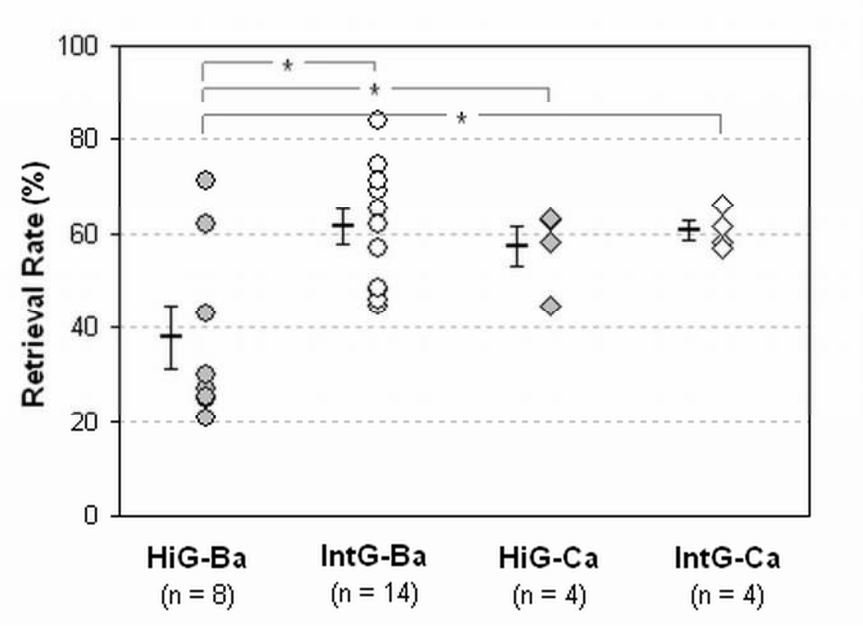


Figure 5.3 Proportion of Ba- and Ca-gel beads of purified alginate retrieved after 2 days in the peritoneal cavity of C57BL/6J mice. Retrieval rates represent the percentage of 500 implanted beads that were recovered intact upon explantation. Each point represents an individual mouse. Bars represent the mean retrieval rate ± SEM. * $p < 0.05$.

5.5.5 Elemental composition of alginates and gels

The survey scans acquired by XPS indicate that the elemental composition of the HiG and the IntG alginate were similar in many aspects (**Table 5.5**). The basic structure of the alginate, which is represented by the relative proportions of carbon and oxygen, was consistent for all the samples tested, as C/O ratios were maintained between 1.2 and 1.6.

The relative quantities of the counterions, namely sodium (Na), barium (Ba), and calcium (Ca) were also measured in each sample, thus providing an estimate of the replacement of Na^+ by Ba^{2+} and Ca^{2+} counterions upon gelation. In the sodium alginates, Na was detected in amounts of 2.0 ± 0.6 at% for the HiG alginate and 5.1 ± 1.4 at% for the IntG alginate. This difference was not statistically significant. As the alginates were converted to gel beads, Na levels increased in most cases, reaching amounts of 4.4 to 6.8 at%. This excess sodium was attributed to NaCl salt deposits on the dehydrated samples since chlorine (Cl) was detected on numerous occasions (results not shown), and crystallization could be observed when the films were examined through a microscope. On the surface of Ba-gel beads, barium was detected only

in minimal amounts (≤ 0.6 at%). These gels were also contaminated with traces of calcium (≤ 0.6 at%), which were assumed to originate from the Ringer's solution used to rinse the beads during sample preparation. As for the Ca-gels, calcium quantities were also quite low, with values ≤ 1.5 at%.

Table 5.5 Results from XPS survey scans of purified sodium alginate (Na-Alg), beads of Ba^{2+}-crosslinked alginate (Ba-gel), and beads of Ca^{2+}-crosslinked alginate (Ca-gel). Values represent the mean ± SEM (n = 3 for Na-Alg ; n = 2 for gels). at% = relative atomic percentage.

		Alginate structure			Counterions		
		C/O ratio	**C at%**	**O at%**	**Na at%**	**Ba at%**	**Ca at%**
Na-Alg	**HiG**	1.5 ± 0.1	59.1 ± 1.9	38.5 ± 1.5	2.0 ± 0.6	-	-
	IntG	1.4 ± 0.1	54.3 ± 1.4	37.9 ± 2.0	5.7 ± 1.6	-	-
Ba-gel	**HiG**	1.4 ± 0.1	48.1 ± 0.1	33.6 ± 1.6	6.7 ± 1.4	0.6 ± 0.0	0.3 ± 0.3
	IntG	1.6 ± 0.1	49.7 ± 2.2	31.2 ± 3.7	6.8 ± 1.0	0.3 ± 0.3	0.6 ± 0.0
Ca-gel	**HiG**	1.2 ± 0.2	43.6 ± 2.2	36.1 ± 3.7	6.4 ± 4.2	-	1.4 ± 0.0
	IntG	1.3 ± 0.1	48.6 ± 3.6	37.1 ± 0.5	4.4 ± 3.1	-	1.5 ± 0.1

5.5.6 Molecular composition of alginates and gels

FTIR spectra of the sodium alginates (**Figure 5.4**) reflected their differing M/G content, as interpreted by the placement and intensity of absorbance peaks that have been previously associated with varying G or M content in alginates [33, 34]. In this case, the higher G content of the HiG alginate was represented by more prominent peaks at 1320 cm^{-1}, 1125 cm^{-1}, 950 cm^{-1} and 940 cm^{-1}, as well as a peak at 905 cm^{-1} and a distinct shoulder at 1000 cm^{-1}. For the IntG alginate, the higher M content was associated with a more prominent peak at 1300 cm^{-1}, a shoulder at 1180 cm^{-1}, increased absorbance at 1150 cm^{-1}, slight shoulders at 1100 cm^{-1} and 1040 cm^{-1}, and a peak at 890 cm^{-1}. Also indicative of the differing M/G content [34], the peak associated with OH bending was at a slightly higher wavenumber in the spectrum for IntG alginate (1027 cm^{-1}) compared to the peak placement in the HiG alginate spectrum (1024 cm^{-1}). Moreover, the ratio of absorbances for the peaks at 1030 cm^{-1} and 1080 cm^{-1} was 1.6 ± 0.1 for the HiG sample and 1.7 ± 0.1 for the IntG sample.

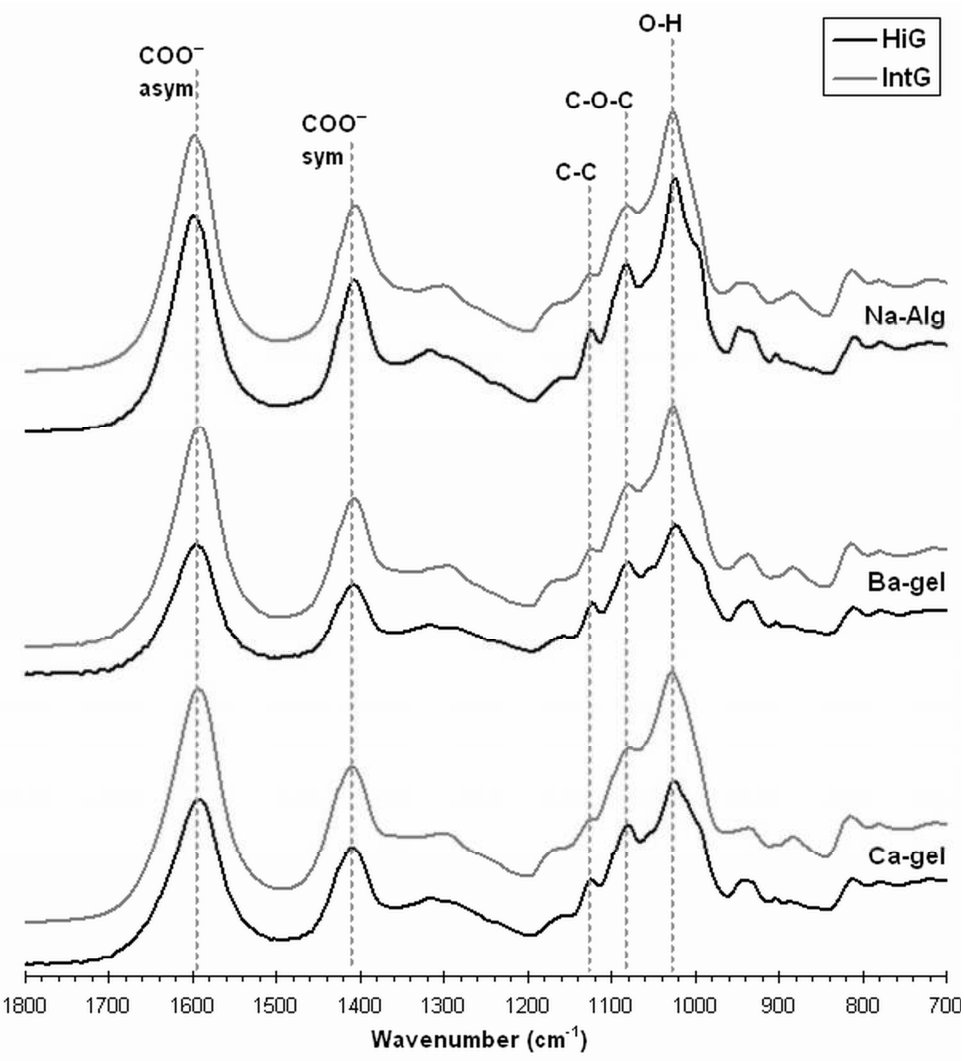


Figure 5.4 Fingerprint region of FTIR spectra for purified sodium alginate (Na-Alg), beads of Ba^{2+}-crosslinked alginate (Ba-gel), and beads Ca^{2+}-crosslinked alginate (Ca-gel). Spectra are representative of each sample type. Positions for labelled peaks are listed in **Table 5.6**.

When the alginates took the form of Ba- and Ca-gels, a number of slight changes in the IR absorbance spectra confirmed the exchange of counterions [33, 35]. The most obvious was a shift of the COO^- asymmetric stretch peak (~ 1600 cm^{-1}) to values 5-6 cm^{-1} lower (**Table 5.6**). Also observed were a slight shift of the COO^- symmetric stretch peak (~ 1410 cm^{-1}) to higher wavenumbers, C-C stretch peak (~ 1125 cm^{-1}) and C-O-C stretch peak (~ 1080 cm^{-1}) to lower wavenumbers, and more pronounced shoulders of the O-H bend peak at ~ 1025 cm^{-1}. The ratio of absorbances of the peaks at 1080 cm^{-1} and 1125 cm^{-1} decreased from 1.7 ± 0.1 (Na-Alg) to

1.5 ± 0.1 (Ba-gel) and 1.6 ± 0.0 (Ca-gel) for the HiG alginate. Similarly, this ratio decreased from 1.8 ± 0.1 (Na-Alg) to 1.7 ± 0.0 (Ca-gel) for the IntG alginate (the ratio remained 1.8 ± 0.0 for the Ba-gel). This is also a sign of the Na^+ ions being exchanged for Ba^{2+} or Ca^{2+} ions [33]. Overall, peak shifts associated with ion exchange during the conversion from sodium alginate to gel were similar in magnitude between the HiG and the IntG samples (**Table 5.6**).

Table 5.6 Characteristic peaks in the FTIR spectra of purified sodium alginate (Na-Alg), beads of Ba^{2+}-crosslinked alginate (Ba-gel), and beads Ca^{2+}-crosslinked alginate (Ca-gel). Values represent the mean wavenumber ± SEM (n=2).

Peak assignment	**Peak wavenumber (cm^{-1})**					
	HiG			**IntG**		
	Na-Alg	**Ba-gel**	**Ca-gel**	**Na-Alg**	**Ba-gel**	**Ca-gel**
COO^- str (asym)	1599 ± 1	1594 ± 1	1593 ± 0	1598 ± 1	1592 ± 1	1593 ± 0
COO^- str (sym)	1408 ± 0	1407 ± 1	1409 ± 1	1407 ± 1	1409 ± 1	1410 ± 0
C-C str	1123 ± 0	1120 ± 4	1121 ± 2	1124 ± 0	1123 ± 0	1123 ± 0
C-O-C str (ring)	1083 ± 1	1080 ± 0	1079 ± 1	1083 ± 1	1080 ± 0	1078 ± 0
O-H bend	1024 ± 0	1023 ± 1	1024 ± 0	1027 ± 1	1027 ± 1	1028 ± 0

5.5.7 Alginate and gel wettability

The contact angle values of 1.0 µL water droplets on sample films are graphed in **Figure 5.5**. Films made from sodium alginate solutions (2.0% w/v in water) produced contact angles of 44.6 ± 2.8° for HiG alginate and 45.7 ± 2.5° for IntG alginate. When the same alginates were crosslinked with Ba^{2+} ions (Ba-gel) or Ca^{2+} ions (Ca-gel), the films became significantly more hydrophilic; the water droplets spread quickly and their contact angles were lower, ranging from 10.0° to 14.2°. The contact angles were statistically equal between the two alginate types, regardless of whether the films were based on an aqueous solution or a gel.

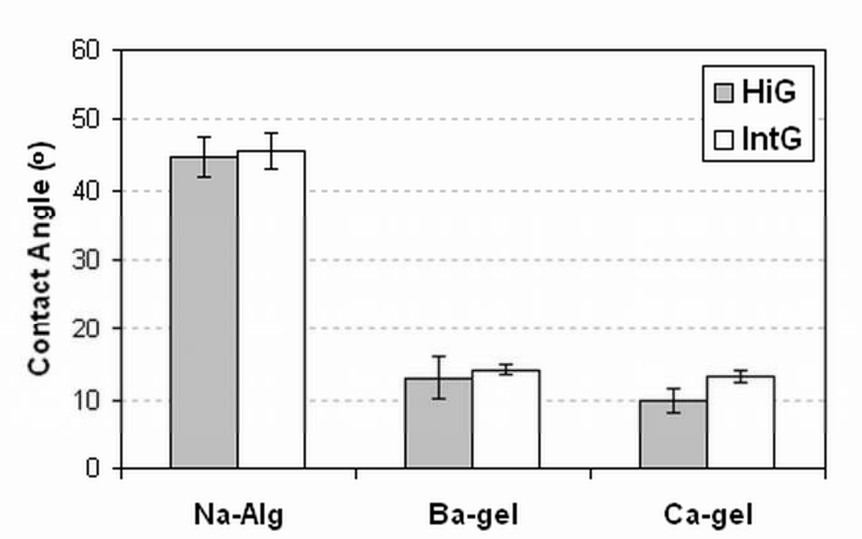


Figure 5.5 Wettability of dry films of purified sodium alginate (Na-Alg), Ba-gel, and Ca-gel. Wettability is represented by the contact angle of a 1.0 μL water droplet on the film, with lower angles indicating a greater wettability. Bars represent the mean contact angle ± SEM (n = 3 for Na-Alg ; n = 5 for the gels).

5.5.8 Swelling and degradation of alginate gel beads

As shown in **Figure 5.6**, all of the gel beads tested behaved similarly as they were immersed in the first rinse of 1 mL of sterile water. That is, all samples swelled to an average of 183 – 197% of their original diameter. Moreover, less than 13% of the beads underwent degradation, as defined by an inability to see the beads or measure their diameters using light microscopy. As the beads were subjected to a second rinse of pure water, the HiG Ba-gel beads showed to be much more stable than the rest. This was the only sample type to not degrade any further. In contrast, the HiG Ca-gel beads as well as both types of IntG beads were > 80% degraded. Also at the second rinse, judging from what remained measureable, the IntG Ba-gel beads were on average much more swollen (351% of the original diameter) than the other gel types (232 - 249% of the original diameter). By the third rinse, the HiG Ba-gel beads were the only bead type that remained relatively intact with 22% degraded, vs. 91% of the HiG Ca-gel beads and 100% of the IntG beads degraded (**Figure 5.6 (b)**).

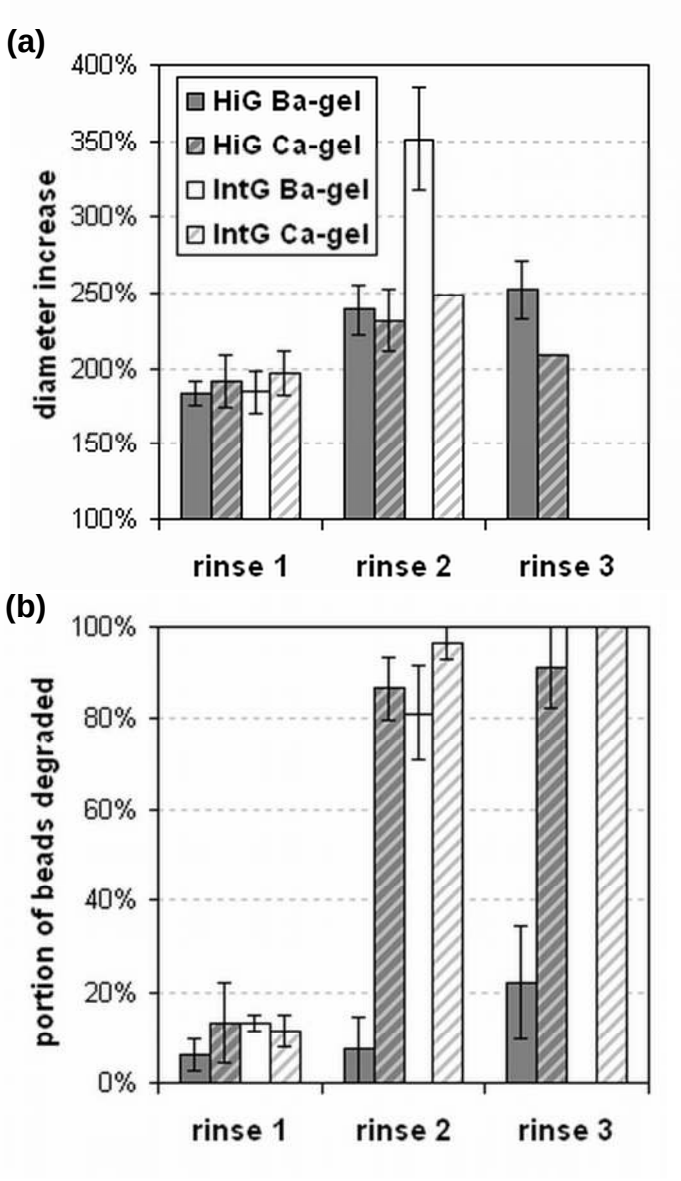


Figure 5.6 Swelling and degradation of gel beads of purified alginate with successive rinses in water. Each rinse consists of immersing 30 ± 5 beads in 1 mL of pure water for 5 – 10 min. **(a)** Swelling is represented by the percentage increase in bead diameter, as measured using optical microscopy. **(b)** Degradation is associated with an inability to see the bead or to measure its diameter using the microscope. Bars represent the mean percentage ± SEM (n = 3).

5.6 Discussion

The *in vivo* biocompatibility of alginate gels was significantly different for the two types of commercially available alginates studied, namely one alginate with a high guluronate content (HiG) and one with an intermediate guluronate content (IntG). Biocompatibility was evaluated in terms of host cell adhesion to alginate gel beads after two days in the peritoneal cavity of male C57BL/6J mice, as well as the proportion of beads that could be retrieved upon explantation. After the implantation period, gel beads of IntG alginate were consistently free of cell adhesion, while gel beads of HiG alginate were almost always covered with host cells (**Figure 5.2**). The use of IntG alginate also led to higher retrieval rates (**Figure 5.3**). This discrepancy between the biocompatibility of HiG and IntG gel beads was observed regardless of the crosslinking ion used (Ba^{2+} vs. Ca^{2+}).

Purity is essential for achieving the biocompatibility of alginates intended for cell encapsulation, as well as to be able to pinpoint the influence of alginate characteristics on biocompatibility without the interference of immunogenic contaminants. Once purified, the alginates used in this study were confirmed to have relatively low levels of proteins (< 5 mg/g) and endotoxins (< 250 EU/g), and no detectable traces of foreign elements. When the same purification method was applied earlier in our lab, protein levels were reduced to as little as 0.5 mg/g in the HiG alginate [9]. Between different batches of purified IntG alginate, we also noticed a tendency for the endotoxin content to vary (by several hundred EU/g). These fluctuations in purity demonstrate one of the potential drawbacks of purifying alginates in-house, as the final quality of the alginate can depend on the person applying the protocol or the specific purification batch. As an alternative option, a number of companies now offer a range of ultrapure alginates that may be suitable for overcoming such limitations. However, we chose to purify own alginates for this study because we have observed that (for HiG alginate) our in-house purified alginate can be more pure than at least one brand of industrially purified alginate [9]. Between the two alginates that were investigated in this study, the IntG alginate tended to have higher endotoxin levels (231 EU/g) than the HiG alginate (9 EU/g), while quantities of detected proteins and foreign elements were similar in both cases. Despite their higher endotoxin content, the purified IntG alginate did not induce a significant immune response, as indicated by the lack of immune cell adhesion to the gel beads *in vivo*. Thus, the possibility that

contamination was responsible for the immune response to the HiG alginate beads was excluded.

One of the most obvious differences between the alginates studied was the fraction and distribution of guluronate (G) and mannuronate (M) residues within the samples (**Table 5.2**). In this case, gel beads based on the alginate with less guluronate (IntG : 44%G), including fewer and shorter blocks of consecutive G residues, were clearly more biocompatible than gels of an alginate with a high G content (HiG : 71%G). Earlier studies have stirred somewhat of a debate about the effect of M/G content of alginates on their biocompatibilities : The M-blocks of soluble alginate have been held responsible for stimulating monocytes to produce cytokines [36, 37] and induce antibody responses [19]. When in the form of implantable microcapsules with a poly-L-lysine (PLL) membrane, samples based on alginates with a higher M content have shown to be more immunogenic by some [19, 38], yet less immunogenic by others [16, 17]. More recently, Duvivier-Kali *et al* compared the biocompatibility of gel beads (without PLL) made with alginates having similar M/G contents to the alginates in this study [18]. Despite their use of mouse species that differed from ours (NOD and BalB/c) as transplant recipients, their results were similar to our own. This included a tendency for less cell adhesion, yet more fragmentation and swelling, for the gel beads of 'High M' alginate (61%M) in comparison with gel beads of 'High G' alginate (70%G). The same group later published another study that included a comparison of the *in vivo* biocompatibility of barium alginate gel beads made from commercially available alginates that varied in M/G content [39]. This time, they observed that the alginate M/G content had only a modest effect on the proportion of explanted beads with adherent cells. Although this shift in perspective could have been explained by the fact that they switched to Lewis rats as transplant recipients, others have also seen that, as long as the alginate is purified, it can be biocompatible, regardless of the M/G content [8, 17, 40]. In this study, various other physico-chemical details of the alginates and their gels were examined in an attempt to either clarify the role of M/G content in influencing biocompatibility, or to offer alternative explanations for their different immunogenicities.

The elemental compositions of the alginates and gels were analyzed in order to verify whether different effects of the alginate structure on counterion exchange could explain their difference in biocompatibility. In particular, there is published evidence that unbound ions

(particularly Ba^{2+}) can be cytotoxic [41] and are capable of leaching out of alginate-based microcapsules [42] , therefore higher concentrations of counterions may hypothetically contribute to non-biocompatibility of the implant. Moreover, alginate with a higher G content is expected to bind more gelling ions because consecutive GG blocks provide the binding sites for crosslinking divalent cations in association with an 'egg-box' structure [4, 43]. However, our XPS results showed that the HiG alginate did not crosslink greater amounts of Ba^{2+} ions (nor Ca^{2+} ions) than the IntG alginate (**Table 5.5**), thus the hypothesis that the HiG alginate gel was more immunogenic because it contained higher concentrations of barium that could potentially leach out was excluded.

As a useful technique for monitoring intermolecular interactions, FTIR was also employed in this study in an attempt to explain the gel biocompatibility results in terms of the efficiency of alginate-counterion binding. Our results (**Figure 5.4**, **Table 5.6**) confirmed that there was indeed counterion exchange during gelation of the alginates, but also that molecular changes during this transformation were fairly minor, as peak alterations associated with ion exchange were quite subtle in this case, at least compared to observations made by others [33, 35]. Although the FTIR results also confirmed the difference in M/G content between the HiG alginate and the IntG alginate, there was no strong evidence that one alginate type interacted with the counterions more strongly than the other. These results supported those of the XPS analyses.

The biocompatibility of alginates and other hydrogels is often said to be attributed to the hydrophilic nature of these materials [2], which explains our interest in measuring the interaction of our samples with water. However, the HiG alginate and the IntG alginate samples showed similar wettabilities, as measured by the contact angle of water on dry films (**Figure 5.5**). This result indicates that both alginates had the same proportion of hydrophilic groups available for rehydration, regardless of the counterion (Na^{+}, Ca^{2+}, Ba^{2+}) involved. While this also confirms our previous observations that the contact angle is directly associated with the purity level of the alginates [32] (which was very similar for the two alginates studied here), this parameter did not correlate with the biocompatibility of the alginates. This situation highlights the limitation of using dry samples to interpret the relationship between alginate hydrophilicity and biocompatibility. Indeed, the alginate gels are fully hydrated when in contact with

biological tissues and fluids *in vivo*. In an attempt to better represent the *in vivo* situation, preliminary investigations of the wettability of hydrated alginate gels have been performed by measuring the contact angle of air bubbles on gels immersed in liquids. We observed that the air bubbles rolled off of all of the alginate gels that were studied (results unpublished), indicating complete hydrophilicity of the gels in their hydrated state regardless of the quality of the alginate, and furthermore, confirming that hydrophilicity is not a determining factor for alginate biocompatibility.

By extrapolating the values of reduced and inherent viscosities of dilute solutions to zero concentration ($c \rightarrow 0$), the intrinsic viscosity of a polymer can be estimated according to relations developed by Huggins [30, 44] and Kraemer [31]. In this study, the plots of η_{red} vs. c were better described by a 2nd order polynomial fit, corresponding to **Eq. 5.3** truncated to the 3rd term, rather than by a linear fit associated with Huggins' equation. The intrinsic viscosity was nevertheless estimated using the $c = 0$ intercept of this plot. This was justified by the fact that the intercepts of the two plots coincided nicely (**Table 5.4**) and that others have also used non-linear fits to estimate the intrinsic viscosity of alginate samples [45, 46]. Using this approach, it was determined that the HiG alginate had an intrinsic viscosity of 213 mL/g, a value that was less than half of that for the IntG alginate (523 mL/g). Assuming the applicability of the Mark-Houwink equation (**Eq. 5.6**) [47], the intrinsic viscosity [η] is directly associated with the viscosity average molecular mass of the sample, $\overline{M}_v$.

Eq. 5.6 $$[\eta] = K\overline{M}_v^{\,a}$$

In **Eq. 5.6**, K and a are constants for a particular polymer, solvent and temperature. For alginates dissolved in 0.1 M NaCl, the value for a is generally close to unity [22]. This implies that, based on the measured values for intrinsic viscosity, the average molecular mass of the IntG alginate is 2.5 times greater than that of the HiG alginate. Overall, this data suggests that a higher average molecular mass, or a higher intrinsic viscosity, could be favourable for the biocompatibility of alginate gels. We are not the first to observe this trend [12]. In our case, however, it is highly unlikely that oligosaccharides are responsible for the immunogenicity of HiG alginate, since the purification process, which included chemical extractions of Ba-gel beads and solution dialysis through a 50,000 Da MWCO membrane, should have rid the alginate

samples of low molecular weight fractions that are not properly bound during the gelation process. Indeed, we have observed that purifying our alginates has the effect of increasing their solution viscosity [32]. Otterlei *et al* observed that higher molecular weights of mannuronic acid favour cytokine production by monocytes [48], but this effect was observed for alginate solutions *in vitro*, and may not be comparable to our results using alginate gels *in vivo*. Zimmermann *et al* have stressed the importance of the use of a ultra-high viscosity (UHV) alginate for the improved biocompatibility of alginate gels, in association with the influence of vicosity on gelation properties, including swelling and surface roughness [15, 49, 50]. Although the alginates used in this study cannot be considered as UHV, our results support the notion that higher viscosity (or higher molecular mass) leads to better biocompatibility when the alginates are in the form of gel beads.

Because of the difference in molecular mass between the two alginates studied, we were obliged to use two different concentrations of alginates (2.0% w/v for HiG ; 1.63% w/v for IntG) in order to target the optimal solution viscosity for spherical bead production (220 ± 20 cps). The alginate concentration is considered to have an effect on the strength and rigidity of the gel [51], which is important to keep in mind because such physical properties of the gel may in turn influence the gel biocompatibility, as discussed in the next paragraph.

The stability of the alginate gels were tested *in vitro* by rinsing gel beads with pure water and observing to what extent they swelled and degraded. It was observed that gels of HiG alginate, particularly the Ba-gels, were remarkably more resistant to swelling and degradation than the IntG gels (**Figure 5.6**). Moreover, after two days in the peritoneal cavity of mice, the IntG alginate gel beads showed signs of damage and fragmentation (**Figure 5.2(c)**), while the HiG gel beads stayed firm and round (**Figure 5.2(b)**). Since it is mainly the longer, and stiffer, blocks of consecutive G residues that participate in the crosslinking of alginate chains [4, 52], these results were not unexpected. As confirmed by NMR (**Table 5.2**), the value for $N_{G>1}$ was 16.8 for the HiG alginate, but only 7.1 for the IntG alginate. Despite the observation that the IntG gel was less stable both in water and *in vivo*, retrieval rates were higher than for the HiG gel. This illustrates that fragmentation of the beads was less of a problem for retrieval rates than the immunogenicity of the HiG gel beads, which tended to be overgrown with cells and adhered to recipient organs and peritoneal wall. While the lower stability of the IntG gel may make this

alginate less suitable for immobilizing cells and tissues, its biocompatibility was not compromised. In fact, it is quite possible that the swelling properties of the alginate gels contributed to their difference in biocompatibility. The biocompatibility of hydrogels is generally attributed to their hydrophilicity, chain mobility at the surface, decreased interfacial tension with surrounding biological fluids and tissues, softness and pliability [53]. These characteristics of hydrogels are believed to minimize the adsorption of proteins and cells that lead to an immune response, as well as reduce frictional forces with surrounding tissues that can lead to fibrosis. For those gels that are more apt to swelling and taking up water, these favourable properties of hydrogels for biocompatibility are perhaps better enhanced. Indeed, it was observed that the IntG gels that swelled more *in vitro* and *in vivo* were also the more biocompatible. As for the HiG alginate gels, although the Ca-gel was more prone to swelling than the Ba-gel upon immersion in pure water, the Ca-gel remained just as firm as the Ba-gel when placed in an *in vivo* environment, explaining why an enhanced biocompatibility associated with swelling was not observed for the Ca-gels of HiG alginate.

An overview of the results of this study suggest that the main factors that determine alginate gel biocompatibility are the relative proportion of guluronate (G) and mannuronate (M) and the intrinsic viscosity (related to molecular mass) of the alginate. Without being able to isolate and change one variable at a time, however, no definite conclusions can be made about the relative importance of these parameters for gel biocompatibility. In most likelihood, it is a combined effect of the alginate's M/G content and molecular mass that ultimately governs its biocompatibility, via the influence of these characteristics on the physical properties of the crosslinked gel such as stability and swelling [52, 54]. Such a statement should nevertheless be verified experimentally in future studies. This would require the use of alginates with tightly controlled properties, such as epimerized alginates with tailored MG composition [55] or alginates that are extracted directly from freshly harvested stipes of algae rather than industrially processed [56].

5.7 Conclusion

Results of this study indicate that the biocompatibility of purified alginate gels is influenced by the proportion and distribution of mannuronate (M) and guluronate (G) residues within the alginate, as well as its intrinsic viscosity, although the relative importance of each

parameter remains to be determined. This can be explained by the effects of both monomer conformation and distribution and polymer chain length on the physical properties of the resulting cross-linked gels, including the swelling ability and stability of the hydrated gel. In contrast, neither the elemental composition of the alginates, including counterion amounts, nor the wettability of the samples, were a predictive factor for their biocompatibility. In this study, the alginate with intermediate G content (44 %G) and higher intrinsic viscosity (523 mL/g) resulted in gel beads that were much more biocompatible than those made from an alginate having 71% G and an intrinsic viscosity of 213 mL/g. The superior biocompatibility of the IntG gel beads was not affected by their susceptibility to swelling and fragmentation; in contrast, the tendency of these beads to swell up with water could be favourable to their biocompatibility. Overall, the use of an alginate of ~ 40% G and high molecular mass is recommended for the production of biocompatible gels, although caution should be made when using such gels for cell immobilization purposes due to its lack of stability *in vivo*.

5.8 Acknowledgements

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5.10 Supplementary data

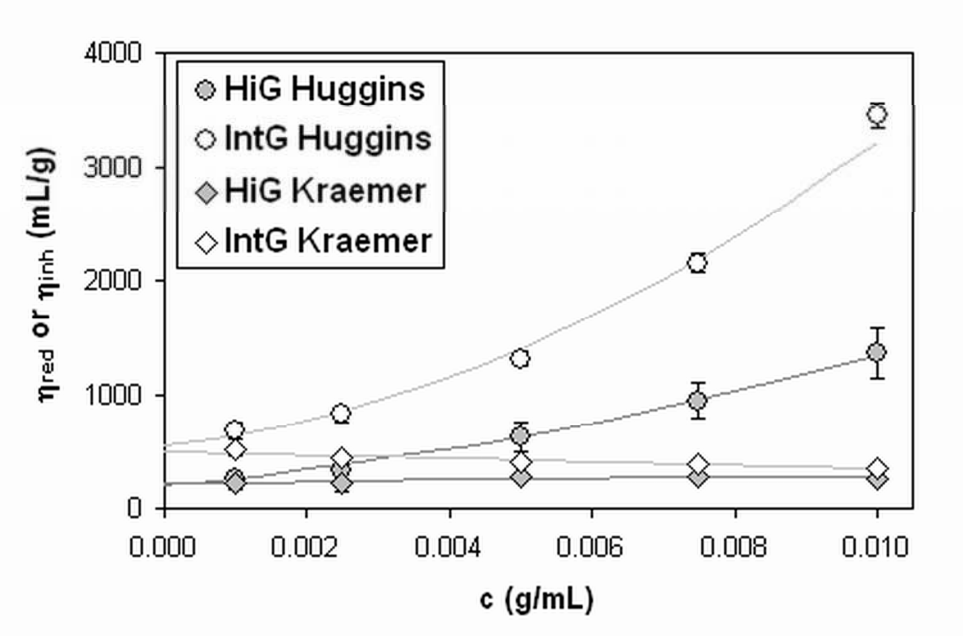

Figure 5.S1 Plots of reduced viscosity, η_{red} (circles), and inherent viscosity, η_{inh} (diamonds), against concentration, *c*, for sodium alginates dissolved ≤ 0.01 g/mL in 154 mM saline (12.5 mM HEPES, pH 7.4). Data points represent the mean value ± SEM for two different purification batches (n = 3).

CHAPTER 6

ARTICLE 3 : BIOCOMPATIBILITY AND PHYSICOCHEMICAL CHARACTERISTICS OF ALGINATE-POLYCATION MICROCAPSULES

IN PRESS : Acta Biomaterialia

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6.1 Abstract

There is a need to better understand the biocompatibility of alginate-polycation microcapsules based on their physicochemical characteristics. Microcapsules composed of alginate with 44% (IntG) or 71% (HiG) guluronate, gelled with calcium (Ca) or barium (Ba), and coated with poly-L-lysine (PLL) or poly-L-ornithine (PLO), followed by IntG alginate were compared. For microcapsules with an IntG(Ca) gel core, using PLO instead of PLL resulted in less immune cell adhesion after two days in C57BL/6J mice. The PLO microcapsules were also characterized by a greater hydrophilicity and superior resistance to swelling and damage under osmotic stress. For microcapsules with a PLL membrane, replacing the IntG(Ca) gel core with IntG(Ba) or HiG(Ca) gel resulted in stronger immune responses ($p < 0.05$). This was explained by poor penetration of PLL into the gel, as demonstrated by FTIR analyses and membrane rupturing during osmotic swelling. XPS analyses show that all microcapsules had the same amount of polycation at their surface. Moreover, alginate coatings had non-significant effects on the biocompatibility and physicochemical properties of the microcapsules. Thus, alginate-

polycation interactions for membrane formation are more important for biocompatibility than each the quantity of polycation at the surface and the alginate coating.

6.2 Keywords

alginate; microcapsule; biocompatibility; poly-L-lysine; poly-L-ornithine

6.3 Introduction

Alginate-polycation microcapsules are still the most commonly studied devices for the immunoprotection of transplanted therapeutic cells and tissues [1-4], yet their biocompatibility remains variable and insufficiently understood [5, 6]. In the case of inadequate biocompatibility, the release of cytokines from nearby activated macrophages, cellular overgrowth and the development of fibrotic tissue surrounding the implant contribute greatly to encapsulated graft failure [7-9]. While at least one group has demonstrated that it is possible to produce alginate-polycation microcapsules that induce a minimal immune response [10], first-hand experience and an overview of published works indicate that such promising results are difficult to reproduce despite the use of similar capsule structures [11, 12]. Indeed, the design and production of microcapsules intended for cell transplantation lacks official standardization, which is the result of in-house protocols and an incomplete understanding the physicochemical details of the microcapsule in relation to bioperformance [13]. This situation has the unfortunate consequence of hindering our ability to predict and reproduce the *in vivo* performance of the microcapsules, and ultimately delaying the progress of encapsulated cell transplantation towards regular clinical application.

Of the various types of the alginate-polycation microcapsules that have been studied over the past three decades, the alginate-poly-L-lysine-alginate (APA) microcapsule, based on the early work of Lim and Sun [14], represents the “classic” design. Other variations of the APA microcapsule have been investigated in more recent years, including the replacement of poly-L-lysine (PLL) with chitosan [15, 16], poly(methylene-co-guanidine) (PMCG) [17], poly-L-ornithine (PLO) [18, 19], or the use of multilayers [20, 21]. However, alginate-PLL-alginate and alginate-PLO-alginate microcapsules have dominated the few clinical trials of microencapsulated cell transplantation that have been attempted to date [22-24]. Using each of these microcapsule designs, the applicability of encapsulated cell transplantation has

successfully been demonstrated in humans, but long-term viability of the grafts has been proven difficult to maintain, thus highlighting the need to continue optimizing the microcapsule design.

As a strategy to optimizing the microcapsule design, we chose to investigate the relationships between the *in vivo* biocompatibility and the physicochemical characteristics of alginate-polycation microcapsules of different design. In addition to characterising the "classic" alginate-PLL-alginate microcapsule, the effect of replacing the PLL with the PLO was examined. PLO is an interesting candidate for biocompatibility studies not only because of its recent role in clinical trials of encapsulated cell transplantation, but also because it is so similarly structured to PLL. Furthermore, we investigated the consequence of altering the gel core properties as a strategy to increasing capsule stability, i.e. by substituting the crosslinking ion (Ba^{2+} for Ca^{2+}) or the type of alginate (high guluronate content for intermediate) before adding the capsule membrane. Samples without a polycation or an alginate coating were also analysed in order to study the roles of these fabrication steps on the microcapsule properties and bioperformance. The aim of this work was to elucidate correlations between the structure, physicochemical properties, and the *in vivo* biocompatibility of alginate-polycation microcapsules, with the intent of developing the necessary tools for optimizing the microcapsule design. The study was designed to provide information about polymer-to-polymer interactions within the capsule membrane and the exposure of functional groups on the capsule surface, as these factors are expected to directly influence the stability and immunogenicity of the microcapsule. Studies that focus on this kind of analytical approach have rarely been published in the case of alginate-polycation microcapsules for cell encapsulation, despite a clear need for this type of work in order to establish standards necessary for the advancement of cell encapsulation technology to clinical trials.

6.4 Materials and Methods

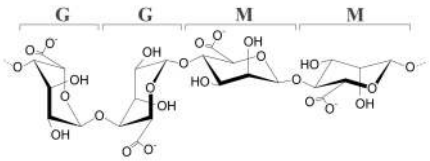
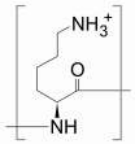
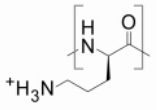
6.4.1 Materials

Sodium alginates. "IntG" alginate (Keltone® LVCR, CAS 9005-383-3, lot 721322) was provided by ISP Corporation (NJ, USA). "HiG" alginate (Protanal® LF10/60, batch S13636) was provided by FMC BioPolymer (Drammen, Norway). The alginates were purified in-house before their use, as previously described [25, 26]. After their purification, the alginates

contained < 5 mg/g of proteins (microBCA assay), < 250 EU/g of endotoxins (LAL assay), and no traces of foreign elements (XPS). The proportion and distribution of guluronate to mannuronate monomers and the intrinsic viscometries of each type of alginate (after their purification) are shown in **Table 6.1**.

Polycations. Poly-L-lysine (PLL) hydrobromide (product no. P7890; CAS 25988-63-0) and Poly-L-ornithine (PLO) hydrochloride (product no. P2533; CAS 26982-21-8) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The degree of polymerization and molecular weights of these polycations are presented in **Table 6.1**.

Table 6.1 Description of polymers used for microcapsule fabrication.

Alginate	**IntG**	**HiG**
Molecular structure [1]		
Composition [2]	F_G = 0.44 ; F_{GG} = 0.27 ; F_{MM} = 0.39 ; F_{GM} = 0.17 ; $N_{G>1}$ = 7.1	F_G = 0.71 ; F_{GG} = 0.60 ; F_{MM} = 0.18 ; F_{GM} = 0.11 ; $N_{G>1}$ = 16.8
Intrinsic viscosity [3]	523 mL/g	213 mL/g
Polycation	**PLL**	**PLO**
Molecular structure		
Degree of polymerization [4]	126 VIS	192 VIS ; 113 MALLS
Molecular weight [4]	26 300 VIS ; 15 000 MALLS	28 900 VIS ; 17 070 MALLS

[1] Example of a –GGMM– segment within an alginate molecule

[2] As determined by proton nuclear magnetic resonance

[3] As determined by rotational viscometry

[4] As specified by the manufacturer

G = guluronate residue ; M = mannuronate residue

VIS = viscometry ; MALLS = multi-angle laser light scattering

All solutions were sterilized by filtration (0.22 μm) before their use. Reusable labware was specially cleaned and sterilized by Pasteur oven (200°C for 2h), ethylene oxide (EO) or autoclave before their use. Disposable plastic materials were considered as sterile. Manipulations were performed under sterile conditions whenever possible.

6.4.2 Microcapsule fabrication

Alginates were dissolved at a concentration of either 1.63% w/v (IntG) or 2.0% w/v (HiG) in 154 mM saline (12.5 mM HEPES, pH 7.4) and filtered 0.22 μm. These concentrations were selected to target a suitable viscosity (220 ± 20 cps) for droplet production. The alginate solution was extruded through a 25G blunt tip needle using a mechanically driven syringe, while droplets were detached from the needle tip using a pulsed electrostatic droplet generator. The droplets were converted into gel beads by immersing them in a crosslinking solution for 30 min, either 10 mM $BaCl_2$ (16 mM HEPES, pH 7.4) or 100 mM Ca Lactate. If no membrane was added, the gel beads were rinsed three times and then stored in Ringer's solution (154 mM NaCl, 5.6 mM KCl, 1.7 mM $CaCl_2$, 5 mM HEPES, pH 7.4), noting that Ringer's solution induced less swelling of the gel beads than saline. To form microcapsules, polycationic membranes were added to the gel beads by immersing them for 5 min in a solution of either PLL or PLO (0.05% w/v in saline, filtered 0.22 μm). For certain microcapsules, alginate was also added to the membrane by rinsing once with saline then immersing for 5 min in alginate solution (diluted 1:10 in saline). Microcapsules were then rinsed three times and stored in saline. Throughout the manuscript, beads and microcapsules are described according to their components, using the notation "alginate(crosslinker)/polycation/alginate".

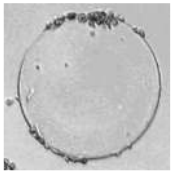
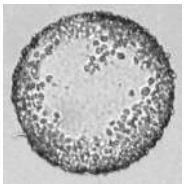
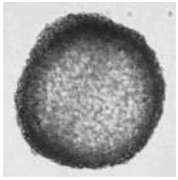
6.4.3 In vivo Biocompatibility

Samples of 500 gel beads or microcapsules were handpicked to exclude individual beads/capsules with defects. The samples were injected, via a 22G catheter, into the peritoneal cavity of male C57BL/6J mice (Jackson Laboratory, Maine, USA). After two days, the beads or capsules were explanted. To do so, the mice were euthanized by cervical dislocation and a small ventral incision was made. The peritoneal cavity was flushed using ~ 30 mL of either Ringer's solution (for beads) or saline (for capsules). After flushing, the cavity was opened and inspected for samples adhered to the organs or wall of the peritoneum. The explanted beads/capsules were

separated from free-floating cells by rinsing the samples over a 41 μm nylon mesh. The explanted samples were then inspected under optical microscope. Each bead or capsule retrieved was classified into one of four categories according to the extent of host cell adhesion to its surface, as described in **Table 6.2**. For each capsule type, at least two separate batches of capsules were tested in a minimum of three mice ($n \geq 3$).

For each explanted sample, a 'cell adhesion score' was calculated using a weighted scoring system described by **Eq. 6.1**. The parameters *A*, *B*, *C*, and *D* are the number of beads/capsules retrieved that fall into categories A, B, C, or D, respectively (**Table 6.2**), and *tot* is the total number of beads that were retrieved for that sample. On average, the value for *tot* was 50% ± 15%, and this value did not change significantly between different microcapsule designs.

Table 6.2 Classification of beads/capsules retrieved after two days in the peritoneal cavity of C57BL/6J mice, according to the extent of host cell adhesion to the bead surface. This classification system is used to calculate the cell adhesion score (**Eq. 6.1**).

Category	**A**	**B**	**C**	**D**
Portion of bead surface covered by host cells	none 	< 50% 	> 50% 	100% 
Score	**0**	**3.3**	**6.6**	**10**

Eq. 6.1 $$cell\ \ adhesion\ \ score = \left(0 \times \frac{A}{tot}\right) + \left(3.3 \times \frac{B}{tot}\right) + \left(6.6 \times \frac{C}{tot}\right) + \left(10 \times \frac{D}{tot}\right)$$

Protocols for *in vivo* studies were approved by the animal care ethics committee of the Maisonneuve-Rosemont Hospital Research Centre and have been stated to conform to the ethical guidelines of the Canadian Council for Animal Care. These guidelines were respected throughout this study.

6.4.4 X-Ray Photoelectron Spectroscopy (XPS)

XPS was used to quantitatively determine the elemental composition of the bead and capsule surfaces (with the exception of hydrogen, H, which is not detectable by XPS). Before their analysis, the samples (~ 1 mL of beads/capsules) were quickly rinsed with 10 mL of sterile water in order to remove excess salts that could interfere with the interpretation of the results. The water was removed and the samples were transferred onto a 1 cm × 1 cm square of silicon wafer. For the polycation controls, 1.0% w/v aqueous solutions of PLL or PLO were spread on the wafer squares to form films. All samples were allowed to dry ≥ 24h under a sterile flowhood followed by ≥ 24h in a vacuum before being analyzed by XPS.

XPS spectra were acquired and processed using an Escalab MKII Surface Analysis System equipped with an Avantage data system (Thermo VG Scientific, West Sussex, UK). An unmonochromated Mg Kα source anode operated at 216 W (18 mA, 12 kV) was used to generate x-rays. Survey spectra were recorded for a single scan of 0-1200 eV binding energy range, at a pass energy of 100 eV. Charge shift corrections of the spectra were made by setting the C1s peak to 285.0 eV. Peaks integrations were calculated with subtraction of a Shirley background. For each bead or capsule type, two separate batches were analyzed (n = 2).

6.4.5 Micro-Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

MicroATR-FTIR was used to estimate the relative proportions of alginate to polycation within the capsule membranes, as well as observe the molecular interactions between these polymers. FTIR spectra were acquired using a Varian 7000 FT-IR Research Spectrometer equipped with a UMA 600 microscope and germanium microATR crystal (Digilab®, Randolph, MA, USA). Samples and controls were prepared and dehydrated onto squares of silicon wafer as described in **section 6.4.4**. Using the microscope view as a guide, the microATR crystal was gently pressed against a single bead/capsule (or a film, for the controls). Sample spectra were recorded for a range of 4000 to 700 cm^{-1} at a resolution of 4 cm^{-1}, with 128 scans co-added per spectrum. Background spectra consisted of the bare crystal under the same experimental conditions. For each bead or capsule type, two separate batches were analyzed (n = 2).

6.4.6 Contact angle technique

Sample films were cast on microscope slides pre-coated with poly-L-lysine (PLL). The PLL enhanced film adhesion to the slides yet had no impact on contact angle values (results not published). To coat the slides, they were cleaned (10 min in 1M HCl at 100°C), rinsed with distilled water, immersed for 10 min in a PLL solution (20 mg PLL-HBr, 296 mL distilled water, 4 mL 1M Tris), then dried under a sterile flowhood. The films were constructed so as to mimic the capsule fabrication process described in **section 6.4.2**. Briefly, 200 μL of alginate solution (1.63% or 2.0% w/v in saline) was spread over a slide. The alginate-covered slide was then immersed in 20-30 mL of the necessary solutions to form a gel or polymer complex mimicking the bead or capsule composition. To remove excess salts that can interfere with the results, the films were quickly rinsed with sterile water before being allowed to dry overnight under a sterile flowhood. For the polycation controls, 1.0% w/v aqueous solutions of PLL or PLO were spread on the slides and allowed to dry overnight under a sterile flowhood.

Contact angle measurements were made using a VCA Optima Contact Angle Surface Analysis System (AST Products, Inc., Billerica, MA, USA). One (1) μL of sterile water was dispensed from a mechanically controlled 100 μL syringe fitted with a 22G blunt tip needle. The sample was raised to touch the suspended water droplet. Care was taken to place the droplet on a clear area of the film in order to minimize the interaction between the water and any residual salts on the samples. Within 2 sec of contact with the water droplet, a photo was taken. The contact angle of the droplet to the surface was measured using the provided software. Each measurement represents the average of 2 to 4 spots on the same film. Five measurements were made for each sample type ($n = 5$), and at least two batches were tested per sample type.

6.4.7 Explosion assay

This assay is a modified version of a published protocol [27]. Samples of 30 ± 5 beads or microcapsules (suspended in Ringer or saline) were transferred to a 24-well plate. Using a gradient mounted in the eyepiece of an optical microscope, their diameters were measured. On average, initial diameters were 339 ± 17 μm for the gel beads, 368 ± 40 μm for the alginate-polycation microcapsules (no alginate coating), and 387 ± 42 μm for the complete microcapsules (with alginate coating), regardless of the choice of alginate, crosslinking ion, or

polycation. Samples were rinsed by carefully aspirating the fluid then adding 1 mL of sterile water, causing the beads/capsules to swell. After 5 min, the diameters were re-measured and beads/capsules were classified as either intact, damaged (i.e. splitting or other membrane defects), or exploded. This procedure was repeated twice more for a total of three rinses. For each sample type, explosion assays were completed for three different batches of capsules or beads ($n = 3$).

6.4.8 Statistical analysis

Samples means were compared using the one-way ANOVA test. In the case of a significant difference between the means, multiple comparisons of the means were carried out using Tukey's HSD post hoc test.. Differences for which $p < 0.05$ were considered to be statistically significant.

6.5 Results and discussion

6.5.1 In vivo biocompatibility

Microcapsule biocompatibility was evaluated by the extent of host cell adhesion to their surfaces after two days in the peritoneal cavity of C57BL/6J mice. Cell adhesion was quantified using a cell adhesion score (**Eq. 6.1**) ranging from 0 (no cell adhesion) to 10 (complete surface coverage by immune cells). **Figure 6.1** compares the cell adhesion scores for different capsule formulations. The majority of the samples tested were fabricated using the IntG alginate because we had observed that, in its gelled form, this alginate is more biocompatible than the HiG alginate. In fact, HiG(Ca)/PLL microcapsules were excluded from the biocompatibility tests since it was judged to be non-ethical to implant mice with capsules that were expected to be immunogenic.

The IntG(Ca)/PLL/IntG microcapsules gave a mean cell adhesion score of 4.5 ± 0.6 ($n = 13$). This corresponded to the observation that the majority of the recovered microcapsules had surfaces partially adhered with cells (**Figure 6.2(a)**). Moreover, a number of the explanted capsules appeared to be collapsed, forming "lemon" shapes. These damaged capsules tended to have more cell adhesion than those that stayed round. Moreover, the biocompatibility of these IntG(Ca)/PLL/IntG microcapsules tended to vary depending on the capsule batch (cell adhesion

scores for individual mice ranged from 1.7 to 7.7). Such inconsistencies in cell adhesion indicate an inhomogeneity among capsule batches induced by the PLL coating process.

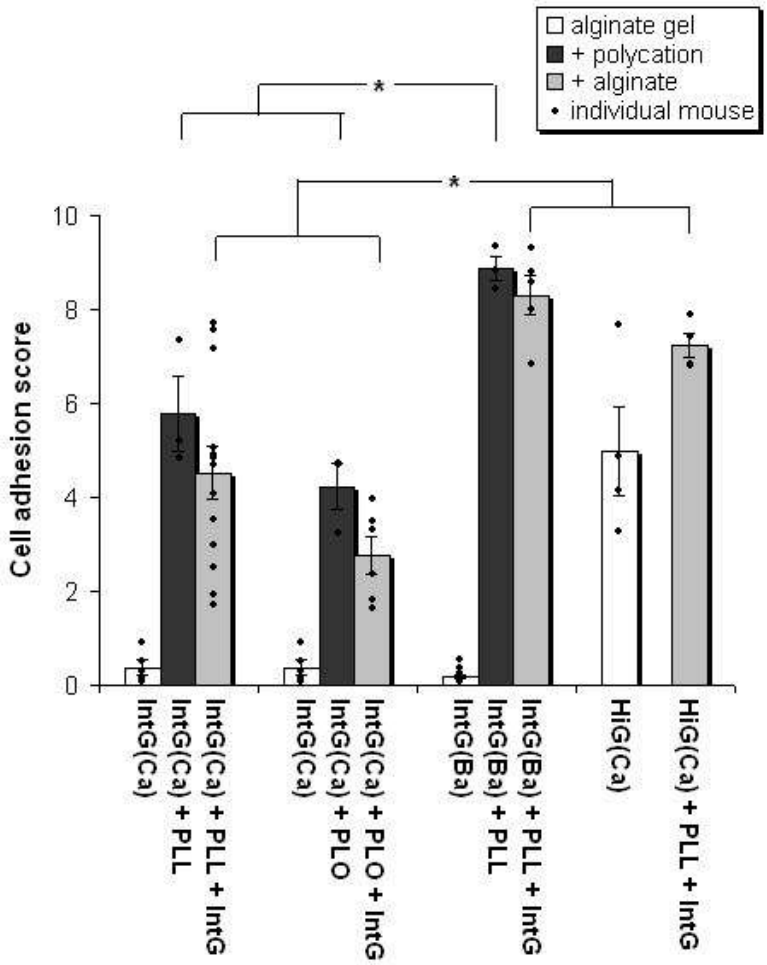


Figure 6.1 Biocompatibility of microcapsules of different design. Biocompatibility is measured by the extent of host cell adhesion after two days implantation in the peritoneal cavity of C57BL/6J mice, and is quantified by a cell adhesion score (0 = no cell adhesion ; 10 = complete coverage by cells). Bars represent the mean cell adhesion score ± SEM ($n \geq 3$). * $p < 0.05$

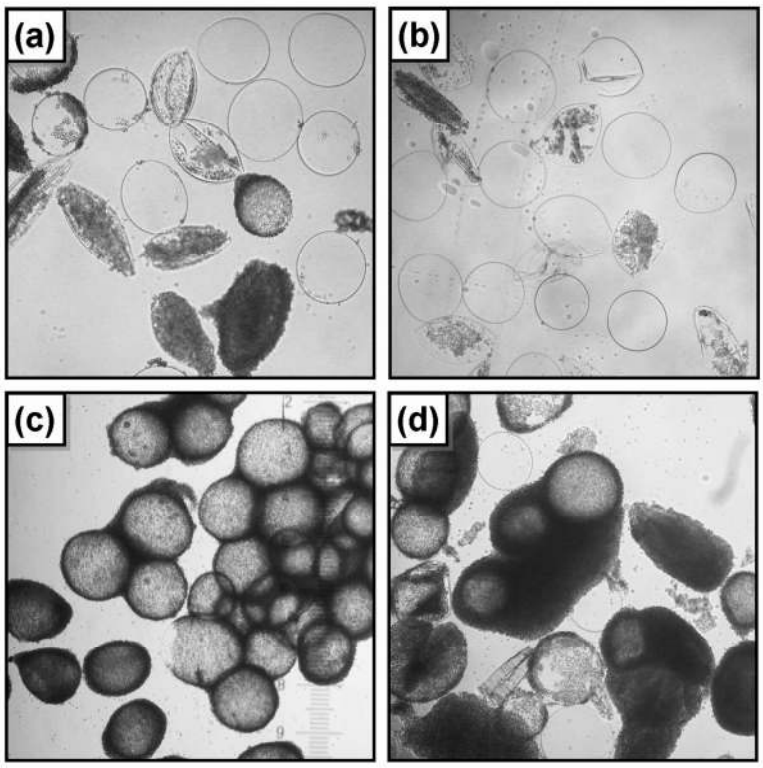


Figure 6.2 Observation of alginate-polycation microcapsules under light microscope after two days in the peritoneal cavity of C57BL/6J mice. Photos represent typical samples of **(a)** IntG(Ca)/PLL/IntG, **(b)** IntG(Ca)/PLO/IntG, **(c)** IntG(Ba)/PLL/IntG, and **(d)** HiG(Ca)/PLL/IntG microcapsules upon their explantation.

When the PLL was replaced with PLO, the extent of cell adhesion to the microcapsules dropped noticeably, yielding a mean score of 2.8 ± 0.4 for the IntG(Ca)/PLO/IntG samples (n = 6). Under the microscope (**Figure 6.2(b)**), the explanted samples looked similar to those with a PLL membrane, including the presence of collapsed capsules with cells adhered to their surfaces. However, cell adhesion scores were more consistent, ranging from 1.6 to 4.0 for individual mice. In at least one other study that directly compared the effect of PLL vs PLO on alginate microcapsule biocompatibility, the opposite result was observed [28], despite their use of similar methods and materials.

In an attempt to minimise the proportion of collapsed capsules that attract immune cells, the gel cores of the alginate-PLL-alginate (APA) microcapsules were replaced with either barium alginate or an alginate having a higher G content. Such modifications are expected to increase the strength and stability of the crosslinked gel core [29], and may therefore protect the

microcapsules from compressive forces or excessive swelling during the implantation period. As expected, the explanted capsules tended to stay round (**Figure 6.2(c)** and **Figure 6.2(d)**). However, biocompatibility clearly worsened ($p < 0.05$), as cell adhesion scores went up to 8.3 ± 0.4 for IntG(Ba)/PLL/IntG ($n = 5$) and 7.2 ± 0.3 for HiG(Ca)/PLL/IntG ($n = 4$). Moreover, these capsules were often found in clusters held together by host cells and the beginnings of fibrosis. These results support de Vos' observations that APA microcapsule biocompatibility is diminished by the use of high G alginate in the core [28, 30]. However, to our knowledge, such a negative effect of switching to a barium alginate gel core on APA capsule biocompatibility has not been seen before [31].

To confirm the respective roles of alginate and polycation in microcapsule immunogenicity, complete alginate-polycation-alginate microcapsules were compared to alginate gel beads (no membrane) and alginate-polycation microcapsules with no alginate coating (**Figure 6.1**). For all the samples tested, adding a polycation clearly augmented the immune response (cell adhesion scores increased by 3 to 8 points when compared to the gel beads ; $p < 0.05$). Immunogenicity of the polycationic membrane has often been suggested in the literature [28, 30, 32, 33]. More interestingly, adding an alginate coating had only the slightest (statistically non-significant) effect of reducing the immunogenicity that was induced by the polycation. This result puts into question the usefulness of adding an alginate coating to "counterbalance" or "neutralize" the positively charged polycation as a strategy to enhance the biocompatibility of alginate-polycation microcapsules.

6.5.2 Surface chemistry

As demonstrated in **Figure 6.1**, the polycationic component of the microcapsules had a profound effect on their biocompatibility. It is therefore of interest to evaluate the quantity of polycation that is present at the surface of each microcapsule type. Using XPS, the elemental composition of the outer 10 nm of the microcapsules was measured. The elements representing the gel crosslinking ions (Ca^{2+} and Ba^{2+}) were undetectable for all samples having a membrane, confirming that only the outermost portion of the capsule surfaces was being analysed.

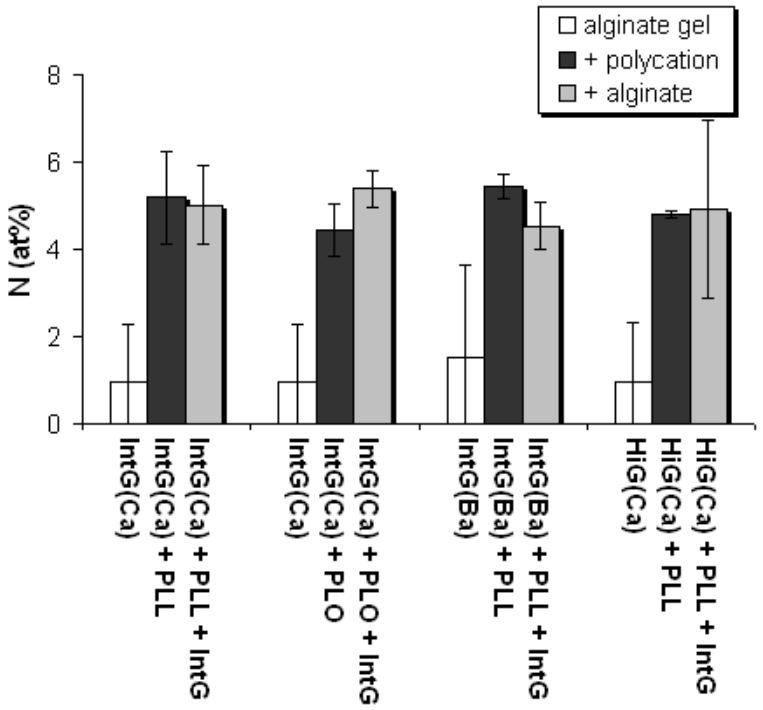


Figure 6.3 Effect of polycation and alginate coating on the quantity of nitrogen (N) detected on the surface of microcapsules of different design, as measured by XPS. Bars represent the mean value ± SD (n = 2) ; at% = relative atomic percentage.

The most direct indicator of polycation quantity is the relative atomic percentage (at%) of nitrogen. The alginate gel beads showed only traces of nitrogen (< 1.5 at% N) that were later attributed to slight contamination of the sample substrates. When polycationic membranes were added to the gel beads, nitrogen levels consistently increased to values between 4.5 and 5.5 at%, regardless of whether the alginate coating was added or not (**Figure 6.3**). Examination of the high resolution spectra confirmed that the nitrogen originated principally from the PLL or PLO molecules (results not shown). In comparison, pure PLL had 9.6 at% N when analysed by XPS, suggesting a blend of polycation and alginate at the microcapsule surfaces [34]. More importantly, surface nitrogen levels did not vary significantly with different formulations of microcapsules, and thus did not correlate with microcapsule biocompatibility.

These results demonstrate that, although the choice of polycation and gel type clearly influenced microcapsule biocompatibility, these parameters did not have an effect on the amount of polycation at the microcapsule surface, which was consistent (5.0 ± 0.5 at% N)

between sample types. In comparison, using similar XPS analyses, Ponce *et al* [28] saw a difference of 3.3 at% N (n = 1) between PLL and PLO capsules (using HiG alginate), while de Vos *et al* [35] saw a difference of 1.4 at% N (n = 4) between IntG and HiG alginate-PLL capsules. In each of these studies, the authors claimed a relationship between the estimated amounts of polycation and the *in vivo* biocompatibility of the microcapsules.

6.5.3 Membrane composition and intermolecular interactions

With an analytical depth of 2-3 μm, ATR-FTIR can provide insights into the relative amount of polycation that diffuses into the alginate gel core to form a membrane a few microns thick [36]. Moreover, shifting of peaks associated with functional groups can provide information about intermolecular interactions within the membrane.

The spectra for the microcapsules with a polycationic membrane always displayed peaks characteristic of both the polycation and the alginate, confirming the co-presence of these polymers within the membrane. Moreover, the addition of an alginate coating had very little influence on the peak heights and shapes, demonstrating once again the minimal influence of this coating on the microcapsule membrane properties.

For the microcapsules with a barium alginate gel core, the characteristic peaks for the polycation were much less prominent than for the other capsule types. This effect can be observed in **Figure 6.4**, which compares the spectra for alginate-PLL microcapsules that differ only in the choice of crosslinking ion in the gel core, Ca^{2+} vs Ba^{2+}. Notice the peak shapes in the areas highlighted in grey. For the capsules with a IntG(Ca) gel core, one can clearly see the presence of peak shoulders associated with the PLL molecule (NH_3^+ at ~ 3050 cm^{-1} ; Amide II at ~ 1530 cm^{-1}), yet these shoulders are barely visible for the capsules with an IntG(Ba) gel core. This is a good indicator that relatively lower amounts of PLL could penetrate into and/or bind to the barium alginate gel.

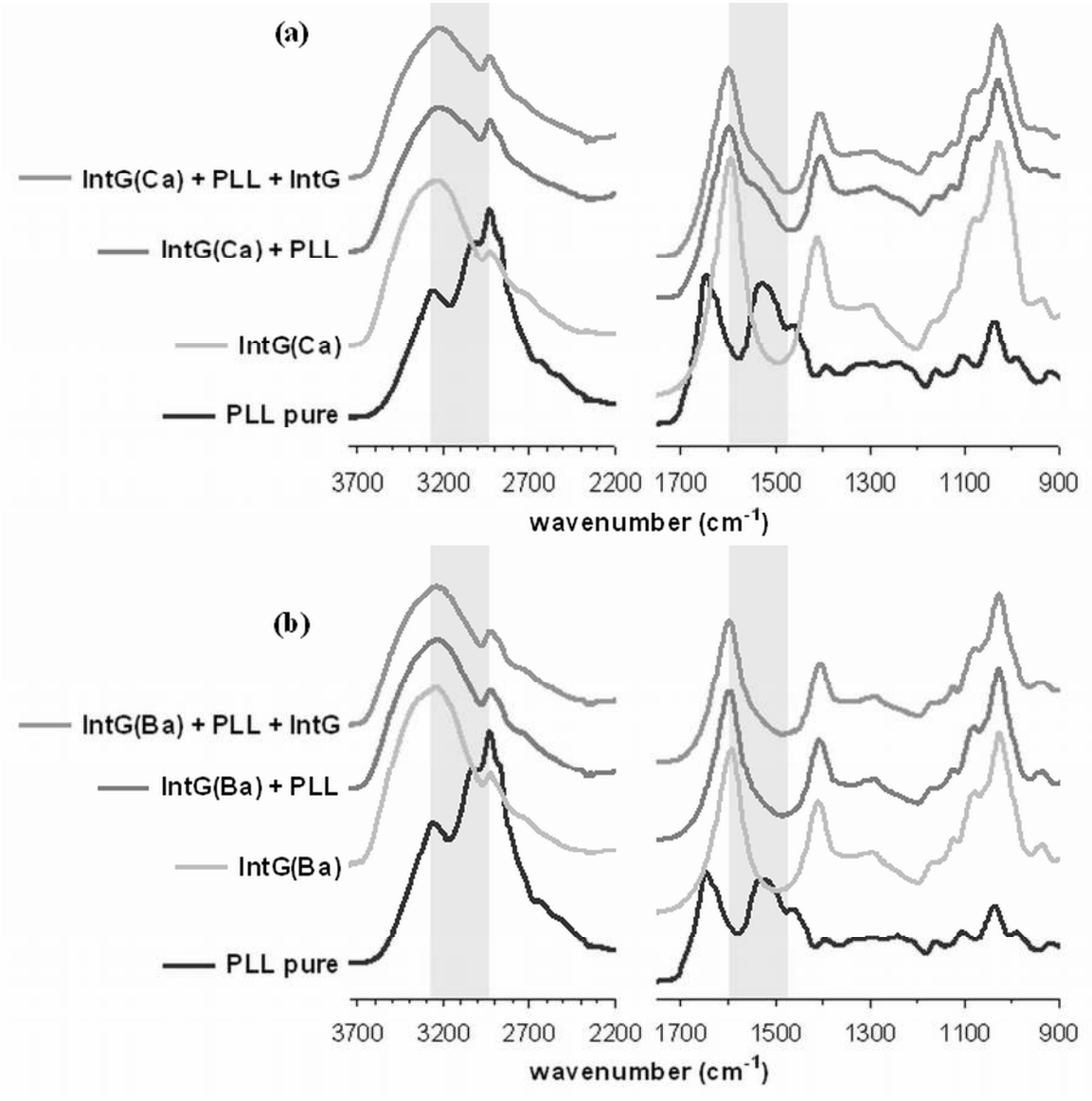


Figure 6.4 FTIR absorbance spectra for alginate-PLL microcapsules having a (a) calcium alginate gel core, or a (b) barium alginate gel core. Regions of particular interest are highlighted in grey. The peak shoulder near 1560 cm^{-1} is quantified in **Figure 6.5**.

The relative quantities of polycation and alginate within the microcapsule membranes were estimated semi-quantitatively using the height ratio of the peaks associated with Amide II of the polycation backbone (~ 1526 cm^{-1}, shoulder) and the COO^- of the alginate (~ 1593 cm^{-1}). The results are shown in **Figure 6.5**, where a higher Amide II / COO^- ratio indicates a greater quantity of PLL or PLO within the membrane. One can now more clearly observe that the microcapsules with a barium alginate gel core had less polycation incorporated in their

membranes than the capsules with a calcium alginate gel core. A poor penetration of PLL into the barium alginate gel can be explained by the higher affinity of Ba^{2+} ions for alginate than Ca^{2+} ions [37], making it energetically less favourable for the PLL to displace the barium in order to electrostatically bind to the alginate. A similar decrease in PLL binding efficiency by alginate gel beads was seen by Thu *et al* when they substituted calcium ions for strontium ions to crosslink the alginate [27]. The poor biocompatibility of the IntG(Ba)/PLL/IntG microcapsules can be explained by this reduced penetration of PLL into the barium alginate gel, as a thinner and/or less stable membrane may present a higher risk of exposing unbound PLL that can attract the immune system.

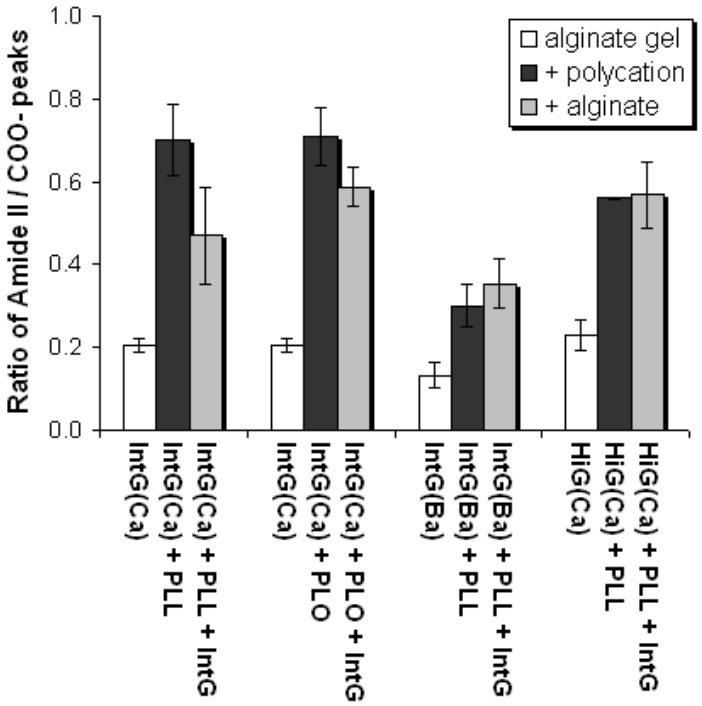


Figure 6.5 Relative heights of the FTIR spectral peaks associated with Amide II (~ 1526 cm^{-1}, shoulder) of the polycation and COO^- (~ 1593 cm^{-1}) of the alginate for microcapsules of different design. Bars represent the mean ratio value ± SD (n = 2).

The FTIR spectra of the two most biocompatible microcapsule types, IntG(Ca)/PLL/IntG and IntG(Ca)/PLO/IntG shared common features. Specifically, the Amide II / COO^- ratios (**Figure 6.5**) demonstrate that the polycation diffused efficiently into their IntG(Ca) gel cores (black bars), and moreover, that the alginate coating had an effect of lowering the

polycation/alginate ratio (grey bars). Since their surface compositions did not change significantly with the alginate coating (**section 6.5.2**), this result suggests that the dilute alginate successfully diffused into the membrane of these two capsule types during the coating process.

An indicator of the strength of ionic interaction between alginate and its counterion is the shift of the FTIR peaks associated with the asymmetric (~ 1593 cm^{-1}) and symmetric (~ 1410 cm^{-1}) stretching vibrations of the COO^- groups of the alginate [39, 40]. The shifting positions (wavenumbers) of these two peaks as the various components of the microcapsules were added are shown in **Table 6.3**. In general, as the polycation was added to the alginate gel bead to form a membrane, the peak near 1593 cm^{-1} consistently shifted to higher wavenumbers, while the peak near 1410 cm^{-1} shifted to lower wavenumbers. These shifts were slightly more pronounced for the two microcapsule types having an IntG(Ca) gel core, supporting the view that this gel type bound the polycation more efficiently than the barium alginate gel or gel of HiG alginate. When alginate coatings were added, however, there were no clear patterns in the peak shifts, leaving any interpretations about the extent of interaction between gel-bound polycation and solubilised alginate ambiguous.

Table 6.3 Placements of FTIR absorbance peaks associated with the assymmetric and symmetric stretching vibrations of the COO^- functional groups of the alginate. Values represent the mean wavenumber ± SEM (n = 2).

	COO^- asym		**COO^- sym**	
	Wavenumber (cm^{-1})	**Shift vs. gel bead (cm^{-1})**	**Wavenumber (cm^{-1})**	**Shift vs. gel bead (cm^{-1})**
IntG(Ca)	1593 ± 0		1410 ± 0	
IntG(Ca) + PLL	1599 ± 2	+ 6	1404 ± 2	- 6
IntG(Ca) + PLL + IntG	1602 ± 3	+ 9	1408 ± 2	- 2
IntG(Ca)	1593 ± 0		1410 ± 0	
IntG(Ca) + PLO	1597 ± 2	+ 4	1402 ± 0	- 8
IntG(Ca) + PLO + IntG	1598 ± 3	+ 5	1407 ± 1	- 3
IntG(Ba)	1592 ± 1		1408 ± 0	
IntG(Ba) + PLL	1596 ± 1	+ 4	1405 ± 1	- 3
IntG(Ba) + PLL + IntG	1595 ± 2	+ 3	1404 ± 0	- 4
HiG(Ca)	1593 ± 0		1409 ± 1	
HiG(Ca) + PLL	1595 ± 0	+ 2	1405 ± 1	- 4
HiG(Ca) + PLL + IntG	1596 ± 1	+ 3	1404 ± 2	- 5

6.5.4 Exposure of hydrophilic groups

It has been stated that, as a hydrogel, alginate is biocompatible because of its superior hydrophilicity, which allows for minimal protein and cell adhesion [6]. It is therefore of interest to know the surface wettability of alginate-based microcapsules. In this study, sample wettability was estimated by the static contact angle of 1 µl water droplets on the surface of dehydrated films mimicking the chemistry of the microcapsules under study. While this measurement may not be directly representative of the wettability of hydrated gels, it is nevertheless a good indicator of the proportion of hydrophilic groups (-OH, $-COO^-$, $-NH_3^+$) that are exposed at the sample surface and are available for rehydration.

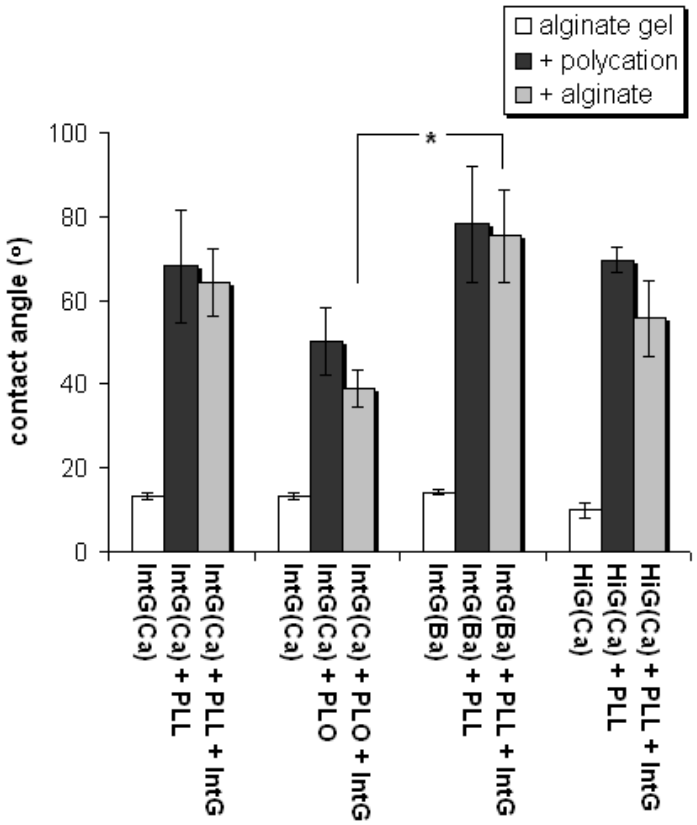


Figure 6.6 Wettability of films representing the composition of microcapsules of different design. Wettability is measured by the static contact angle of 1 µl droplet of pure water on the dry film surface. Bars represent the mean contact angle ± SEM (n = 5). * $p < 0.05$.

As can be seen in **Figure 6.6**, the alginate gels were indeed very hydrophilic with average contact angles measuring below 15°. The water droplets immediately spread along the film

surfaces as the gel rehydrated. When polycations were incorporated into the films, the water droplets spread less and, for all sample types, contact angles increased significantly ($p < 0.05$) to values above 50°. Adding the polycation also induced a large variation in the results, revealing an inhomogeneity of the polyelectrolyte complexes that can also explain the variability in cell adhesion scores between individual capsules. In comparison, pure PLL and PLO had contact angles of 20.4 ± 0.2° and 24.6 ± 0.6°, respectively. Therefore, it is not the polycation itself, but rather the alginate-polycation complex that has hydrophobic qualities. This can be visualized as the oppositely charged functional groups of the polymers are busy interacting with eachother and are therefore unavailable for interaction with water. Adding an alginate coating lowered the contact angles by 3° to 14°, but this effect was not statistically significant and the hydrophilic properties of the alginate gels were never completely restored. Similar results for alginate-PLL complexes have been seen before in the literature [40].

The films containing PLO were slightly more hydrophilic than those samples containing PLL. When the alginate coating was added, this difference was statistically significant ($p < 0.05$) in one case, as indicated on **Figure 6.6**. This result may be a reflection of the slightly higher ratio of hydrophilic NH_3 to hydrophobic CH_2 in the PLO side chain (1:3) compared to PLL (1:4). It may also suggest a less efficient binding between the PLO and alginate, thus allowing for the charged groups to interact with the water molecules, but the FTIR results contradict this hypothesis. In any case, the more wettable PLO-based samples also represent the most biocompatible microcapsules, thus supporting the opinion that hydrophilicity is indeed important for minimizing biomaterial immunogenicity.

6.5.5 Microcapsule swelling and membrane strength

When alginate-based microcapsules are immersed in pure water, the extent to which they swell is dependent on the gel characteristics, while the tendency of the capsule membrane to deform or split when swelling occurs is a reasonable indicator of membrane strength and homogeneity. The reactions of complete alginate-polycation-alginate microcapsules to water rinses are shown in **Figure 6.7**.

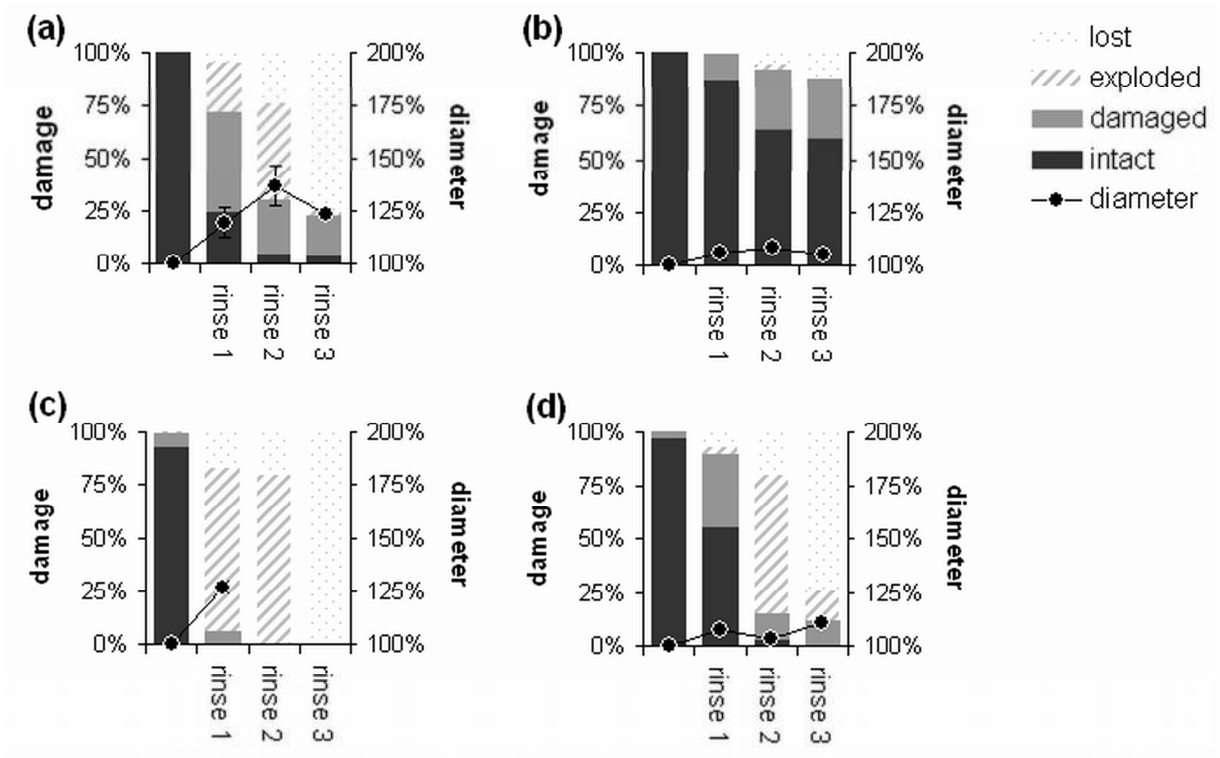


Figure 6.7 Resistance of microcapsules of different design to swelling and damage when rinsed with pure water : (a) IntG(Ca)/PLL/IntG, (b) IntG(Ca)/PLO/IntG, (c) IntG(Ba)/PLL/IntG, (d) HiG(Ca)/PLL/IntG microcapsules. Bars show the mean proportion of capsules within a sample that are intact ■ , damaged ■ , exploded ▨ , or lost ⋯ before and after each of three water rinses (n = 3). The line graph −●− indicates mean the percentage increase in diameter of intact and damaged capsules ± SEM (n = 3). Each rinse consists of immersing 30 ± 5 microcapsules in 1 mL of pure water for 5 - 10 min.

As indicated by the proportion of undamaged capsules with each water rinse (black bars in **Figure 6.7**), the PLO microcapsules were clearly the most resistant to swelling and damage. Even after three water rinses, these microcapsules never swelled more than 108% of their original diameter, and less than 15% of the capsules were exploded or lost. At least two other studies have compared the osmotic swelling and stability of PLO vs PLL alginate microcapsules and have concluded that PLO membranes performed better [19, 41], although the opposite result has also been published [28]. The resistance of the PLO membrane to swelling and rupture is in concordance with the FTIR results that suggest that PLO penetrates well into the gel core to form a membrane (**section 6.5.3**). More importantly, these damage-resistant capsules were also the most biocompatible when implanted (**Figure 6.1**), thus emphasizing the importance of

membrane stability and strength for avoiding an immune response. Ironically, PLO capsules showed a tendency to collapse during their implantation period (**Figure 6.2(b)**) despite their superior stability in the explosion assay. Since IntG(Ca) gel is quite pliable, this implies that the damage that was induced *in vivo* was due to compression forces and shear stresses rather than to osmotic swelling.

On the other end of the spectrum, microcapsules having a barium alginate gel core were clearly the most prone to rupture when swelling was induced (**Figure 6.7(c)**). With the first water rinse, diameters increased to 127% and over 90% of the capsules were destroyed; in no cases did these capsules survive the second rinse. The fragility of these microcapsules is likely to be a result of the poor incorporation of the PLL into the gel core that was observed by FTIR. More importantly, these microcapsules were also the most immunogenic despite that most of them stayed round during their implantation period (**Figure 6.2**). This observation emphasizes once again that the quality and strength of the polycationic membrane is more important in governing biocompatibility than the ability of the gel core to resist compression forces *in vivo*.

In between these two opposites were the PLL microcapsules with calcium alginate gel cores. Of these samples, those with an IntG alginate core tended to swell more (up to 137%) than those with a HiG alginate core, as could be expected since high guluronate alginate forms stiffer gels that are less prone to swelling [42]. Additionally, the higher concentration of HiG alginate within the capsule core could have contributed to its greater resistance to swelling. Yet, those capsules with a HiG alginate core had membranes that more readily split despite minimal swelling, demonstrating a stiffer and less elastic membrane than their IntG counterparts. Similarly to the case of the capsules with an IntG(Ba) gel core, the poor resistance of the membrane to swelling is possibly related to an inadequate interaction between the HiG alginate gel and the PLL, either because of the higher proportion of GG blocks (which are involved in binding the Ca^{2+} ions, leaving less alginate available for binding the PLL) or the higher proportion of low molecular weight fractions within this alginate (as reflected by its low intrinsic viscosity). As far as the implications for biocompatibility, the stiffer membranes of the microcapsules with the HiG alginate core seem to be the greater problem for inducing immunogenicity.

When the alginate coatings were omitted, the results (not shown) were almost identical as those presented in **Figure 6.6**. Thus, the alginate coating had no effect on the microcapsule's resistance to swelling and damage in water. In contrast, alginate beads without a membrane swelled to over 200% of their original diameter before disintegrating (results not shown), demonstrating the role of the alginate-polycation membrane in limiting the swelling of the capsule.

6.6 Conclusions

The results of this study provide evidence that the biocompatibility of alginate-polycation microcapsules is influenced by each the alginate composition, gelling ion, and polycation. In our case, the use of PLO rather than PLL as the polycation resulted in microcapsules with better biocompatibility *in vivo*. The PLO capsules were also associated with a greater hydrophilicity and adequate diffusion of polycation into the gel to form a membrane that resisted swelling and rupture when subjected to osmotic stress. These features can be attributed to the shorter side chains of the PLO compared to PLL, which may influence hydrophilic/hydrophobic (NH_3^+/CH_2) balance as well as the manner in which the polymer interacts with the alginate gel. As for the influence of crosslinking ion and alginate type on biocompatibility, the use of a barium alginate gel or an alginate with a higher guluronate content as the capsule core resulted in a stronger immune response to the microcapsules. This was explained by a poor diffusion and integration of the PLL into these gel types, particularly the barium alginate. In this case, it was probably energetically unfavourable for the polycation to displace the gelling ion in order to crosslink with alginate and form a membrane. This is a natural consequence of the stronger affinity of the barium ions to alginate, and the stronger affinity of the guluronate blocks to bind the divalent cation. While these microcapsules better resisted the compressive forces *in vivo*, as evidenced by their rounder appearance upon explantation, their membranes were more prone to splitting when the capsules swelled under osmotic stress. Indeed, a low quality of membrane is expected to be more susceptible to inhomogeneity and defects that increase the risks of unbound PLL of being exposed at the capsule surface and attracting proteins and cells of the immune system. In contrast, it was shown that the quantity of polycation at the surface had little to do with biocompatibility, and moreover, that the alginate coating had very little effect on each the biocompatibility and

various physicochemical properties that were investigated in this study. Thus, it is not the alginate coating's ability to "neutralize" the positively charged groups of the membrane that governs the biocompatibility and physicochemical properties of alginate-polycation microcapsules, but it is rather the membrane forming step of polycation diffusing into and binding to the alginate gel that is of importance.

6.7 Acknowledgements

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CHAPTER 7

INDUCING NORMOGLYCEMIA IN DIABETIC MICE USING MICROENCAPSULATED ISLETS: WITH OR WITHOUT A MEMBRANE

NOT SUBMITTED FOR PUBLICATION

7.1 Abstract

The polycationic membrane is supposed to give alginate-polycation microcapsules a controlled permeability and increased stability, but its biocompatibility is not optimal. This study seeks to determine whether a modestly immunogenic membrane hinders or helps the ability of the microcapsule to immunoprotect. We thus tested the ability of 300 rat islets, encapsulated in either alginate-PLO microcapsules or in barium alginate beads, to normalize the blood glucose levels of immunocompetent diabetic mice. One of the four mice that were transplanted with islets in PLO microcapsules became normoglycemic (for 14 days), while the other three remained diabetic. All four of the mice that received islets immobilized in gel beads were normoglycemic for periods ranging from 16 to 50+ days. Once hyperglycemia was established, the samples were explanted and examined under microscope in an attempt find out why the grafts failed. In the case of PLO microcapsules, graft failure seemed to have been due to fragmentation and degradation of the samples *in vivo*. In the case of gel beads, reasons for graft failure were less clear. Many of the explanted beads were damaged and/or adhered with host cells. As well, ~10% of the islets could be recovered, although some were unhealthy or degraded. The length of graft survival did not correlate with the proportion of recovered islets nor with the extent of host cell adhesion. In conclusion, the PLO membrane was clearly not beneficial for islet function *in vivo*, although it remains uncertain as to whether some type of membrane is still necessary for immunoprotection.

7.2 Introduction

In our research lab, the main purpose for developing alginate-polycation microcapsules is to allow the safe transplantation of islets of Langerhans (i.e. clusters of insulin-secreting cells) for the treatment of insulin-dependent diabetes. Ideally, our encapsulated islet system should normalize the blood glucose levels of diabetic recipients without the administration of exogeneous insulin (by syringe) and without the use of immunosuppressive agents.

The purpose of adding a polycationic membrane to alginate-based microcapsules is to reduce and control their permeability as well as enhance their stability. These qualities are expected to enhance the immunoprotective abilities of the microcapsules. However, as we have shown, the biocompatibility of the polycationic membrane has not yet been optimized (**CHAPTER 6**). We were interested to know whether a mild immune response to the microcapsules is actually enough to hinder their performance as immunoprotective devices, or if the membrane was even necessary at all. In order to answer these questions, we encapsulated islets in gel beads (without a membrane) or microcapsules (with a PLO membrane), and then tested their ability to normalize the blood glucose levels of immunocompetent, diabetic mice.

7.3 Materials and Methods

7.3.1 Materials

Keltone® LVCR sodium alginate, lot #721322, was provided by ISP Corp (New Jersey, USA). The alginate was purified according to protocols that were optimized in our lab [1, 2]. The purity and biocompatibility of the alginate was verified (**CHAPTER 5**) [3]. Further details about this alginate can be found in **Table 6.1**.

Poly-L-ornithine (PLO) hydrochloride (product no. P2533; CAS 26982-21-8) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Characteristics of this polymer are described in **Table 6.1**.

All solutions were sterilized by filtration (0.22 μm) before their use. Solution formulae are described in **APPENDIX I**. Reusable labware was specially cleaned and sterilized by Pasteur oven (200°C for 2h), ethylene oxide (EO) or autoclave before their use. Disposable

plastic materials were considered as sterile. Manipulations were performed under sterile conditions whenever possible.

7.3.2 Islet isolation

Islets were isolated from the pancreata of adult male Wistar rats (250 - 400 g, Charles River Institute, St-Constant, QC). The islet isolation procedure, based on methods published by Lacy *et al* [4], has been previously described [5]. Briefly, type V collagenase (diluted 1 mg/mL in Hank's balanced salt solution) was infused into the pancreas via the common bile duct. The inflated pancreas was excised and further digested for 30 min at 37°C. Islets were purified by consecutive filtration (800 µm ; 250 µm) followed by a discontinuous Euroficoll gradient. Islets were then handpicked to > 95% purity under light microscope and then cultured overnight in a supplemented RPMI 1640 medium at 37°C and 5% CO_2. Approximately 400 islet-equivalents (ieqs) were isolated from each rat. These manipulations were done under the approval of the Maisonneuve-Rosemont Hospital Animal Ethics Committee.

7.3.3 Islet encapsulation

Islets were suspended in a viscous solution of purified alginate (1.63% w/v in saline). The suspensions were extruded from a 25G blunt-tip needle using a mechanically controlled syringe and electrostatic pulsed generator. This formed tiny droplets that were immersed for 30 min in a crosslinking solution (10 mM $BaCl_2$ for beads ; 100 mM Ca Lactate for microcapsules) to form gel beads with islets immobilized within. To form a membrane, the beads were immersed for 5 min in a PLO solution (0.05% w/v in saline), rinsed 3 min in saline, immersed 5 min in dilute alginate solution (0.163% w/v in saline), and rinsed twice in saline. Gel beads without a membrane were rinsed twice with Ringer's solution. Finally, beads and microcapsules were rinsed and cultured overnight (37°C ; 5% CO_2) in Ultraculture medium supplemented with 1% v/v penicillin-streptomycin-glutamine.

7.3.4 Transplantation

Male C57BL/6J mice weighing 14-26 g (Jackson Laboratory, Maine, USA) were used as transplant recipients. Diabetes was induced by intraperitoneal injection of Streptozotocin, STZ (22.5 mg/mL in sodium citrate, pH 4.5) at a dosage of 150 mg/kg of body weight. Glucose

levels in blood samples taken from the tail vein were measured using an Accu-Chek® meter. Mice were transplanted if two subsequent blood glucose readings were ≥ 15 mmol/L. To do so, samples of 300 encapsulated islets (i.e. 280 ± 50 islet equivalents, ieqs, thus representing a marginal mass islet cell transplantation) were handpicked and injected, via a 22G catheter, into the peritoneal cavity. Throughout the *in vivo* studies, blood glucose levels and body weights were monitored as often as every second day. Four mice were transplanted per condition (barium gel beads vs PLO microcapsules), divided over two batches of mice (n = 4). Empty beads and microcapsules, as well as non-encapsulated islets (300), served as controls.

7.3.5 Explantation

Mice that displayed signs of hyperglycemia (blood glucose levels ≥ 20 mmol/L, difficulty in weight gain, excessive urination) were euthanized by cervical dislocation and samples were explanted by peritoneal washings. Explanted samples were examined by light microscopy and their biocompatibility was quantified using a cell adhesion score, as described in **section 5.4.4** and **section 6.4.3**.

7.4 Results

7.4.1 Normalization of blood glucose

Blood glucose levels of the transplanted mice, along with the controls, are graphed in **Figure 7.1**. The length of graft survival for each mouse is indicated in **Table 7.1**. Within 48h of transplantation, normoglycemia (i.e. blood glucose levels ≤ 12.0 mmol/L) was induced in all four of the mice that received islets immobilized in gel beads. Graft survival periods for this group varied from 16 days to 50+ days. In contrast, only one of the mice that received islets encapsulated in PLO microcapsules achieved normoglycemia (for 14 days), while the other three mice in this group remained hyperglycemic after the transplantation.

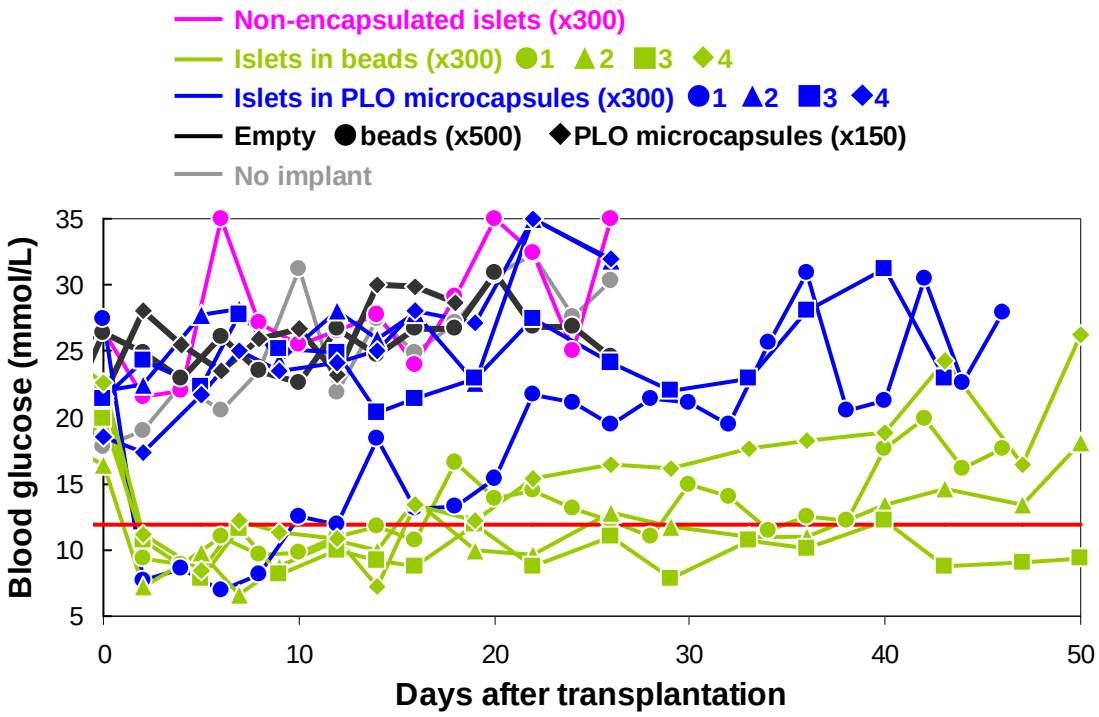

Figure 7.1 Blood glucose levels of STZ-induced diabetic C57BL/6J mice after being transplanted with encapsulated islets (n = 4), non-encapsulated islets (n = 1), or empty beads or microcapsules (n = 1). Each line graph represents an individual mouse. The upper limit for normoglycemia (12 mmol/L) is indicated by a red line.

7.4.2 Examination of explanted samples

At the end of each transplantation period, the samples were explanted and examined under light microscope. With the exception of one mouse, samples were explanted after the recipient experienced prolonged hyperglycemia, i.e. after the transplanted islets had already begun to fail.

As shown in **Table 7.1**, only a small portion of the encapsulated islets were recovered upon explantation. On average, of 10 ± 1% of the islets immobilized in beads were recuperated. In contrast, islets encapsulated in PLO microcapsules could be retrieved from one mouse only, and this represented just 2% of the islets that were implanted. In the case of zero islet recovery, nothing recognizable was recovered by peritoneal washings (not even empty microcapsules).

Table 7.1 Evaluation of samples explanted from the peritoneal cavity of STZ-induced diabetic C57BL/6J mice after graft failure. Parameters evaluated include the proportion of islets recovered and the immune response to the transplants. TX = transplantation.

Transplant	Mouse	Graft survival (days)	Explantation (days after TX)	Islets recovered	Cell adhesion score	
					Empty	With islets
Non-encapsulated islets (×300)		< 2	26	0%	n/a	n/a
Islets in beads (×300)	1	18	46	7%	7.62	9.05
	2	40	50	13%	3.07	3.58
	3[a]	> 50	50[a]	9%	3.15	2.69
	4	16	50	10%	2.46	2.18
Islets in PLO microcapsules (×300)	1	14	46	2%	5.65	6.19
	2	< 2	26	0%	n/a	n/a
	3	< 2	43	0%	n/a	n/a
	4	< 2	26	0%	n/a	n/a
Empty beads (×500)		< 2	26	n/a	1.32	n/a
Empty PLO microcapsules (×150)		< 2	18	n/a	6.31	n/a
No implant		< 2	n/a	n/a	n/a	n/a

[a] normoglycemic upon explantation

Several empty beads or microcapsules were also explanted. Although a number of these were empty because the islets within were lost *in vivo*, most were actually empty when they were implanted (they were picked up during the hand-picking process). Overall, more beads than PLO microcapsules were retrieved. The beads tended to be deformed while the PLO microcapsules were rather collapsed or squished, although fragments were commonly observed in both cases (**Figure 7.2**). The extent of host cell adhesion to empty vs. islet-containing beads/capsules was compared for each sample explanted (**Table 7.1**). For one mouse only, the cell adhesion score was considerably higher for islet-containing beads/capsules than for empty ones (Δ = +1.43). Otherwise, the presence of islets within the beads/capsules did not appear to have an influence on the extent of host cell adhesion to those beads/capsules.

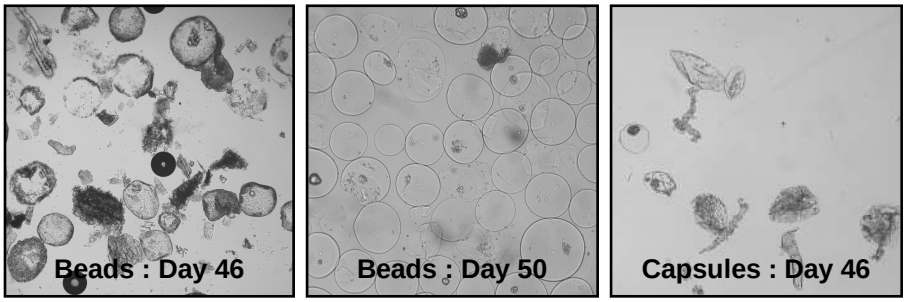

Figure 7.2 Examples of explanted samples as viewed under light microscope.

On several occasions, a “phantom” was observed in the alginate gel where the islet used to be, as shown in **Figure 7.3**. In place of the islet, there was a hole in the gel along with single dispersed cells. In some cases, the islet was still intact but had been displaced within the gel.

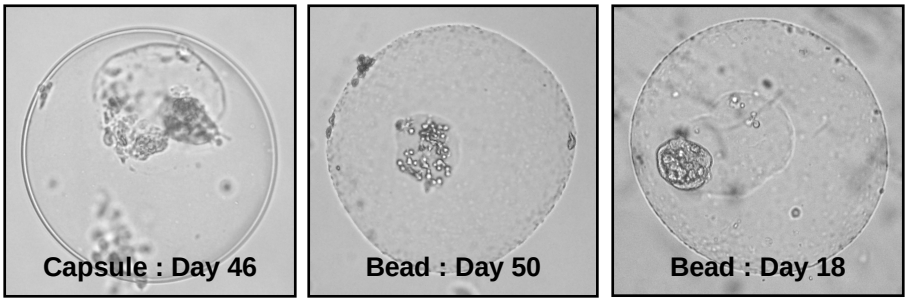

Figure 7.3 Observations of “phantoms” left by islets that were immobilized in the alginate gels.

Fissures could also be observed in the explanted gel beads. These fissures would lead into the bead center towards the islet, which would generally be unhealthy or degraded into single cells. Moreover, host cells could often be seen to be adhered along the inside walls of the fissure. This phenomenon is shown in **Figure 7.4**.

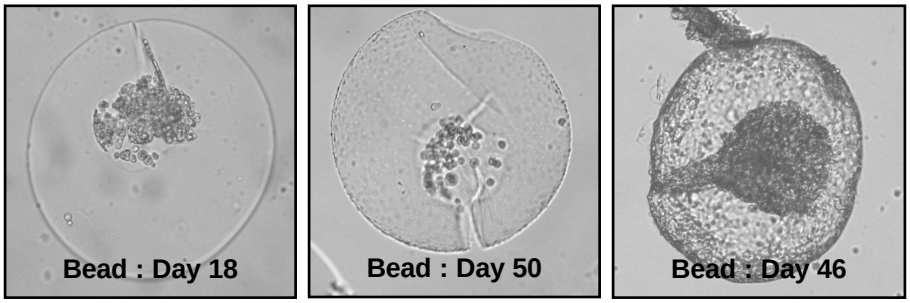

Figure 7.4 Observations of fissures leading into the centers of beads that contained islets.

7.5 Discussion

The primary function of the polycationic membrane is to reduce and control the permeability of the microcapsule, as well as improve its stability [6]. In principle, this should lead to improved immunoprotection by the microcapsule. However, it has been difficult to optimize the biocompatibility of the polycationic membrane. For this reason, a handful of research groups have started advocating the use of alginate gel microbeads (without a membrane) for cell encapsulation [7-10]. Despite some promising results of islet transplantation using uncoated alginate gel beads, the higher permeability of these devices (vs. microcapsules with a membrane) remains a concern as adequate immunoprotection might not be achievable despite manipulations of the alginate and gelation techniques [11, 12].

And yet, in this study, the gel beads without a polycationic membrane were clearly more efficient than the microcapsules with a PLO membrane for protecting the viability and function of islets transplanted into immunocompetent mice. We are not the only ones to have observed that adding a polycationic membrane hinders the success of encapsulated islet transplantation rather than enhances it. For example, Safley *et al* [13, 14] showed that, compared to barium alginate gel beads without a membrane, the use of a PLL membrane greatly reduced the efficiency of adult porcine islets to induce normoglycemia in non-obese diabetic mice.

Once the recipients became hyperglycemic, we explanted and examined the transplants in order to understand the possible reasons for graft failure. In the case of the PLO microcapsules, the extremely low retrieval rates and observations of collapsed or fragmented capsules covered with host cells seems to suggest that the microcapsules simply started to fall apart after a few days *in vivo*. If this was indeed the case, the islets within would quickly have been unprotected and attacked by the host immune system. Similarities between the *in vivo* performance of islets in PLO microcapsules and non-encapsulated islets support this view.

In the case of the alginate gel beads, the results were quite variable. First of all, graft survival ranged from 16 to 50+ days. Upon explantation, we saw signs of an immune response to the transplants, particularly host cell adhesion to the bead surfaces. In some cases, it appears that the host cells managed to enter the bead via fissures in order to directly attack the islet within. That said, there were no correlations between the extent of cell adhesion and the length of graft survival, suggesting that host cell adhesion was not the main contributor to graft failure.

We are not the first to fail to see a correlation between the duration of graft function and extent of overgrowth to microencapsulated islets [15]. Similarly, although we observed a massive loss of islets (averaging 90% loss), there were no clear correlations between the proportion of islets recovered and the length of graft survival.

These results tell us that factors other than host response and islet loss (i.e. that were invisible by optical microscope) were likely the main cause for the eventual dysfunction and/or death of the islets encapsulated within alginate gel beads. Such factors could have been the penetration of tiny cytokines into the porous gel [7, 11] or the poor diffusion of oxygen into the center of the beads and islets [16, 17]. However, until a closer examination of the biological state of the islet cells (including cellular stress signals, apoptosis, and necrosis) is carried out, we have no definite proof of either of these phenomena.

7.6 Conclusions

As immunoprotective devices for transplanted islets, barium alginate gel beads (without a membrane) were much more effective for inducing normoglycemia in immunocompetent diabetic mice than alginate microcapsules with a PLO membrane. It appears that the PLO microcapsules failed to protect the transplanted islets because they fragmented and degraded *in vivo*. The reasons for eventual graft failure of islets immobilized in gel beads could not be clearly determined based on the results of this study.

7.7 Acknowledgements

A special thanks to Stéphanie Bilodeau for helping me excise the pancreases, for having done all of the islet isolations, and for helping me check the blood glucoses of my mice.

7.8 References

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CHAPTER 8

GENERAL DISCUSSION

Within the field of encapsulated islet transplantation, the biocompatibility of alginate-polycation microcapsules is still both difficult to control and reproduce. This situation stems from the combination of a lack of standardization for microcapsule fabrication and an insufficient understanding of what physical and chemical properties influence the biological response to these devices. With the intention of contributing to the optimization of microcapsule biocompatibility, this research aimed to elucidate the relationships between the fabrication parameters, physicochemical properties, and *in vivo* biocompatibility of alginate-based microcapsules.

8.1 Can protein adsorption predict APA microcapsule biocompatibility ?

Protein adsorption was chosen as a starting point for this research, for practical purposes (related to my internship in Groningen) as well as for scientific reasons. Not only is protein adsorption considered to be the first event to occur upon implantation [1, 2], thereby governing all subsequent biological events [3, 4], but the Groningen lab had previously demonstrated that proteins adsorb to alginate-polycation microcapsules *in vivo* (according to an increasing nitrogen signal detected by XPS), and this seemed to correlate with the severity of inflammatory response towards the implants [5, 6]. In a few short months, we managed to prove, for the first time, that *human* immunoglobulin adsorbs to alginate-PLL microcapsules *in vitro*. Not only this, we demonstrated how to quantify protein adsorption using a simple and accessible technique that also allows direct visualization of the proteins. While I was in Groningen, Julie was simultaneously using an elution technique in combination with mass spectrometry to prove that not only immunoglobulins, but a whole range of proteins (from mouse serum and plasma) adsorb to the surface of APA microcapsules *in vitro* [7]. Since this research, few other studies have been published that demonstrate protein adsorption to alginate-based microcapsules [8, 9].

We clearly showed that the polycation is mainly responsible for adsorption of immunoglobulins. This implies that the positive electric charge of the surface played an important role in binding the proteins. This supports the theoretical view that electrostatic attraction or repulsion is one of the main interactions between biological proteins and surface [3]. However, when the same microcapsules were previously analyzed using zeta potential measurements, the HiG microcapsules had a greater net surface charge than the IntG microcapsules [10]. This difference in net surface charge did not correlate with a different extent in immunoglobulin adsorption, implying that immunoglobulin adsorption is also affected by other factors other than electrostatic forces, such as hydrophobic binding.

Most importantly, we could not prove a correlation between immunoglobulin adsorption and microcapsule biocompatibility. That is, all the microcapsules (with a membrane) showed equivalent adsorption despite that only one of the four capsule types has shown adequate biocompatibility *in vivo* [11, 12]. This may be explained by the fact that we tested the adsorption of human Ig while the biocompatibility studies were performed on rodents. However, a more reasonable explanation is that the combination of IgG, IgM, and IgA adsorption does not determine the cellular response to the microcapsules. IgG is the most abundant of the immunoglobulins in plasma and serum [13], but it is also the most potent since its adsorption to surfaces makes the surface a target for phagocytosis by immune cells [14, 15]. Upon returning to Montréal, we attempted to continue the adsorption studies using more specific proteins, namely IgG, the complement factor C3, and albumin. However, preliminary experiments were not successful and experimental protocols were not optimized due to time constraints. That said, there has been some evidence of a correlation between total protein adsorption and cellular overgrowth on APA microcapsules [5, 6], but any other evidence has been sparse.

So the question remains, can protein adsorption predict APA microcapsule biocompatibility ? We still don’t know for sure, but if the answer were ‘yes’ than this would be a very interesting option for screening microcapsules *in vitro* before having to use animals. The answer to this question risks being unanswered for a while yet, given the difficulty that others have had establishing clear correlations between protein adsorption and “ideal” biomaterials with highly specific, functionalized surfaces [16-18].

8.2 So what properties govern alginate-polycation microcapsule biocompatibility ?

8.2.1 All purified alginates are not equal

The results of our study on the factors influencing alginate gel biocompatibility came to us as a bit of a surprise. Leading research groups have suggested that, as long as the alginate is purified, then it is biocompatible [11, 19, 20]. Therefore, we purified our alginates, and verified that they were pure. Yet, the alginate with higher guluronate (G) content produced gels that were not biocompatible. This told us that the conformational differences between the M and G residues played some role in determining biocompatibility, probably via their influence on the physical properties (stiffness, swellability) of the resulting gels. This viewpoint, however, has not been frequently mentioned, and so the results of this study, when they are published, will undoubtedly just add to the debate about what M/G composition of alginate is ideal for biocompatibility.

Our results also indicated that the intrinsic viscosity (molar mass) of the alginate also plays a role in determining alginate gel biocompatibility. Perhaps this is the better explanation for why the HiG alginate gels were immunogenic. After all, at least one group of researchers also promotes the use of high viscosity alginate for ultimate performance [21-23]. However, the design of our study did not allow us to determine which of the two factors was the more important for biocompatibility : M/G content or molar mass. Resolving this problem would require access to alginates with highly controlled properties, which can be obtained (ideally) by extracting the alginate directly from the plant to control polydispersity and viscosity [24, 25] and then tailoring the M/G composition by epimerization [26-28]. While this would be interesting for future studies, we had opted to stick to two kinds of commercially available alginates that are commonly used in the cell encapsulation field.

Finally, it must be noted that the biocompatible alginate is not necessarily the most suitable choice for cell encapsulation purposes, neither for biocompatibility nor for immunoprotection reasons. First of all, considering our results concerning microcapsule biocompatibility, the biocompatible alginate must also be able to interact with the polycation in an optimal manner in order to achieve biocompatibility of the microcapsule (with a membrane).

Furthermore, as we have seen, the biocompatible alginate produced a gel that was fragile. When we used this alginate to encapsulate islet transplants, a number of islets managed to move around within the gel, even escape the microcapsule or bead, and were rendered vulnerable to attack by the host immune system. Perhaps this problem could be overcome by using an alginate with a higher molecular weight or degree of polymerization [29, 30].

8.2.2 Can optimal biocompatibility be achieved when using a polycation?

Our results suggested that the polycation is the ultimate problem for microcapsule biocompatibility. This supports many previous studies that came to the same conclusion [31-35]. For this reason, a couple of research groups have already decided to move forward using uncoated alginate gel beads for encapsulating transplanted islets [33, 36-38]. However, this does not necessarily mean that the biocompatibility of microcapsules with a polycationic membrane cannot be optimized. The Groningen group and the Perugia group both use alginate-polycation microcapsules successfully for islet encapsulation, and seem to have overcome most problems associated with biocompatibility [39-42]. This proves that it is possible to achieve optimal biocompatibility when using a polycationic membrane, but the question remains how.

Our results indicated that the most important factor influencing biocompatibility is the ability for the polycation to diffuse into and bind to the alginate gel bead. It follows that both the alginate gel properties (including alginate M/G content and choice of crosslinking ion) and the polycation structure determine the stability and biocompatibility of the complete microcapsule. Previous studies have also investigated the interactions between alginate gel and polycation [43-47], but most did not directly correlate their results with biocompatibility. Ideally, the polycation must be tightly complexed, so that no unbound (charged) part of the polymer is exposed to the tissues at the implantation site [48]. We showed that the combination of PLO and IntG alginate can achieve this effect to a certain extent, possibly due to the shorter lengths of the PLO side chains (in comparison with PLL) that can affect its conformational preferences in each solution and upon complexation [49-51], and the greater flexibility of MG blocks which are less involved in alginate gelation [28, 52, 53], a combination that may lead to better binding between the two polymers. However, further studies must be pursued in order to confirm such a hypothesis.

Historically, researchers have been adding an alginate coating to the microcapsules in order to enhance their biocompatibility. The idea is that the alginate 'neutralizes' the surface as it binds to any unbound portions of the polycation, and as we know, unbound PLL is immunogenic [54]. We and others have recently provided evidence that the alginate coating does not exist as a distinct outer layer, but rather diffuses into the alginate-polycation membrane [12, 40, 46, 48]. However, the research presented in this thesis is the first study to use physicochemical analyses in combination with *in vivo* studies to emphasize that this alginate coating is almost completely ineffective for enhancing microcapsule biocompatibility, at least for our capsule formulations. The general immunogenicity of complete APA microcapsules supports this notion, in contrast to a single study that has directly shown that their alginate coatings did have an effect in reducing microcapsule immunogenicity [31]. Perhaps with the use of an enzymatically tailored alginate, the coating could be more effective, as King *et al* suggested [26]. However, relying on the alginate coating to optimize microcapsule biocompatibility seems unwise, based on the results of this study. Besides, any defects in the membrane would likely result in exposure of unbound polycation, despite the alginate coating [39, 55].

In addition to inducing host cell adhesion, the polycation membrane proved to be problematic for microcapsule stability. This is quite ironic, since one of the main reasons that researchers use the membrane (besides to reduce permeability) is to increase the stability of the microcapsules, since alginate gels are soft an susceptible to biodegradation by chelating agents [53]. In our case, adding a membrane prevented gel swelling (as expected), but resulted in capsules that were poorly resistant to shear stresses or compressive forces that were induced in the *in vivo* environment. At least one other group [33] also observed that adding a membrane reduced the *in vivo* stability of their microcapsules. When we used complete microcapsules to transplant islets into immunocompetent mice, almost all of the samples degraded after several weeks. This is unfortunate because the fragility of the microcapsules interfered with our ability to judge whether these membranes could successfully immunoprotect islets despite their moderate immunogenicity.

8.2.3 The missing pieces of the puzzle

While the relationships between the microcapsules chemistry, physicochemical characteristics, and *in vivo* biocompatibility have been clarified to a new level with this research, there are still a few physicochemical properties that were not characterized but could have added more pieces to the biocompatibility puzzle.

Surface morphology

As a general rule, smooth implant surfaces lead to better biocompatibility [14, 56]. Others have also started to prove this point, or at least emphasize the importance of smoothness, using atomic force microscopy (AFM) [57-59]. We attempted to measure this property (using AFM) on our own microcapsules, but we encountered numerous difficulties immobilizing the microcapsules in a liquid. Despite two years of trying, we never succeeded to keep the capsules still enough to generate reproducible results. We tried, without success, all of the fixation techniques that the other groups have tried, as well as a handful of others. Perhaps it was the smaller size (and thus smaller mass) of our microcapsules that made things problematic.

Surface charge density

Net surface charge of alginate-polycation microcapsules has been explored by two other research groups using zeta potential measurements [8-10]. This proved to be a good method to prove previous suspicions that polycation exposure leads to microcapsule immunogenicity due to the exposure of positive charges. Neither of these groups, however, tested the effect of the alginate coating for reducing this positive charge.

Initially, we proposed to investigate local charge densities on the microcapsule surface, as opposed to the net charge of several microcapsules. This type of measure could highlight inhomogeneities in the alginate coating or polycation distribution, and therefore explain microcapsule immunogenicity even if the net charge is neutral. The proposed methodology was to immerse the microcapsules into solutions of different pH, thereby inducing changes in the ionization degree of the polyelectrolytes, and then measure the force between the AFM tip (which is charged in an ionic solution) and the microcapsule surface, thereby producing force maps that indicate the local distribution of the polycation and polyanion [60]. Such a study has

never been attempted before. However, as previously mentioned, we unfortunately never got past the sample fixation step.

Wettability of the hydrated microcapsules

Almost all microcapsules designed for cell transplantation are based on hydrogels, due to the pliability and hydrophilicity of these gels, which act to minimize protein and cell adhesion [3, 61]. In our research, we evaluated wettability of our samples by measuring the static contact angles of water droplets on dehydrated films that mimic the chemistry of our microcapsules. However, this is rather an evaluation of the sample's capacity to rehydrate, and may not be representative of the hydrophilicity of the hydrated samples. To overcome this shortcoming, we started to measure the contact angle of air bubbles on hydrated films. Our preliminary results (not shown) showed that the air bubbles rolled off of all of the hydrated samples, independent of the composition of the film (type of alginate, with or without a polycation). Unfortunately, these studies were not pursued due to a lack of time. It would be interesting, however, to elaborate on this concept to show demonstrate any possible correlations between the hydrophilicity of the hydrated samples and their biocompatibility.

8.3 Standardization of microcapsules for encapsulation

No "winning formula" for achieving optimal microcapsule biocompatibility has been found, and no official standards have been written to this effect. This is a direct consequence of the many in-house protocols that have been developed for the fabrication of alginate-polycation microcapsules [62]. This situation has made it difficult to directly compare results between different study groups, thus hindering our progress towards the rational design of the optimal microcapsule. That said, this problem was addressed by researchers in the bioencapsulation field a few years back. As part of a COST865 funded project [63], a number of European research groups collaborated together in an effort to characterize and standardize the properties and characterization methods of microcapsules designed for bioencapsulation. The results of this collaborative effort have been recently published in a paper that 'strongly advises' the use of specific characterization techniques in future studies in order to improve reproducibility and help researchers understand variations in outcomes of biocapsules [64].

8.4 Biocompatibility of the encapsulated islet system

This research focused on the biocompatibility of empty microcapsules, since studies have shown evidence that an immune response to the microcapsules contributes to the death and dysfunction of the cells enclosed within [65-67]. It should be briefly mentioned, however, that once an islet is enclosed within the microcapsule, the system is changed. This should absolutely be kept in mind when considering the biocompatibility of the complete encapsulated islet system. Factors that should be considered include:

Microcapsule stability : Broken or damaged capsules expose the islets and render them vulnerable to recognition and attack by the host immune system.

Membrane permeability **:** Cytokines, nitric oxide, and other small molecules that can possibly penetrate the microcapsule membrane can enter the microcapsule and induce cell damage [65-68]. Damaged or dying cells, as well as xenografts, may release chemical substances and shed antigens that can attract the host immune system [69-71].

Microcapsule size **:** Smaller capsules have been associated with bulging, i.e. protrusion of the islets due to inadequate encapsulation [72, 73]. Again, exposed islets can attract a response from the host immune system.

Transplantation site **:** Some transplantation sites may be more suitable than others for accessibility reasons, proximity to the insulin-producing organs, or the partial pressure of oxygen (pO_2), but these sites also differ in terms of the mechanical stress than can be induced on the microcapsules and the concentration of macrophages and other immune cells [74-79].

Animal model **:** Of course, the ultimate goal is to transplant into humans, but pre-clinical trials necessarily involve animal models. The sensitivity of the animal (i.e. the severity of its immune response to implants) is strain and species dependent [80-82], and conclusions about “biocompatibility” of the system should therefore be stated with caution.

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CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The biocompatibility of alginate-polycation microcapsules could not be optimized. They consistently showed immunogenicity, as characterized by host cell adhesion to the microcapsules *in vivo*, and supported by the adsorption of immunoglobulin *in vitro*. These foreign body reactions were clearly moderated by the presence of the polycation. Moreover, the alginate coating, intended to improve the biocompatibility of the microcapsules, had no significant effect on the physicochemical properties, nor on the biocompatibility of the samples. Although all microcapsule designs tested showed signs of being immunogenic, we showed that it may be possible to moderate the severity of response by simply choosing a combination of materials (alginate, crosslinking ion, polycation) that optimizes the penetration and binding of the polycation into the alginate gel. When tested with islets within, it was not the moderate immune response to empty microcapsules that was most problematic for graft survival, but the tendency of the microcapsules to degrade.

In contrast, the alginate was proven to be perfectly biocompatible under specific conditions. In our case, this was an intermediate guluronate content, higher molecular mass and adequate purity level. The biocompatibility of these alginate gels, however, was dampened by their fragility, which will be a problem for cell immobilization purposes. Nevertheless, graft survival was improved with the use of alginate gel beads without a membrane.

Recommendations

In order to improve the biocompatibility of the alginate-polycation microcapsule, it is recommended to optimise the diffusion and the binding of the polycation to the alginate gel. In order to find the most suitable combination(s) of parameters to optimise polycation-alginate interactions (choice of materials, gelling dynamics, etc.), it is suggested to start with a thorough evaluation and screening process of different formulations using FTIR or similar techniques to monitor the intermolecular interactions. After getting through this initial step, it is recommended

to support this data by verifying the various other physicochemical properties that have been seen to correlate with biocompatibility, notably the membrane wettability (contact angle technique), surface charge (zeta potential), and surface morphology (atomic force microscopy or AFM).

In contrast, it is not recommended to focus on improving the alginate coating technique as a strategy to improve microcapsule biocompatibility. Although it has been demonstrated that dilute alginate does indeed penetrate into the polycationic membrane and may even alter the surface morphology of the microcapsules, there has been little experimental evidence that this coating is effective for 'neutralizing' the surface of the device. Indeed, instead of trying to "cover" the problems associated with the polycation, it seems more wise to concentrate directly on the polycation so that there's no problem to cover.

It must be noted that even if the microcapsule biocompatibility is optimized, an immune response to the device is not necessarily, even unlikely, to be completely eliminated. Injury caused by the implantation surgery as well as physical imperfections of individual microcapsules are two scenarios that are essentially unavoidable yet likely to stimulate an inflammatory response. For this reason, incorporation of anti-inflammatory agents into the microcapsule system, which are effective short-term and work locally (vs. systematically), should be considered [1-3].

Once biocompatibility of the microcapsule has been established, it is absolutely necessary to verify the effects of any modifications to the microcapsule design on its stability, permeability, and diffusion properties, since a biocompatible microcapsule is not very useful if it is incapable of immunoprotecting the transplanted cells enclosed within.

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APPENDIX I

SOLUTION FORMULAE

Solution name	**Formula**
$BaCl_2$, 10 mM (Mtl)	10 mM $BaCl_2$, 16 mM HEPES, pH 7.4
$BaCl_2$, 10 mM (Gron)	10 mM $BaCl_2$, 2 mM KCl, 135 mM NaCl, 10 mM HEPES, pH 7.4
Ca^{2+}-free KRH, 220 mOsm	90 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.4
Ca^{2+}-free KRH, 310 mOsm	135 mM NaCl, 4.7 mM KCl, 25 mM HEPES, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$, pH 7.4
$CaCl_2$, 100 mM	100 mM $CaCl_2$, 2 mM KCl, 10 mM HEPES , pH 7.4
Ca Lactate, 100 mM	100 mM Ca Lactate
KRH (Krebs-Ringer-Hepes), 25 mM	133 mM NaCl, 4.69 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 25 mM HEPES, 2.52 mM $CaCl_2$, pH 7.4
Ringer's solution	154 mM NaCl, 5.6 mM KCl, 1.7 mM $CaCl_2$, 5 mM HEPES, pH 7.4
Saline, 154 mM	154 mM NaCl, 12.5 mM HEPES, pH 7.4
Spoelhepes	132 mM NaCl, 4.7 mM KCl, 1. mM $MgCl_2$, 25 mM HEPES, 2.5 mM $CaCl_2$

APPENDIX II

BOOK CHAPTER

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La microencapsulation pour la thérapie cellulaire

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Résumé

La microencapsulation permet la transplantation sécuritaire de cellules et de tissus qui sécrètent des produits thérapeutiques pour le traitement d'un grand nombre de maladies. En principe, en microencapsulant les cellules avant de les transplanter, elles sont protégées contre le rejet par le système immunitaire de l'hôte. Ce chapitre résume le concept de l'immunoprotection des cellules, incluant les avantages et inconvénients d'utiliser les microcapsules comme choix de dispositif immunoprotecteur. Les méthodologies pour la microencapsulation de cellules vivantes sont discutées en détail. En plus, les caractéristiques des microcapsules requises pour l'application en thérapie cellulaire sont présentées. Des commentaires sur les perspectives dans le domaine de la microencapsulation pour la thérapie cellulaire sont également présentés.

Mots-clés : microencapsulation, thérapie cellulaire, transplantation, immunoprotection, biocompatibilité

1. Introduction

La microencapsulation permet la transplantation sécuritaire de cellules ou de tissus biologiques pour la thérapie potentielle d'un grand nombre de maladies, particulièrement celles qui sont causées par une déficience d'une hormone, d'un facteur ou d'une enzyme. Actuellement, des recherches sont en cours sur l'application de la microencapsulation des cellules pour le traitement du diabète [1], de l'hémophilie [2], du nanisme [3], du cancer

[4], de l'insuffisance rénale [5] et hépatique [6], de l'anémie [5], de l'insuffisance pituitaire [7], des maladies du système nerveux central (tels que la maladie de Parkinson ou la chorée Huntington) [8], des désordres ophtalmiques [9], et la douleur chronique [10], pour en nommer seulement quelques exemples.

La thérapie cellulaire représente une approche très prometteuse pour traiter ces genres de désordres. Souvent, les patients affectés par ces maladies sont traités par l'administration de l'hormone ou de la protéine manquante, par exemple, par injection. Par contre, il est souvent extrêmement difficile de mimer avec précision les dosages naturellement présents, ainsi que les rôles complexes de l'hormone/enzyme/facteur original, avec un substitut qui est produit et introduit *ex vivo*. Dans d'autres cas, l'administration du produit thérapeutique par injection à certains sites n'est pas évidente (par exemple à cause d'une barrière hémato-encéphalique). Avec la thérapie cellulaire, des lignées cellulaires ou des cellules/tissus allogéniques ou xenogéniques sont transplantés chez le patient pour lui fournir une source du produit thérapeutique qui sera libéré dans les dosages contrôlés biologiquement, et donc précisément en fonction du volume, du temps, et des facteurs endogènes (comme la glycémie dans le cas du diabète).

La microencapsulation est une approche qui vise à assurer l'immunoprotection des cellules ou des tissus transplantés, un concept qui est expliqué en détail dans la section 2.1. En principe, en immobilisant le greffon à l'intérieur d'une membrane semi-perméable et biocompatible, une réponse immunitaire contre le transplant est évitée grâce à une barrière physique entre les composants du système immunitaire et les cellules ou tissus greffés. L'avantage principal de l'immunoprotection est qu'elle permet la thérapie cellulaire sans nécessiter l'administration à vie de médicaments immunosuppresseurs puissants. Des tels médicaments sont relativement toxiques, sont associés à de nombreux effets secondaires, et laissent le patient susceptible à de graves infections et même le cancer. De plus, si l'immunosuppression est efficace sur le rejet aigu, elle n'a qu'un faible intérêt sur la prévention du rejet chronique qui reste une cause notable de rejet du greffon. Parmi les différentes formes géométriques des dispositifs immunoprotecteurs, les microcapsules offrent des avantages (et quelques inconvénients) spécifiques qui sont présentés dans la section 2.2.

La microencapsulation pour la thérapie cellulaire représente un système complexe puisque les capsules contiennent des cellules vivantes, sont implantées dans le corps animal ou humain, et ont généralement pour but une fonction sur un long-terme. Les méthodes de production de ces dispositifs, ainsi que de nombreuses caractéristiques physiques et chimiques des microcapsules, doivent alors répondre à des conditions spécifiques dont plusieurs ne sont pas requises pour les applications industrielles ou non-médicales. Les matériaux de fabrication et les principales méthodes de production des microcapsules pour la thérapie cellulaire sont présentés dans la section 3. En plus, des caractéristiques des microcapsules qui sont considérées comme nécessaires pour assurer la survie et le fonctionnement des cellules encapsulées, incluant, la perméabilité sélective, la taille contrôlée, la stabilité, et la biocompatibilité, sont décrits dans la section 4.

Finalement, des perspectives dans la domaine de la microencapsulation pour la thérapie cellulaire sont discutées dans le section 5.

2. La microencapsulation pour l'immunoprotection

2.1. Le concept d'immunoprotection

Le terme « immunoprotection » se réfère à la protection des cellules ou des tissus transplantés contre le système immunitaire de l'hôte. Ce terme est souvent remplacé dans la littérature par « immunoisolation » ou même « immunomodulation ». Par contre, les auteurs jugent que immunoprotection est le terme le plus approprié puisque, par définition, il inclut les deux autres termes. C'est à dire que l'immunoisolation fait référence à l'utilisation d'une membrane semi-perméable pour empêcher les composants du système immunitaires de reconnaître et d'attaquer subséquemment le greffon encapsulé, tandis que l'immunomodulation suggère une interaction ou une intervention proactive avec le système de l'hôte. L'utilisation du terme immunoprotection est encore justifiée du fait qu'il n'est pas encore confirmé si les microcapsules sont capables d'immunoisoler le greffon avec 100% efficacité ou si au contraire elles sont innocentes quant à leur participation dans l'immodulation [11].

Peu importe le choix du terme, le but de la microencapsulation est de protéger les cellules encapsulées contre la reconnaissance et l'attaque subséquente du système immunitaire en présentant une barrière physique qui, en principe, bloque le contact direct entre le greffon et les principales composantes du système immunitaire de l'hôte, surtout celles de haut poids moléculaire, incluant les cellules immunitaires et les anticorps. En plus, la membrane de la microcapsule peut moduler la diffusion des cytokines, des antigènes, et autres molécules immunologiques. Pour assurer la survie et le fonctionnement des cellules encapsulées, la membrane doit simultanément assurer la diffusion libre des nutriments et de l'oxygène qui proviennent du sang, des déchets du métabolisme et du produit thérapeutique (e.g. l'hormone déficiente) qui sont produits par les cellules encapsulées, et, s'il y a lieu, de la substance qui stimule la libération de l'hormone (e.g. glucose). Le concept de l'immunoprotection par la microencapsulation est representé schématiquement dans la Figure 1.

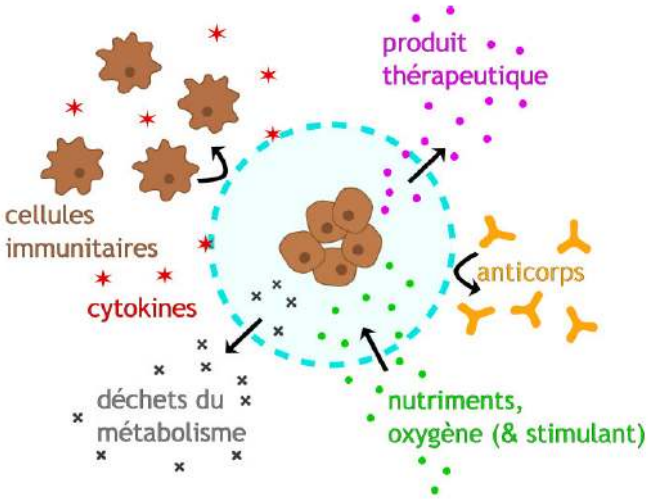


Figure 1 : Représentation schématique du concept d'immunoprotection par la microencapsulation des cellules/tissus transplantés

2.2. Les avantages et les inconvénients des microcapsules comme dispositifs immunoprotecteurs

Comme immunoprotectrices, les microcapsules offrent des avantages spécifiques, ainsi que quelques inconvénients, en comparaison avec les macrocapsules ou autres dispositifs macroscopiques. Quoique tous ces dispositifs aient le même but, à savoir celui d'offrir l'immunoprotection aux cellules ou aux tissus transplantés, il existe des différences fondamentales entre les microcapsules et les macrodispositifs, surtout en ce qui concerne la géométrie, la taille et le matériel composant, qui ont un impact important sur la bioperformance finale du transplant. Les dispositifs pour la macroencapsulation sont généralement conçus pour l'implantation soit par voie intravasculaire ou par voie extravasculaire. Ils ont typiquement une géométrie tubulaire, celle d'une fibre creuse ou planaire sous forme de disque, avec des dimensions supérieures à 1 cm, la plupart étant fabriqués en thermoplastiques (e.g. PAN-PVC). Par contre, les microcapsules ont une géométrique sphérique, avec un diamètre qui peut varier de 0,2 à 1 mm, et elles sont souvent formées de composés à base d'hydrogel (voir la section 3.1).

Quelques différences importantes entre les microcapsules et les macrodispositifs en termes de leurs bioperformances et de leurs capacités comme immunoprotecteurs des cellules transplantées sont résumées dans le Tableau 1. On considère généralement que les microcapsules sont la meilleure option pour la thérapie cellulaire, malgré quelques inconvénients qui justifient des études qui sont encore en cours actuellement. Les principales caractéristiques des microcapsules et des macrocapsules sont aussi décrites de

manière exhaustive dans l'article récemment publié par Uludag et al [12], raison pour laquelle les auteurs de ce chapitre réfèrent les lecteurs à cet article pour plus de détails sur la macroencapsulation.

Tableau 1 : Comparaison des caractéristiques entre des microcapsules et des macrodispositifs immunoprotecteurs

Microcapsules	Macrodispositifs
Rapport surface-volume élevé, ce qui améliore la diffusion à travers la membrane	Membranes épaisses et la géométrie limitent la diffusion à travers la membrane
Degrés de biocompatibilité variables entre laboratoires	Tendance à être recouverts par la fibrose après l'implantation
Faible volume de transplant permet l'accès à plusieurs sites d'implantation par chirurgie minimale ou injection	Grande taille limite les sites et la facilité d'implantation
Difficiles à récupérer 100% du volume implanté	Généralement faciles à explanter, et possibilité de recharger le dispositif
Résistantes à des forces mécaniques, mais la stabilité à long-terme est variable selon le type de microcapsule	Matériaux de fabrication sont forts mécaniquement, mais les dispositifs ont une tendance à se casser
Possibilité d'ajuster la perméabilité, mais la distribution de la taille des pores est large	Possibilité de percer des pores de taille uniforme [13]

En plus des microcapsules conventionnelles et des macrocapsules, il y a actuellement des études menées sur des microcapsules ultra-petites, appelées microcapsules cohérentes, nanocapsules, ou capsules de taille sous-tamis [14-18]. Ces genres de microcapsules sont souvent produits par la technique du **revêtement conforme** (« **conformal coating** »). Cette technique vise à minimiser l'espace vide entre les cellules et l'extérieur de la microcapsule en enrobant le greffon directement avec le polymère protecteur pour former une couche mince qui est généralement de 10 à 25 µm d'épaisseur. Les avantages des microcapsules d'une telle taille sont décrits dans la section 4.3.

3. Techniques de microencapsulation de cellules

La microencapsulation de cellules en vue d'une transplantation comporte des exigences spécifiques qui ne se posent pas dans la plupart des applications industrielles ou non-médicales. Celles-ci concernent surtout la nécessité de ne pas compromettre la viabilité ou le fonctionnement des cellules pendant la production des microcapsules. Cette exigence élimine toutes les méthodes d'encapsulation qui requièrent des conditions non-compatibles avec la survie cellulaire (telles que l'exposition prolongée à des pH ou à des températures non physiologiques ou à l'utilisation de solvants toxiques), ce qui conduit obligatoirement à l'utilisation de matériaux non-cytotoxiques.

Le choix des matériaux et de la technique de production des microcapsules déterminent essentiellement toutes les caractéristiques et par conséquent la bioperformance finale des microcapsules obtenues. Ces exigences ont conduit inexorablement, ces dernières années, au développement de nombreuses méthodes de microencapsulation de façon à optimiser les caractéristiques des microcapsules. Il est important de noter également que la sélection des matériaux et de la technique d'encapsulation dépend grandement de l'application spécifique du transplant. Parmi les facteurs à prendre en considération lors de la sélection des paramètres pour réaliser l'encapsulation, il faut mentionner d'une part le type de greffon à encapsuler (e.g. les cellules individuelles comparées aux agrégats ou aux cellules d'ancrage dépendantes) et d'autre part le site d'implantation. Ce dernier détermine les conditions physiologiques d'exposition du transplant ainsi que le volume maximum du transplant. Des matériaux et des techniques d'encapsulation qui sont actuellement employés pour la microencapsulation des cellules/tissus sont décrites ci-dessous.

3.1. Choix des matériaux

La liste des matériaux qui sont étudiés pour la microencapsulation des cellules et des tissus s'est considérablement allongée dans le but de trouver de nouvelles formules pour optimiser la bioperformance de ces dispositifs immunoprotecteurs. La plupart des matériaux ou des polymères qui ont été étudiés comme candidats potentiels pour l'encapsulation ont été référencés dans des articles de revue [19-21]. Ceux qui sont utilisés les plus fréquemment ou les plus récemment sont présentés ci-dessous.

Les hydrogels

La grande majorité des microcapsules destinées à la thérapie cellulaire est à base d'hydrogels. Les hydrogels sont des réseaux tri-dimensionnels composés de chaînes polymères qui sont très hydrophiles et réticulées. Les hydrogels présentent plusieurs avantages comme matériau de base pour les microcapsules [12,20] :

- Ils forment facilement des billes
- Ils sont transparents, ce qui permet la visualisation des cellules à l'intérieur
- Plusieurs hydrogels naturels peuvent être formés dans des conditions « douces » qui n'ont pas d'effet néfaste sur la viabilité cellulaire
- Les propriétés physicochimiques molles et la souplesse des gels minimisent l'irritation des tissus locaux entourant l'implant
- Leur hydrophilie leur assure une tension interfaciale minimale entre la surface de la capsule et les fluides/tissus de l'hôte, ce qui a pour effet d'empêcher l'adsorption des protéines et l'adhésion des cellules
- Ils sont très perméables aux substances de faibles poids moléculaires, tels que les nutriments et les métabolites, ce qui assure leur libre diffusion.

Des exemples de polymères qui se transforment en hydrogels et qui sont utilisés pour l'immobilisation et la microencapsulation de cellules vivantes comprennent les alginates (Alg), l'agarose, les copolymères d'hydroxyéthyl méthacrylate-méthyl méthacrylate (HEMA-MMA), et le polyéthylène glycol (PEG) ou ses dérivés. Les caractéristiques

principales de ces polymères en tant que matériaux destinés à la microencapsulation, ainsi que quelques exemples de microcapsules constituées de chacun de ces hydrogels, sont présentées dans le Tableau 2.

Tableau 2 : Polymères qui se transforment en hydrogels et qui sont utilisés pour la microencapsulation de cellules ou de tissus

Polymère	Caractéristiques principales	Exemples de microcapsules
Alginate	• Polyanion naturel • Gélifie rapidement en présence des cations divalents (Ca^{2+}, Ba^{2+}) • Utilisé principalement pour la matrice au cœur de la microcapsule et pour la couche extérieure biocompatible • Se complexe facilement avec des polycations par liaisons électrostatiques pour former une membrane • Gel susceptible à une dégradation chimique par des agents chélateurs non complexés avec un polycation	• Barium-Alg [22] • Alg/Poly-L-lysine/Alg [23] • Alg/Poly-L-ornithine/Alg [24] • Alg/Poly(méthylène-co-guanidine)/Alg [25] • Alg/Cellulose sulfate/Poly(méthylène-co-guanidine) [26] • Alg/Aminopropyl-silicate/Alg [27] • Alg/Chitosan [28] • Alg/Chitosan/PEG/Poly-L-lysine/Alg [29] • Alg/Poly-L-lysine/(short-chain alginate)-co-mPEG [30]
Agarose	• Polymère neutre et naturel • Gélifie par une baisse de température (vers 25-30ºC) • Utilisé principalement pour la matrice au cœur de la microcapsule • Forme des gels relativement faibles mécaniquement	• Agarose [31] • Alginate-Agarose [32] • Alginate-Agarose/Chitosan [33] • Agarose- Polystyrene sulfonic acid [34] • Agarose- Polystyrene sulfonic acid/Polybrene/Carboxyméthyl cellulose [35] • Agarose/HEMA-MMA [36]
HEMA-MMA	• Copolymère synthétique • Non-soluble dans l'eau, ce qui oblige de recourir à l'utilisation des solvants organiques cytotoxiques • Se transforme en gel par la précipitation (l'extraction du solvant) • Gel très stable dans les fluides aqueux/physiologiques • Utilisé principalement pour le revêtement conforme	• HEMA-MMA [37] • Collagène/HEMA-MMA-MMA/Alumina ou Chitosan [38] • Collagène/HEMA-MMA-MAA [39] • Agarose/HEMA-MMA [36]

PEG et derivés	• Connu pour défavoriser l'adsorption des protéines et l'adhésion cellulaire à la surface des biomatériaux • Se transforme en gel par la photopolymerization in situ • Utilisé principalement pour le revêtement conforme ou pour la couche extérieure biocompatible	• PEG diacrylate [40] • Alg/Poly-L-lysine/PEG [41] • Alg/Chitosan/PEG/Poly-L-lysine/Alg [29] • Alg/Poly-L-lysine/(short-chain alginate)-co-Mpeg [30]

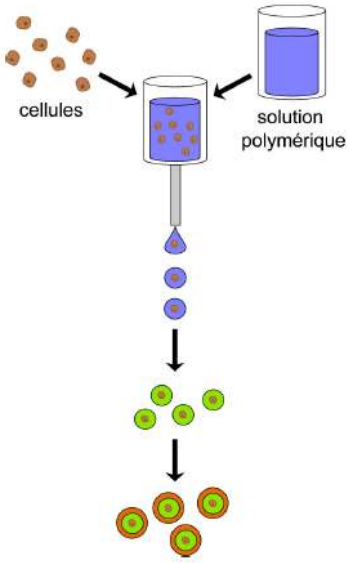
Parmi ces polymères, **l'alginate** (et des variations de formules basées sur l'alginate) est encore celui qui est le plus étudié comme constituant des microcapsules. L'alginate a des caractéristiques uniques qui le rendent très approprié pour l'encapsulation de cellules vivantes, à savoir sa biocompatibilité, sa capacité de se transformer en gel très rapidement et dans les conditions physiologiques, ainsi que sa capacité de complexer facilement avec d'autres polymères. D'excellents articles décrivent en détail les caractéristiques d'utilisation des alginates en tant que matrice d'immobilisation des cellules [42]. Pour ces raisons mais également parce qu'elles sont largement étudiées, les microcapsules à base d'alginate présentent probablement la gamme d'application la plus étendue dans le domaine de la thérapie cellulaire. Encore aujourd'hui, de nombreux travaux sont menés afin d'optimiser encore plus les caractéristiques d'utilisation de l'alginate pour l'encapsulation des cellules. Ceux-ci comprennent des études destinées à l'amélioration de leur pureté [43] et à la production d'alginates avec une composition chimique (ç-à-d la distribution d'acide guluronique/mannuronique) contrôlée [44]; ces caractéristiques du polymère ont une influence significative sur les propriétés de gonflement, de la porosité ou de la perméabilité, de la biocompatibilité, et de la résistance mécanique et chimique des microcapsules à base d'alginate.

D'une manière générale, il n'est pas réaliste de penser qu'un seul polymère ou qu'une seule combinaison de polymères soit approprié pour tous les cas de figures. Pour encapsuler des cellules avec succès, les matériaux sélectionnés pour la fabrication des microcapsules doivent simultanément satisfaire une liste de critères, qui peuvent inclure la résistance mécanique, la taille des pores, l'uniformité des capsules, les caractéristiques de dégradation, de biocompatibilité, et présenter une immunogénicité négligeable [19]. Souvent ces caractéristiques sont conflictuelles et, de plus, les caractéristiques recherchées dépendent de l'application spécifique du transplant.

3.2. Techniques de production de billes et de microcapsules

Il existe de nombreuses techniques qui sont actuellement utilisées ou étudiées pour la microencapsulation des cellules ou de tissus. La diversité des méthodes est une conséquence des différents choix potentiels de polymères disponibles (voir la section précédente) et des différentes exigences des applications spécifiques du transplant.

D'une manière très générale, le procédé qui permet la microencapsulation de cellules vivantes est réalisé en 4 étapes, illustrées dans la Figure 2:



Étape 1
Répartition homogène des cellules/tissus dans une solution polymère

Étape 2
Production de gouttelettes de la suspension cellulaire

Étape 3
Gélification des gouttelettes pour former des billes

Étape 4
Création d'une membrane autour de la bille

Figure 2 : Etapes générales pour la microencapsulation de cellules ou de tissus

Les 4 étapes décrites ci-dessus sont représentatives d'un procédé de microencapsulation typique qui implique surtout des interactions électrostatiques de polyélectrolytes pour la production de microcapsules [45]. D'autres méthodes de microencapsulation sont décrites dans la dernière partie de cette section.

Étape 1 : La répartition homogène des cellules/tissus dans une solution d'un polymère

Cette étape requiert une répartition homogène des cellules dans la solution du polymère pour former une suspension cellulaire. Évidemment, la solution employée doit être compatible avec les cellules vivantes en termes de composition chimique, de pH et de température. En général, les polyanions sont moins cytotoxiques que les polycations, raison pour laquelle ils sont les plus utilisés. La solution doit également être capable de gélifier ou de se solidifier dans des conditions douces qui ne compromettent pas la viabilité des cellules (Étape 3). De plus, la viscosité de la solution (qui est déterminée par le poids moléculaire du polymère et de la concentration de la solution) a un effet important sur la forme, la taille et la morphologie des gouttelettes/billes produites au cours des étapes ultérieures. À la fois, la concentration de la solution influence la densité du réseau, la stabilité et la perméabilité des billes gélifiées ou solidifiées [45].

Étape 2 : La production de gouttelettes de la suspension cellulaire

Pour produire des gouttelettes, la suspension cellulaire est généralement extrudée à travers une buse ou une aiguille à l'aide d'une seringue mue par une pompe ou motorisée. Le débit de la suspension est ensuite interrompu pour créer des gouttelettes. Il est important que ce processus ne communique pas de stress mécanique ou de forces de cisaillement excessives aux cellules, ce qui pourrait les endommager. En plus, pour des raisons expliquées dans la section 4, il est nécessaire que les gouttelettes formées aient une taille contrôlée (généralement 100-1000 µm de diamètre) et uniforme, et possèdent une forme parfaitement sphérique.

Les méthodes habituelles de production de gouttelettes comprennent l'utilisation d'une force de cisaillement (crée par un jet d'air [46] ou l'écoulement d'une solution non miscible [31]), une tension électrostatique [47], ou des vibrations [48] pour détacher les gouttelettes du bout de l'aiguille ou de la buse. Dans d'autres cas, le jet laminaire de la solution peut être coupé physiquement à l'aide d'un fil [49]. Parmi ces méthodes, les deux plus répandues sont d'une part l'utilisation d'un jet d'air propulsé dans la partie externe d'une aiguille co-axiale et d'autre part un système d'impulsions électrostatiques. Ces deux systèmes, décrits en détail ci-dessus, sont illustrés dans la Figure 3. Ils sont particulièrement utiles pour former des gouttelettes (et donc des billes et des microcapules) de petite taille et d'une façon reproductible.

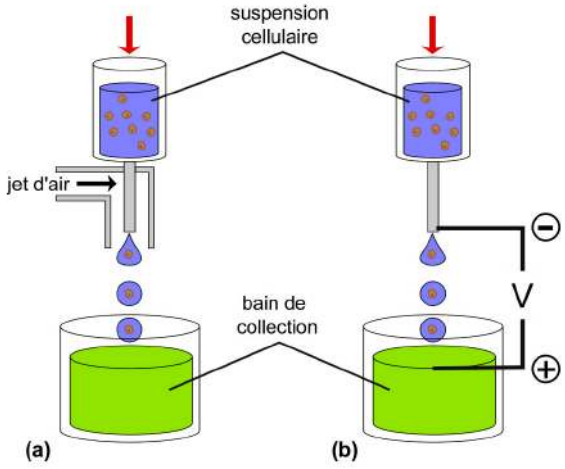


Figure 3 : Production de gouttelettes d'une suspension cellulaire à l'aide de (a) un jet d'air, et (b) une tension électrostatique

La production des gouttelettes par un jet d'air. Cette méthode, illustrée dans la Figure 3a, requiert l'utilisation d'une aiguille coaxiale. La solution de polymère dans laquelle les cellules sont mises en suspension (typiquement l'alginate de sodium) est poussée à travers le cylindre creux interne de l'aiguille. Le jet d'air circule dans la partie externe, autour de l'aiguille, pour exercer une traction sur la gouttelette en cours de formation et la

détacher précocement du bout de l'aiguille avant qu'elle n'ait atteint sa taille définitive. Un jet d'air plus rapide entraîne la formation d'une gouttelette plus petite. À l'inverse, un débit plus élevé de la solution polymère permet à la gouttelette d'atteindre une taille plus importante avant d'être détachée et conduit également à une augmentation de la vitesse de production. Le calibre de l'aiguille et la viscosité de la solution polymère ont également un effet sur la taille des capsules, leur sphéricité, la morphologie de leur surface et leur uniformité. En général, les billes formées par un jet d'air sont plus grandes (> 600 µm diamètre) car la formation de billes sphériques requiert une viscosité assez élevée de la solution polymère. La viscosité de la suspension détermine le diamètre interne de l'aiguille puisque cette dernière pourra être bloquée s'il est trop petit pour laisser passer facilement la suspension visqueuse. Pour cette application, plusieurs chercheurs préfèrent utiliser le système de jet d'air, croyant avantageux d'utiliser des polymères de très hautes viscosités pour la production de microcapsules implantables [50].

La production de gouttelettes par tension électrostatique. Dans ce système, illustré dans la Figure 3b, la solution polymère est forcée au travers de la cavité interne d'une simple aiguille. La gouttelette suspendue au bout de l'aiguille est chargée d'une tension statique et l'électrode de polarité opposée est soit attachée à la cuvette de collection ou placée juste au-dessus. La différence de potentiel entre les deux points, quand elle dépasse un seuil spécifique, attire la gouttelette vers le deuxième point, soit vers la cuvette, avant qu'elle n'ait atteint sa taille définitive. À chaque impulsion électrostatique, une gouttelette se détache. Le débit de la solution polymère ainsi que la fréquence des impulsions électrostatiques sont les deux principaux facteurs déterminant la taille et la vitesse de production des gouttelettes. En effet, un plus grand débit permet à la gouttelette d'atteindre une plus grande taille entre deux impulsions. À l'inverse, une fréquence rapide des impulsions limite la taille des gouttelettes. Différentes combinaisons débit-fréquence peuvent par conséquent conduire à la production de gouttelettes de tailles désirées et qui peuvent de surcroît être prédites de façon précise à l'aide d'un modèle mathématique [47]. Inversement, le calibre de l'aiguille a peu d'effet sur la taille des gouttelettes. Toutefois, une aiguille trop petite se bloquera facilement alors qu'avec une aiguille de plus gros calibre, la gouttelette se fragmentera en gouttelettes difformes. Une viscosité minimale est requise pour que la gouttelette reprenne sa forme parfaitement sphérique avant de toucher la solution de collection. Cependant, cette viscosité est beaucoup plus basse avec le système par impulsions électrostatiques qu'avec le système par jet d'air. En conséquence, l'avantage principal de la technique par impulsions électrostatiques, comparée à celle de la production par jet d'air, est la production de billes de petites tailles, à savoir de l'ordre de 180 µm de diamètre.

Étape 3 : La gélification des gouttelettes pour former les billes

La gélification des gouttelettes permet la création de billes qui pourront immobiliser et protéger des cellules vivantes. Dans le cadre de la microencapsulation des cellules, la transformation des solutions polymères en gel est réalisée, le plus souvent par gélification ionotropique. Cette méthode de gélification est basée sur la capacité d'un polyélectrolyte d'être réticulé en présence de contre-ions pour former un hydrogel. Les alginates peuvent

être gelifiés de cette manière en présence de cations divalents (ou trivalents). Le Ca^{2+} est habituellement utilisé pour réticuler les alginates puisqu'il est non-toxique, alors que l'utilisation de Ba^{2+} conduit à l'obtention de gels plus résistants. L'obtention d'une bille d'alginate par gélification ionotropique peut être réalisée par la simple chute d'une gouttelette d'alginate (sous forme de solution aqueuse) dans une deuxième solution aqueuse contenant les contre-ions, comme le $CaCl_2$. Le gel est formé presque immédiatement après contact entre les deux solutions, ce qui permet à la bille de garder la forme sphérique de la gouttelette.

Les gels thermoréversibles sont formés par un changement de température. Un exemple important d'un tel gel pour l'immobilisation de cellules est l'agarose. L'obtention d'un tel gel se fait généralement de la manière suivante. Dans un premier temps, une solution d'agarose est préparée à la température corporelle (37ºC). Dans un second temps, des gouttelettes de la solution sont extrudées à travers une aiguille dans un fluide non miscible avec l'agarose (par exemple la paraffine) et les gouttelettes ainsi obtenues sont gélifiées dans un troisième temps en diminuant la température de la solution (< 30ºC) [31].

Étape 4 : La création d'une membrane autour de la bille

La membrane est habituellement créée à la surface d'une bille d'hydrogel suite à l'intéraction électrostatique (la complexation) entre la surface de la bille et un polyélectrolyte de charge opposée. A titre d'exemple, des polycations comme le poly-L-lysine ou le poly-L-ornithine peuvent se lier électrostatiquement à la surface des billes de gels d'alginate (qui sont chargées négativement) pour créer la membrane. L'ajout d'une telle membrane est important non seulement pour réduire la porosité de la microcapsule, mais également pour améliorer la stabilité mécanique et chimique des microcapsules obtenues. Sans cette « consolidation », les hydrogels (plus précisément, ceux qui sont réticulés par des contre-ions) sont susceptibles d'être dissous par des chélateurs dans l'environnement physiologique.

Après l'ajout d'un polycation, il est souvent nécessaire de neutraliser ou d'équilibrer les charges libres à la surface des microcapsules en immergeant les capsules dans une solution polyanionique biocompatible qui se lie électrostatiquement à la membrane (par exemple l'alginate dilué). Cette étape est généralement nécessaire pour améliorer la biocompatibilité des microcapsules puisque les surfaces neutres ou chargées négativement ont tendance à empêcher l'adsorption des protéines et l'adhésion des cellules, comparé à ce qui est observé avec des surfaces chargées positivement.

Notons que quelques chercheurs optent de ne pas ajouter une membrane distincte à la surface des billes d'hydrogel. Dans ces cas, la matrice gélifiée (spécifiquement, l'alginate de baryum) doit présenter une porosité assez petite pour donner l'immunoprotection requise par les cellules encapsulées. La perméabilité est contrôlée par un gradient de densité de la concentration de l'alginate ainsi que par l'utilisation d'alginates de hauts poids moléculaires. De façon impressionnante et inattendue, des résultats *in vivo* très prometteurs ont été publiés en ayant recours à cette approche [51].

Autres méthodes de microencapsulation de cellules ou de tissus

La précipitation par l'extraction d'un solvant. Cette méthode s'applique à des polymères non-solubles dans l'eau, en particulier pour l'hydroxyéthylméthacrylate-méthylméthacrylate (HEMA-MMA). En général, le polymère est dissous dans un premier temps dans un solvant organique puis, dans un deuxième temps, au contact d'une solution aqueuse, le polymère précipite. Pour enrober des cellules ou des billes dans un gel d'HEMA-MMA, elles peuvent être propulsées (par exemple à l'aide d'une force centrifuge) à travers un gradient de liquides qui comprend des solutions organiques et aqueuses [16].

La coacervation complexe. La coacervation complexe fait appel à l'interaction de deux polyélectrolytes de charges opposées qui conduit à la création d'un complexe de solubilité réduite et donc qui précipite. En pratique, cette méthode a été utilisée, par exemple, pour produire des microcapsules à base d'alginate et de chitosan [28] ou à base de collagène et d'HEMA-MMA-MAA [39]. Dans ces cas, des gouttelettes d'un polyanion (ou d'un polycation) sont immergées dans une solution d'un polycation (ou d'un polyanion) pour former une membrane mince autour de la cellule ou du cœur du gel.

La photopolymérisation. Cette méthode s'applique surtout aux revêtements conformes des cellules ou pour modifier la surface des microcapsules par le polyéthylène glycol (PEG). Par exemple, un agent photosensibilisant est adsorbé à la surfaces des îlots, qui sont ensuite mis en suspension dans une solution pré-polymérisée de diacrylate de PEG. La polymérisation radicalaire est induite par l'irradiation des îlots pour former une couche de gel de PEG directement sur la surface de l'îlot [52].

4. Caractéristiques requises pour des microcapsules destinées à une application en thérapie cellulaire

Les chercheurs dans ce domaine sont généralement d'accord que les microcapsules doivent avoir nécessairement au minimum certaines caractéristiques pour assurer la réussite de la transplantation de cellules. Il est important de noter que non seulement ces caractéristiques sont influencées par le choix des matériaux et des techniques d'encapsulation (voir la section 3), mais en plus, qu'elles sont hautement interdépendantes. À cause de cette interdépendance, ç'est encore un défi d'ajuster les paramètres indépendamment les uns des autres afin d'obtenir des microcapsules qui possèdent l'équilibre délicat des caractéristiques optimales pour une application médicale spécifique.

4.1. Perméabilité sélective

L'efficacité d'une microcapsule pour immunoprotéger des cellules ou des tissus transplantés dépend principalement de la perméabilité de sa membrane (voir la section

2.1) [53]. La perméabilité est étroitement liée à la moyenne et à la distribution de la taille des pores de la membrane. Afin d'être efficace, en principe, l'immunoisolation requiert que la plupart des pores aient une taille inférieure à toutes les substances potentiellement toxiques pour les cellules, à savoir les cellules immunitaires, les anticorps (e.g. l'immunoglobuline G), les composantes du système du complément (par exemple le facteur C1q), et les cytokines produites par les immunocytes. D'autre part, la perméabilité devrait être assez grande pour assurer la diffusion libre des nutriments, du produit thérapeutique, et des métabolites. Ceci requiert un équilibre très précis entre la perméabilité et le seuil de rétention des molécules (MWCO) [12]. Il est important de noter que la valeur optimale de perméabilité/ MWCO/ taille de pores dépend de l'application spécifique du transplant, surtout si la transplantation est xénogénique ou allogénique.

Malgré l'importance évidente de la perméabilité sélective des microcapsules pour la thérapie cellulaire, les connaissances scientifiques de cette caractéristique sont encore bien limitées. Bien que les pores des microcapsules puissent être visualisés directement à l'aide d'un microscope électronique [54,55], ceci n'est qu'une observation qualitative des pores. D'autre part, le MWCO des membranes des microcapsules est régulièrement estimé grâce à des méthodes indirectes. Généralement, celui-ci est déterminé soit en mesurant la vitesse de perméation des molécules de poids moléculaires connus (surtout les protéines et les dextrans) vers l'intérieur des capsules (par exemple par chromatographie d'exclusion en diffusion inverse [56,57]) ou en piégeant des molécules bien caractérisées dans les microcapsules et en mesurant leur capacité à diffuser dans le surnageant [58,59]. En utilisant de telles mesures de perméabilité, la taille des pores peut aussi être estimée à l'aide d'équations mathématiques [60]. Cependant, ces techniques sont limitées par le fait que le poids moléculaire n'est pas le seul paramètre qui détermine la capacité d'une molécule à diffuser au travers d'une membrane poreuse; sa charge, son volume/conformation 3D, ainsi que sa concentration de chaque côté de la membrane jouent un rôle aussi important que celui du poids moléculaire. Par conséquent, certaines molécules traceuses ne sont seulement qu'indicatives de la situation *in vivo*. En plus, la perméabilité des microcapsules est probablement modifiée *in vivo* à cause du gonflement ou du développement de fibroses ou de vascularisation qui entourent le transplant. Ces facteurs, associés au fait qu'il manque de standards actuellement pour mesurer le MWCO, contribuent aux résultats discordants de certaines études sur un même type de microcapsules (particulièrement alginate-PLL).

4.2. Stabilité mécanique et chimique

La résistance des microcapsules aux attaques chimiques et au stress mécaniques doit être prise en considération puisqu'il suffit d'une faible proportion de microcapsules brisées pour que la libération de substances immunogènes ou l'attraction de protéines ou de cellules immunitaires ne conduisent à l'inefficacité de tout le transplant. Connaissant ce fait, les chercheurs ont développé des méthodes variées pour évaluer la résistance mécanique des microcapsules *in vitro*. Celles-ci comprennent la mesure de la proportion de microcapsules brisées après que celles-ci aient été soumises à un stress mécanique. Les stress standardisés utilisés pour une telle évaluation incluent une charge uniaxiale

[61], un choc osmotique [62], ou une agitation avec des billes de verre [63]. Bien qu'il soit possible de quantifier la proportion de microcapsules brisées par l'observation visuelle (par exemple à l'aide d'un microscope optique), la charge de travail peut être diminuée et la précision des méthodes améliorée en encapsulant de grosses molécules marquées d'un colorant puis en mesurant la quantité de ce colorant relarguée des microcapsules endommagées à la suite du stress appliqué.

La stabilité des microcapsules est dépendante de leur tendance à gonfler dans les solutions salines, de l'épaisseur et de la résistance de la membrane, et de plusieurs propriétés du cœur gélifié. Afin d'optimiser la résistance mécanique et chimique des microcapsules à base d'alginate, de nombreuses approches ont été développées et visent toutes à contrôler ou à modifier des telles caractéristiques. Ces approches (résumées dans la référence [45]) consistent en l'addition de couches multiples, la diminution de la taille, l'application d'une couche de silicate, l'utilisation de contre-ions qui ont une haute affinité pour l'alginate, l'augmentation de la concentration du polymère à la surface, et l'utilisation de la photoréticulation pour créer des liaisons covalentes entre l'alginate et le polycation.

4.3. Contrôle de la taille

La taille moyenne des microcapsules ainsi que leur distribution des tailles sont des caractéristiques très importantes pour leur bioperformance et ce, sous plusieurs aspects. En général, les microcapsules de petites tailles (< 400 µm) sont préférées pour la thérapie cellulaire puisque celles-ci [64]:

- donnent un meilleur (plus élevé) rapport de surface sur volume, ce qui favorise la diffusion ou le transport moléculaire à travers la membrane et donc permet une meilleure oxygénation/nutrition des cellules encapsulées
- ont tendance à présenter une meilleure résistance mécanique
- ont tendance à générer une meilleure biocompatibilité
- donnent accès à un grand nombre de sites de transplantation puisque le volume total de l'implant est diminué d'une manière exponentielle avec le diamètre de la microcapsule

Il est également souhaitable d'avoir une distribution de taille uniforme. Comme il y a des inconvénients à disposer d'une part de capsules trop petites (par exemple l'exposition des cellules à la surface) et d'autre part de capsules trop grandes, il est important que la totalité des capsules ne s'éloigne pas trop de la médiane afin d'éviter un échec général du transplant total.

Comme il a été exposé dans la section 3.2, la taille des capsules est surtout déterminée par la méthode de fabrication. En particulier, l'utilisation d'un système électrostatique permet la production de microcapsules de diamètres allant de 180 à 700 µm. Afin de maximiser les effets bénéfiques de microcapsules de petites tailles, plusieurs chercheurs ont essayé de réaliser cela en suivant l'approche d'un revêtement conforme des cellules (voir la section 2.2). D'un point de vue théorique, l'avantage principal de cette approche est de minimiser le volume vide entre la cellule encapsulée et l'extérieur de la capsule et

donc de réduire la distance de la vascularisation. De telles capsules peuvent également améliorer les propriétés de diffusion, ce qui est important pour la survie des cellules mais aussi pour assurer la libération rapide du produit à visée thérapeutique (par exemple l'insuline) [12]. Néanmoins, l'avantage apporté par la technique d'application d'un revêtement conforme n'a pas encore été clairement démontré en termes de performance métabolique et de compétences immunoprotectrices. Il est aussi important de noter que, d'un point de vue théorique également, cette méthode ne permettra pas la néogenèse des cellules (comme les îlots de Langerhans), alors que celle-ci peut se faire à l'intérieur de microcapsules. Leur efficacité à long terme reste donc aussi à démontrer.

4.4. Biocompatibilité avec les cellules encapsulées

Évidemment, la microcapsule et ses constituants issus de biomatériaux ne doivent avoir aucun effet néfaste sur le fonctionnement et le maintien en vie des cellules encapsulées. Dès que les cellules ou les tissus sont immobilisés dans la microcapsule, il y a une interaction importante entre les cellules encapsulées et la matrice constituant le cœur de la microcapsule ainsi qu'avec la membrane de celle-ci.

Des études expérimentales ont permis de conclure que lors de la microencapsulation, le choix de la nature du coeur de la microcapsule (par exemple choix d'un coeur liquide plutôt que solide) a une influence importante sur la croissance, la viabilité et le fonctionnement des cellules encapsulées [32,65,66]. Dans le cas particulier des îlots de Langerhans, certaines études ont suggéré un effet néfaste de la microencapsulation sur l'efficacité des cellules encapsulées. En effet, jusqu'à récemment, le nombre d'îlots de Langerhans microencapsulés requis pour normaliser la glycémie était beaucoup plus important que pour les îlots non-encapsulés. Plusieurs explications ont été proposées pour expliquer ce fait, à savoir la technique de microencapsulation elle-même peut altérer les îlots et initier la cascade de l'apoptose ou le fait que, contrairement aux îlots libres, les îlots encapsulés ne sont pas revascularisés, ce qui peut interférer avec la nutrition optimale des cellules encapsulées et causer leur nécrose [67]. Inversement, des études plus récentes ont démontré que, l'immobilisation et la microencapsulation permettait d'augmenter la viabilité des îlots. Il a été démontré *in vitro* et *in vivo* qu'il y a une meilleure survie et meilleur fonctionnement des îlots lorsqu'ils sont encapsulées comparé aux îlots libres [68]. Dans cette étude, cette meilleure survie *in vivo* ne s'explique pas seulement par l'immunoprotection puisqu'elle a été observée également dans des modèles de souris immunodéficientes. La meilleure viabilité des îlots encapsulés est expliquée généralement par le fait que la configuration tri-dimensionnelle des microcapsules crée un microenvironnement favorable en reproduisant la matrice extracellulaire naturelle.

En plus du fait que la microencapsulation puisse favoriser la survie et le fonctionnement des cellules encapsulées, elle offre également la possibilité de concevoir des interventions qui peuvent ajouter un gain supplémentaire. A titre d'exemples, certains chercheurs ont incorporé à la matrice des transporteurs d'oxygène pour mieux oxygéner les cellules et ainsi améliorer leur viabilité [69] ou encore d'autres ont co-encapsulé des cellules

produisant des effets bénéfiques dans le but de prolonger la survie des cellules transplantées [70].

4.5. Biocompatibilité chez l'hôte

La biocompatibilité des microcapsules chez l'hôte est un des sujets les plus étudiés dans le domaine puisqu'il représente un des plus grands défis à surmonter avant d'atteindre l'application clinique régulière de la thérapie cellulaire par microencapsulation. Quelques articles, publiés récemment, concernent spécifiquement la biocompatibilité des microcapsules [23,71]. Bien que le terme « biocompatibilité » puisse être défini de plusieurs façons, dans le contexte des dispositifs immunoprotecteurs, l'optimisation de leur biocompatibilité comporte, en général, deux exigences :

1) Minimiser ou éliminer le développement d'une couche fibreuse qui entoure la microcapsule. Le développement de la fibrose ou surcroissance cellulaire est une conséquence de l'inflammation chronique. Cette réaction est stimulée par la présence du transplant, qui est reconnu par le système immunitaire en tant que corps étranger. La fibrose est problématique puisqu'elle peut empêcher la diffusion libre des nutriments, de l'oxygène et des métabolites à travers la membrane de la microcapsule et donc interférer avec le fonctionnement et la viabilité des cellules encapsulées. Récemment, il a été démontré qu'il est possible de produire des microcapsules qui n'induisent pas de fibrose sévère [72]. Par conséquent, il est tentant de dire qu'il s'agit plutôt d'un problème du passé. Toutefois, ce degré de biocompatibilité n'est pas facilement reproductible entre différents laboratoires, ce qui permet de conclure qu'il existe encore un manque de connaissance des différents facteurs qui contribuent au développement de la fibrose autour des microcapsules implantées.

2) Minimiser l'inflammation aiguë qui est associée avec la chirurgie d'implantation. Cette réaction inflammatoire est indésirable puisque les cytokines et d'autres substances réactives produites par les cellules immunitaires sont cytotoxiques et leurs tailles sont assez petites leur permettre potentiellement de diffuser au travers de la membrane et d'endommager les cellules encapsulées. De plus, des changements environnementaux locaux associés à l'inflammation (par exemple une baisse du pH) peuvent modifier les propriétés de surface et membranaires de la microcapsule et par conséquent compromettre sa bioperformance. Malheureusement, il n'est probablement pas possible de complètement éliminer l'inflammation seulement en modulant les propriétés des microcapsules puisqu'elle est initialement induite par l'incision ou la blessure associée à la chirurgie d'implantation. Toutefois, des mesures peuvent être prises pour minimiser la sévérité et les effets néfastes de cette réaction.

En outre, il est important également de noter que dans cette section, la biocompatibilité des microcapsules est discutée sans tenir compte des cellules encapsulées (donc, de capsules hypothétiquement vides). Ceci n'est qu'un aspect de la biocompatibilité du système microcapsule/cellule; une discussion plus en profondeur de ce dernier aspect dépassant quant à lui largement le sujet du présent chapitre.

Généralement, la biocompatibilité des microcapsules, selon la définition présentée ci-dessus, est influencée par de nombreux facteurs dont la stabilité mécanique et la taille des microcapsules (voir les sections 4.2-4.3). La qualité des matériaux de base et surtout les caractéristiques de la surface de la microcapsule ont également un impact important sur la biocompatibilité, tel que décrit ci-dessus. Les facteurs discutés ne sont que des exemples de toutes les caractéristiques qui peuvent potentiellement influencer la biocompatibilité des microcapsules implantées. L'application récente des techniques d'analyses à haute performance, et des nouvelles connaissances de la nature de la réaction biologique envers des implants permettent une meilleure compréhension des mécanismes et une meilleure explication de la biocompatibilité, ce qui amène à la découverte continue de nouveaux paramètres importants pour la bioperformance des microcapsules pour la thérapie cellulaire.

La qualité du biomatériau

Il est absolument nécessaire que les matériaux utilisés pour la fabrication des microcapsules soient dépourvus de tout contaminant et/ou impureté qui pourraient être relargués de la matrice polymère après l'implantation et potentiellement déclencher une réaction immunitaire. Ceux-ci comprennent des agents de réticulation, des précipités ou des solvants, des petits fragments de polymères, des additifs, et des bactéries (surtout dans les polymères naturels). Dans le cas de l'alginate, des études ont mis en évidence que non seulement les contaminants peuvent déclencher une réaction inflammatoire directement, mais qu'ils ont aussi une influence sur les propriétés physiques des alginates, par exemple sur leur hydrophilie [73]. En reconnaissant l'importance de la qualité du matériau mis en œuvre pour assurer à la fois une bonne biocompatibilité et bioperformance générale de l'implant, il est devenu incontournable de bien purifier les polymères avant de les utiliser pour la production de microcapsules implantables.

La composition chimique de la surface

Puisque c'est la surface des microcapsules qui entre en contact avec les molécules et les cellules de l'hôte, il semble évident que ce paramètre est important à prendre en considération pour la biocompatibilité des microcapsules. Pourtant, les résultats expérimentaux qui ont apporté la preuve de ce concept sont relativement récents. Depuis quelques années, des applications de technologies à hautes performances pour analyser la composition chimique et moléculaire de la surface des microcapsules sont de plus en plus décrites dans la littérature. Des techniques telles que la spectroscopie photoélectronique des rayons-X (XPS) et la spectroscopie infrarouge (FTIR) sont maintenant reconnues comme essentielles pour caractériser et évaluer de manière préliminaire des microcapsules et permettre de prédire ou d'expliquer leur bioperformance *in vivo* [72,74,75]. D'autres techniques d'analyses avancées, comme la spectrométrie de masse à émission ionique secondaire (ToF-SIMS), ont été appliquées plus récemment dans la domaine. Le ToF-SIMS démontre avoir un potentiel énorme pour fournir des détails chimiques de microcapsules implantables en combinaison avec l'imagerie [76].

La morphologie/topographie/rugosité de la surface

La morphologie de la surface a été une des premières caractéristiques physiques qui ont été étudiées pour les microcapsules implantables. Rapidement, l'importance de la rugosité de la surface pour la biocompatibilité des microcapsules implantées est devenue évidente. Les surfaces lisses et l'absence de fractures ou crevasses sont importantes à obtenir. Les défauts physiques ont tendance à favoriser l'accumulation des protéines et l'adhésion cellulaire ou même modifier la configuration des microcapsules, ce qui augmente la probabilité que celles-ci soient reconnues comme corps étranger par le système immunitaire de l'hôte. Depuis quelques années, la morphologie de la surface est étudiée par les chercheurs régulièrement, principalement à l'aide de la microscopie à force atomique (AFM) [77,78]. Cette technique permet, entre autre, une visualisation tri-dimensionnelle de la topographie de la surface à haute résolution et l'analyse de microcapsules intactes immergées dans un fluide.

La charge de surface

La charge de surface concerne la charge nette et la distribution locale des charges électriques à la surface de la microcapsule. Depuis longtemps, il est admis que la charge nette à la surface des microcapsules est un paramètre important pour leur biocompatibilité. C'est la raison pour laquelle il une deuxième « couche » d'alginate est fréquemment ajoutée aux microcapsules d'alginate-poly-L-lysine pour lier les charges positives de la poly-l-lysine et de créer de ce fait une charge nette négative à la surface de la microcapsule. Il est généralement admis que les biomatériaux dotés d'une charge nette négative ont tendance à être plus biocompatibles que ceux qui ont des surfaces chargées positivement, ces dernières ayant tendance à favoriser l'adsorption des protéines et l'adhésion cellulaire. Malgré l'importance reconnue de la charge nette de la microcapsule pour la biocompatibilité, ce paramètre n'est pas facile à mesurer à cause de la forme et de la taille de ces objets. C'est la raison pour laquelle la charge nette n'a été étudiée avec succès que récemment par de Vos et Coll., qui ont adapté la technique du potentiel zêta à des capsules de tailles relativement grandes (comparé aux nanoparticules) [79]. A ce jour, à la connaissance des auteurs de ce chapitre, la distribution locale des charges électriques à la surface des microcapsules n'a pas encore été mésurée. Pourtant, il est bien possible que les surfaces pour lesquelles la charge n'est pas répartie de manière homogène jouent un rôle important dans l'attraction et l'adsorption des protéines à leur surface.

L'hydrophilie de la surface

Comme expliqué ci-dessus dans la section 3.1, les hydrogels sont les biomatériaux de choix pour la fabrication de microcapsules destinées à la thérapie cellulaire. Une des raisons principales est qu'ils sont hydrophiles, ce qui minimise la tension interfaciale entre la capsule et les fluides/tissus biologiques. Par conséquent, ils ont tendance à défavoriser l'adsorption des protéines et l'adhésion cellulaire. A la suite de la découverte de cette caractéristique des surfaces hydrophiles, des chercheurs ont développé des méthodes permettant de greffer du polyéthylène glycol (PEG) à la surface des

microcapsules [30,41] ou directement à la surface des agrégats cellulaires [80]. Ce concept d'ajouter du PEG à la surface permet de créer une surface très hydrophile qui est connue pour empêcher l'adsorption des protéines et l'adhésion cellulaire.

L'incorporation d'agents anti-inflammatoires

Quelques chercheurs utilisent la technique de microencapsulation pour développer de nouvelles microcapsules qui renferment des agents anti-inflammatoires qui sont co-encapsulés avec les cellules ou incorporés dans la membrane. L'idée consiste à fournir une libération temporaire ou à court terme de ces agents immédiatement après l'implantation pour diminuer ou contrôler l'inflammation aiguë qui est induite à la suite du processus d'implantation. Récemment, des résultats prometteurs ont été rapportés dans ce sens [81,82].

5. Perspectives

A la suite des travaux scientifiques de T.M.S. Chang, le concept d'immunoprotection des cellules en les encapsulant dans une membrane semi-perméable avait pu être proposé, il y presque 50 ans [83], et ce concept a été appliqué expérimentalement pour la xenotransplantation des îlots de Langerhans afin de corriger le taux de glucose chez des rats diabétiques au début des années 1980 [84]. Depuis lors, il est apparu que même si le concept fut initialement simple, l'application pratique d'un tel système n'est pas du tout évidente. Ceci est démontré par le fait que les réussites exceptionnelles qui ont été observées sur des modèles de grands animaux et sur des êtres humains, n'ont pas pu être reproduites ou bien il existe encore à l'heure actuelle des incertitudes quant à leur reproductibilité [74]. Des efforts actuels de recherche nécessitent une collaboration multi-disciplinaire et multi-laboratoire pour comprendre et résoudre les problèmes qui apparaissent et retardent l'application clinique de cette approche thérapeutique. Ce point est souligné par des commentaires sur les progrès de l'encapsulation cellulaire récemment publiés dans des journaux scientifiques tels que *Nature Medicine* [74,85].

Heureusement, cette approche collaborative commence à se transformer en réalité. Depuis le début de l'année 2006, plusieurs chercheurs, principalement européens, collaborent sur un projet international (COST 865) qui vise à approfondir les connaissances et à créer des standards dans le domaine de la bioencapsulation, qui comprend l'établissement des standards pour la caractérisation des microcapsules pour la thérapie cellulaire [86]. Ce projet représente une étape importante à la fois pour lever les barrières entre les différents laboratoires et pour permettre de travailler par complémentarité plutôt que par concurrence pour exploiter les savoir-faire et les expertises de chaque groupe de recherche impliqué.

Un aspect de la nouvelle approche multi-disciplinaire est le rôle croissant des ingénieurs en matériaux ou biomédicals, des chimistes dans le domaine des polymères, et des physiciens dans un domaine qui a été dominé dans le passé par les biologistes, les biochimistes, et les médecins. Les technologies à haute performance, par exemple le ToF-

SIMS, qui dans le passé était utilisée uniquement dans le domaine du génie des matériaux, commence à démontrer leur utilité pour améliorer la conception et la bioperformance des biomatériaux et des dispositifs médicaux, incluant les microcapsules pour la thérapie cellulaire. Actuellement, les techniques de caractérisations telles que XPS, FTIR, AFM et la microscopie confocale à balayage laser (CLSM) sont plutôt considérées comme des techniques standards pour la caractérisation des microcapsules implantables.

Il est certain que l'utilisation régulière de techniques d'analyses à haute performance associées aux efforts de collaboration vont permettre un approfondissement de nos connaissances des paramètres qui influencent la bioperformance des microcapsules. Ceci devrait nous permettre de contrôler ces paramètres pour optimiser la conception de la microcapsule et de ce fait d'accélerer le progrès de la microencapsulation pour la thérapie cellulaire vers l'application clinique régulière.

6. Reférences

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APPENDIX III

BOOK CHAPTER

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3 Microencapsulation technologies for a bioartificial endocrine pancreas

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Abstract

This contribution offers an overview of the technologies suitable for pancreatic islet encapsulation. It proposes a comprehensive outline of the technologies from the point of view of the physico-chemist and engineer. After presenting methods for producing droplets of appropriate size, it classifies techniques for converting droplets into microcapsules.

Introduction

The concept of encapsulating islets of Langerhans for solving problems of immune rejection was published as early as 30 years ago. Starting with the work of Lim and Sun [1] on the entrapment of islets within alginate beads, the principle has remained relatively

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the same over the years. However, many groups around the world have worked intensively to understand the limitations of this system, not only from the point of view of the encapsulation technique itself, but also of the biocompatibility of the system and the biology surrounding and inside the microcapsules. The object of this contribution is to concentrate on the description of the encapsulation technologies from the point of view of the engineer and/or the physico-chemist. The biomedical aspects will not be treated directly in this chapter.

We provide a few (simple) equations to build basic theory, but we combine them as much as possible with drawings and graphs for easier understanding.

Microcapsule size

What may be one of the most important parameters of a capsule for cell immuno-protection is its diameter, or its size. If the capsules are to be injected, the diameter must be, at maximum, a third of the internal needle diameter. Larger capsules will sediment more in the syringe, creating risk of blockage. However, larger capsules are easier to recover for post-transplantation analysis, such as in the case of a foreign body reaction.

Clinical islet transplantation usually involves about 600 000 islets, i.e. a volume of 5 to 10 ml [2]. If an equivalent number of islets are to be immobilized in capsules of 250 to 500 μm diameter, this represents a volume of 20 to 160 ml to be transplanted. Because of this increased volume, the transplantation of large capsules is practical only in the peritoneal cavity [3]. In addition, it has been demonstrated that the use of larger capsules created a delay in the systemic response to glucose level [4] as well as in the transfer of oxygen into the capsules. These mass transfer problems are attributed to the increased distance between the capsule membrane and the encapsulated islet, which may be one to several hundred micrometers. As a consequence, authors [5] report that oxygen (as an example) decreases radially in the capsules, leading to a lowering of the islet activity.

For capsules smaller than 320 μm diameter, transplantation in the portal vein is possible without observing an important and permanent increase of the portal pressure [6]. Small capsules also offer improved oxygen and nutrient supply (including glucose) and good release properties of the insulin. One problem that may be presented with using smaller capsules is an increased probability of islet protrusion, which can induce a foreign body reaction [7], and a higher rate of empty capsule production.

Concerning the size of the capsules for islet encapsulation, one must also consider:

- Size distribution. Capsules that are too large or too small may lead to problems as described above. A wide size distribution also requires more work when handpicking capsules that are suitable for implantation.
- Homogenous distribution of the islets within the capsules. Most other authors refer to a single islet per capsules. However, in practice, islet distribution may follow Poisson's law with the effect that all capsules are assumed to fill a mean of 2 or 3 islets [8]. Some artifacts (such as sedimentation in the extrusion system) may improve this situation.

Conventional capsule diameters typically range between 250 and 800 micrometers. Thus, there is a tendency to develop technologies that target this capsule size [3, 6]. Yet, some authors propose to coat or graft polymers directly to the islets [9, 10] by putting the

islets into contact with a limited volume solution containing reagents for interfacial precipitation or polymerization. Wilson and Chaikof propose a very good review that discusses the selection of the microcapsule size to optimize the cell encapsulation and transplantation process, from the point of view of both the production and the performance of the system for diabetes treatment [11].

Droplet production (making the capsule core)

Many books classify methods for producing capsules as chemical, physicochemical or mechanical methods. However, many technologies combine such aspects and thus similar methods can be placed in more than one of these categories.

Let us therefore use a more appropriate method of classification based on the three steps for producing the capsules [12]:

- **Incorporation**: The active component (the islets of Langerhans in this case) has to be incorporated into a solution that will serve as the future capsule core (for example an alginate solution).
- **Dispersion**: The capsule core is dispersed. In the case of a liquid, this involves forming droplets. In the case of a solid core, the dispersion consists in mixing the particles with a coating solution.
- **Stabilization**: The droplets (or the liquid coating) have to be solidified.

Methods could be classified based on the dispersion steps (dripping, spraying, emulsification, fluidization…). Most of the works devoted to the encapsulation of fragile

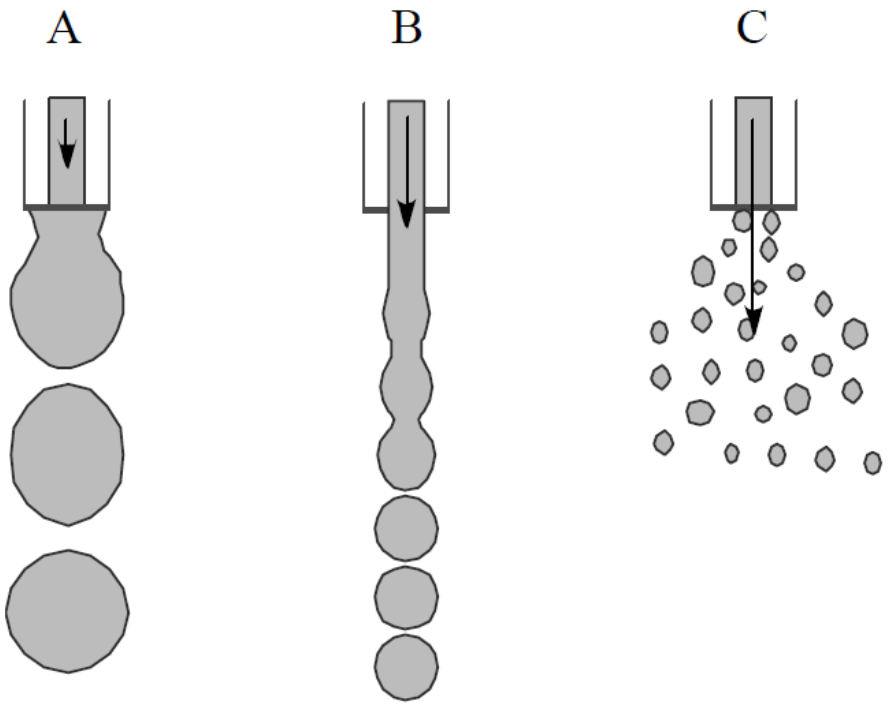

Figure 1. From dropping to spraying methods (Poncelet ©).

islets refers to dripping technologies. While a liquid is extruded from a simple needle or nozzle, one could observe that:

- For a very low flow rate, droplets are formed and detached one by one from the needle. We are in **simple dropping** conditions (Fig 1a).
- Above a certain flow rate, the liquid is ejected from the needle as a jet, which could break into droplets naturally (Raleigh's theory) or be broken up using a moderate external force. The latter may be a mechanical force to physically "cut" the jet, a vibrational force to "shake off" the droplets, a limited pressure co-axial air flow to add drag, or an electrostatic force to "pull" the droplets down. We are in **jet breakage** conditions (Fig 1b).
- For a high flow rate, or if a high external force is applied (such as high speed coaxial air flow), the liquid is exploded into number of small droplets. We are in **spray** conditions (Fig 1c).

Spraying involves high energy, shear forces, cavity effects and high pressure. Obviously, such conditions would be detrimental for the survival of the cells. We then have to mainly consider dropping and jet breakage for the encapsulation of islets of Langerhans.

Simple dropping

In simple dropping conditions, the detachment of the droplet occurs when the gravity force (linked to the mass of the droplet) becomes higher than the surface tension force maintaining the droplet on the needle [13]. The rupture point could then be defined by:

Gravity forces = surface tension forces

$$m\ g = \pi\ d_e\ \gamma \tag{1}$$

where m is the mass of the droplet, g the gravity force, d_e the external needle diameter, and γ the surface tension. From the mass of the droplet, we could deduce the diameter:

$$m = \frac{\pi}{6} d^3 \rho \tag{2}$$

where d is the droplet diameter, and ρ is the density of the droplet.

To reduce the size of the droplet, one would propose to reduce the diameter of the needle. However, the droplet diameter is only affected by the cubic root of the needle diameter. Moreover, the detachment of the droplet does not happen directly on the needle and the diameter of the liquid at the breakage point is only quasi constant. More importantly, Figure 2 shows that in practical simple dropping conditions, the droplet size will be too large for cell transplantation purposes, i.e. above ~1.8 mm in diameter.

To obtain a capsule diameter lower than 1 mm, one has to add some moderate external force. Three options that are commonly applied include the use of the co-axial airflow, an electrostatic potential, and a vibrating nozzle:

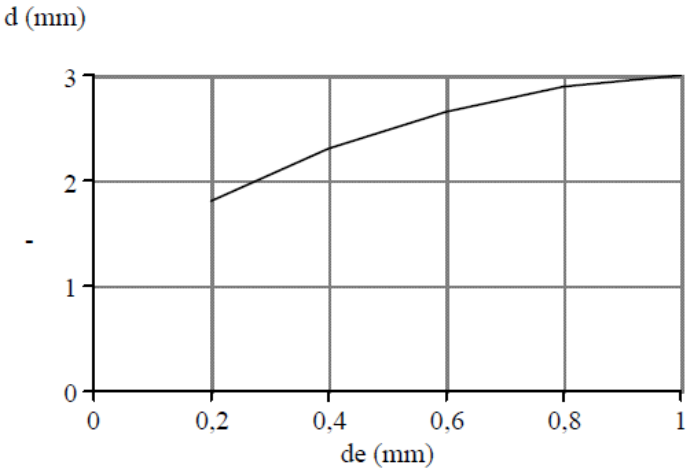

Figure 2. Size of falling droplet versus external nozzle diameter.

Jet-breaking by co-axial air-flow

A coaxial airflow may be applied around the needle to create a drag force and reduce size of the droplet. In this case, the droplet falls from the needle when:

Gravity forces = surfaces tension forces – drag forces

$$m\ g = \pi\ d_e\ \gamma - F_g \tag{3}$$

wherein Fg is the drag force [8].

If the coaxial airflow is not well controlled or is increased too much, it will create turbulence, droplet size dispersion and finally spraying. Such a system is therefore suitable to reduce the size of the droplet to a minimum of 500 micrometers. There now exists some commercial devices to produce microcapsules using a coaxial airflow (as an example consult www.nisco.ch).

That said, a very promising device supporting multi-60µm-nozzles surrounded by micro-channels with a controlled air flow has been used to produce 150µm capsules [14]. Building such system is based on microfluidic engineering, a relatively complex technology. However, because the nozzle diameters are so small, pancreatic islets would have to be carefully screened for their size in order to avoid blockage of the nozzles.

Jet-breaking by an electrostatic potential (electro-dripping)

An alternative method to reduce the size of droplets is to apply an electrostatic potential between the nozzle and the receiving solution [6]. The existence of this electrostatic potential leads to charge accumulation on the surface of the droplet, creating repulsion and consequently reducing the surface tension [15]. In this case, the force balance is described by :

$$m\ g = \pi\ d_e\ \gamma_o\left(1 - \frac{U^2}{U_{cr}^2}\right) \tag{4}$$

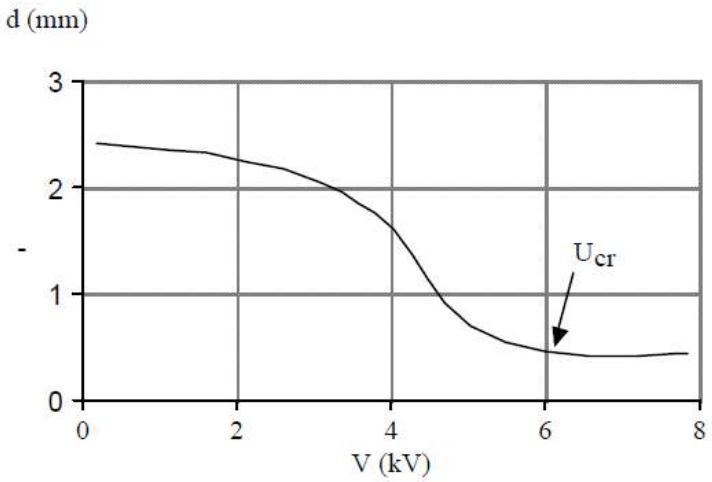

Figure 3. Droplet size versus electrostatic potential (adapted from [16]).

where γ_o is the surface tension without electrostatic potential, U is the electrostatic potential and U_{cr} the critical electrostatic potential. Figure 3 shows the evolution of the size in function of the electrostatic potential (denoted by V in the figure). Equation 4 and Figure 3 suggests that:

- For very low electrostatic potentials, the size of the droplet is similar to when no electrostatic potential is applied.
- For medium electrostatic potentials, the droplet size decreases quickly with increasing potential.
- As the electrostatic potential approaches its critical value, the droplet size stabilizes towards a minimum value. In fact, at the critical electrostatic potential, the surface tension is null and the liquid leaves the needle as a jet. Thus, we are no longer in the dropping mode, and the droplet size is defined by Raleigh's theory, which is presented in the next section.
- Note that others have used electrostatic pulses instead of a continuous electrostatic potential. This approach is described in chapter 5.

With the electrostatic droplet generator, depending on the design of the system, the manipulator could produce droplets (and then capsules) as small as 50 micrometers in diameter. One may be concerned by the high electrostatic potential applied to droplet. However, it has been shown that even high electrostatic potential does not significantly affect cell survival [17]. Improvements of the reliability of the electrostatic droplet generator and the control of the electrodripping process (size, size distribution of the capsules, productivity …) are still in development, but commercial equipments already exist (as an example, consult www.nisco.ch).

Jet breaking by a vibrating nozzle (nozzle resonance dripping)

By increasing the liquid flowrate leaving a nozzle, it forms a liquid jet [18] whose velocity can be described by

$$u_j \geq 2\sqrt{\frac{\gamma}{\rho\ d_i}} \tag{5}$$

where u_j is the linear velocity of the liquid leaving the nozzle as a jet, γ is the surface tension of the liquid, ρ is the liquid density and d_i is the internal nozzle diameter. Generally, for the encapsulation of islets, the liquid is an aqueous solution with a fixed surface tension (except when using an electrostatic generator) and density. The minimum jet velocity is therefore mainly a function of the nozzle internal diameter.

Equation 5 also suggests that there is a maximum flowrate limit for dropping methods. To scale-up droplet production, one has to use jet-breaking methods.

A capillary liquid jet has a tendency to absorb certain vibrations and break into small droplets [19]. The transition from liquid jet to droplets can be described by the Rayleigh break-up theory [20]. According to this theory, the optimum conditions for droplet formation occur when the jet enters in resonance with an applied vibration (Figure 4), which is defined by [20]:

$$f = \frac{u_j}{\lambda} \quad \text{and} \quad \lambda = 4.058\ d_j \tag{6}$$

where f is frequency of vibration applied to the liquid jet, u_j is the jet linear velocity at the exit of the nozzle, λ is the natural resonant wavelength of the fluid jet and d_j is the jet

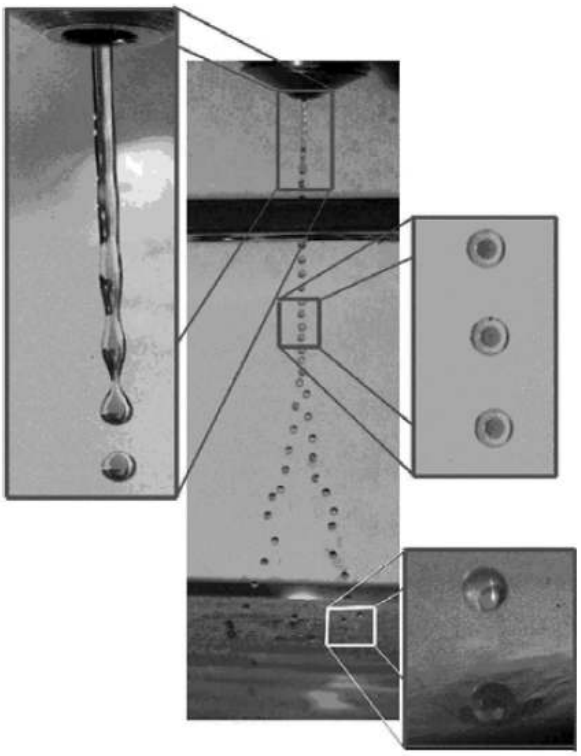

Figure 4. Jet breakage by resonance (Inotech ©).

diameter. The jet breaks in cylinders with a length equal to λ and diameter d_j. The jet linear velocity may be 2 to 5 times the minimum jet velocity (and should be, to get a good flow regime). The flowrate, Q, is then given by the product of the jet cross-sectional area times the jet linear velocity :

$$Q = \left(\frac{\pi d_j^2}{4} \right) u_j \tag{7}$$

The volume of the liquid cylinder formed by breakage of the jet is equal to the volume of resulting droplet. Mathematically, this is represented by :

$$\frac{\pi}{6} d^3 = \frac{\pi}{4} d_j^2 \lambda \tag{8}$$

Combining Equations 6 and 8, and subsequent simplifying, leads to a very simple rule relating the droplet and jet diameters:

$$d = 1.89 \ \ d_j \tag{9}$$

Equation 9 provides a mean droplet diameter because the jet is submitted to many vibrations that, in practice, can cause the formation of smaller or larger droplets. That is, even if the applied vibrational frequency is constant, one is expected to observe a droplet size distribution due to interfering vibrations. However, by isolating the jet from external vibrations and applying a specific vibration, one can obtain quasi mono-dispersed droplets (standard deviation between 5 and 10 % of the mean diameter) [21].

The factor 4,058 in equation 6 is defined for ideal liquids. Viscosity variations (especially for non-Newtonian fluids such as alginate solutions) or the presence of particles (islets) may slightly affect this value. In practice, Schneider and Hendrick [22] observed that uniform droplets could be formed in the range of wavelength defined by:

$$3.5 \ \ d_j < \lambda < 7 \ \ d_j \tag{10}$$

To simplify the production of mono-dispersed capsules, most commercial equipments (www.nisco.ch, www.inotech.ch, www.brace.de) are equipped with diodes that flash at the same frequency as the jet. Setting the system is then easy.

- Select a nozzle to obtain the correct droplet size (in practice the internal diameter of the nozzle should be half of the desired droplet diameter).
- From the data provided by the supplier (or from Equations 6 and 7) define an appropriate flowrate and frequency of vibration.
- Fine-tune the frequency by observing the jet. When the frequency is correct, the droplets appear to be immobile in the diode flashing light.

With such equipment, production rates may be as high as liters per hour for 1 mm diameter beads. However, the production rate decreases proportionally with the square of the droplet diameter (Equation 7). For beads around 250 micrometers in diameter,

productivity is thus limited to a few hundred milliliters per hour, which is nonetheless sufficient for creating a bioartificial pancreas.

The main limitation of the jet-breaking approach to droplet formation is that the liquid viscosity must be lower than 200 mPa·s. For higher viscosity fluids, the breakage could be provoked by a mechanical force (as an example, using a wire to cut through the jet, see www.geniaLab.de) in place of a vibration. This approach is generally not used for medical applications, however, because the mechanical forces tend to injure the living cells to be encapsulated.

Converting droplets to microcapsules

In the previous paragraphs, we described methods that are suitable for producing droplets for the encapsulation of islets of Langerhans. The conversion of these droplets into microcapsules requires a "stabilization" process. This may be based on different processes such as solidification by cooling, jellification, polymerization, coacervation (see below), drying or solvent evaporation. However, for islet encapsulation, ionic jellification (or gelation) and interfacial coacervation are most suitable, since the other methods generally require strong temperature fluctuations, the use of harsh chemicals, or pH ranges that are not compatible with living cells.

Gelation

The most common method of pancreatic islet encapsulation consists of mixing the islets in an alginate solution and dropping this suspension into a calcium chloride solution. An example of a protocol to produce calcium alginate (gel) beads is described as follows:

1. In a beaker, place 2 g of sodium alginate powder
2. Add a small quantity of water and mix with a glass rod to obtain a homogeneous paste
3. Slowly add the rest of water (100 ml) while mixing gently to avoid air incorporation
4. Let stand for overnight to allow complete re-hydration of the alginate
5. Add the islet suspension by gentle mixing
6. Introduce the mixture into the droplet generator
7. Allow droplets to fall into a 50 mM $CaCl_2$ solution agitated gently by a magnetic stirrer
8. Allow droplets to continue gelling in the $CaCl_2$ solution for 30 min
9. Filter the capsules on a 40 micrometer nylon mesh
10. Keep the capsules in adequate buffer solution (avoid phosphate, a chelating agent)

Calcium is sometimes replaced by barium to obtain stronger gel [23]. Other materials have also been proposed to encapsulate the pancreatic islets [24] such as agarose [25], poly(ethylene glycol) [26] or the more exotic chitosan-polyvinyl pyrrolidone [27]. The real future of such materials is yet to be evaluated. However, more than 90 percent of the literature on islet (and more generally cell) encapsulation is based on entrapment in alginate beads. The alginate system has strong advantages over most other methods:

- The surface of the alginate droplets jellify instantaneously upon contact with the calcium ion solution, allowing to obtain smooth and spherical beads. Most other materials would jellify at slower rates.
- Alginate jellification takes place at room temperature (or a little bit higher), at neutral pH, without toxic cross-linkers, without harsh chemical reactions, and at a physiological osmotic pressure and ionic strength. These conditions are compatible with living cells.
- It is one of the most well known systems, both in terms of the jellification process and of materials involved.
- In the case of islet encapsulation, a key point for success is not the encapsulation process itself, but the purity and quality of the materials. Alginate has been thoroughly analyzed and purified. Moreover, some groups have developed alginates with tailored properties and improved biocompatibility using enzymatic modification [28]

However, the domination of the calcium-alginate system over the literature has unfortunately limited the development of other alternatives. If, in the future, alginate beads are discovered not to be the ideal system for cell encapsulation, it is unlikely that some other system will be advanced enough to immediately replace it. Despite its apparent simplicity, producing alginate beads suitable for pancreatic islet transplantation requires a very careful selection of the materials and precise conditions of production [29]. As the purification and the modification of the alginate are probably the key to success, research groups have historically kept their alginate production process a secret [30]. Overall, drawing conclusions from the literature is difficult as there are many differences and discrepancies between published studies. These discrepancies are partially linked to the complexity of the biology involved, but are also the result of the different alginates that are being used for producing the microcapsules.

Coating of hydrogel beads

In general, alginate beads are not expected to offer adequate protection of the islets of Langerhans against the host immune system. That said, some groups do not agree and have reported successful immuno-protection using simple barium-alginate beads with a density gradient [31]. As a strategy to improve the immuno-protective capabilities of the system, alginate beads are more commonly coated with other polymers. This coating acts to provide desirable surface properties, a more selective permeability, and a greater mechanical and chemical stability of the capsule. The initial alginate beads generally serve as the inner core for the complete microcapsule system.

The coating process is relatively simple. Alginate beads (charged negatively due to the carboxyl group of alginate) are suspended in a low concentration (e.g. 0.2 %) solution of a positively charged polymer. Initially, poly-L-lysine was selected as the polycation [1]. Alternatives have since been proposed, including chitosan [32] or poly-L-ornithine [33], but polylysine remains the most commonly applied of them all.

It must be pointed out that the term "coating" is not appropriate as it has been demonstrated that the cationic polymer in fact interpenetrates the alginate gel [34]. The thickness of the polymeric membrane increases with the time of incubation in the polycation solution, and some of the polycation may potentially even reach the center of

the beads [34]. Physico-chemical analyses of the microcapsule surface have also supported the notion that the polycation does not form a distinct layer [35].

Poly-L-lysine (PLL)-coated alginate beads are expected to have a positively charged surface that is suspected to provoke a foreign body reaction. PLL-coated beads are thus re-suspended in a low concentration alginate solution [36]. The process is sometimes repeated to reach optimum membrane properties.

Interfacial coacervation

Probably the main alternative to the alginate bead system is based on interfacial coacervation, also called "polymer complex membrane formation". Developed originally by Dautzenberg in Germany [37], it consists of dropping a polymer solution (cellulose sulfate) into a polymeric solution of opposite charge (poly(dimethyldiallylammonium chloride)). If well selected, the two polymers interact at the droplet interface to form insoluble complexes that are still hydrated, called "coacervates". The coacervates coalesce to form a membrane surrounding the droplet and thusly form the capsules.

Hunkeler has tested more than 1 500 combination of polymers, defining rules to obtain strong and biocompatible capsules [38]. However, most of the research is focused on the coupling of alginate with chitosan [39].

In comparison to alginate beads (whether coated by a polycation or not), microcapsules formed by interfacial coacervation have a more specific molecular cut-off (down to 3000 Daltons) [40]. Authors reported that even though some polymers may independently have some cytotoxicity, the formed membrane shows high biocompatibility [41]. Interfacial coacervation allows the formation of a capsule in one step, while coated beads may require many steps (particularly in consideration of the washing steps between the coating steps). However, it is a more delicate technology to drive.

Co-extrusion

The co-extrusion technology consists of concentrically extruding two liquids as droplets then solidifying the external layer (Figure 6). This approach for encapsulation has been tested for mammalian cells and is of interest for islet encapsulation [42]. This technology allows a lot of freedom in selecting the membrane material (even if the polymer or its solvent could have some toxicity) [43].

Even if the principle is simple, optimizing such a system is complex. To obtain nice, spherical, core-centered capsules as presented in Figure 6, one has to consider the viscosity of each phase, their surface tension, their flow rates, the size of the concentric nozzles, the breaking process, and the properties of the collecting solution. The most advanced system of this kind is currently based on the nozzle resonance method [44]. The main limitation in applying this method to the encapsulation of islets is the size of the capsules (which actually cannot be smaller than 500 μm diameter).

Coated islets

To minimize the transplant volume, a coating may be deposited or grafted directly onto the islets. The simplest solution is to suspend the islets in a reactive solution. PEG derivatives are generally used as the grafting material as PEGacylation has been shown to

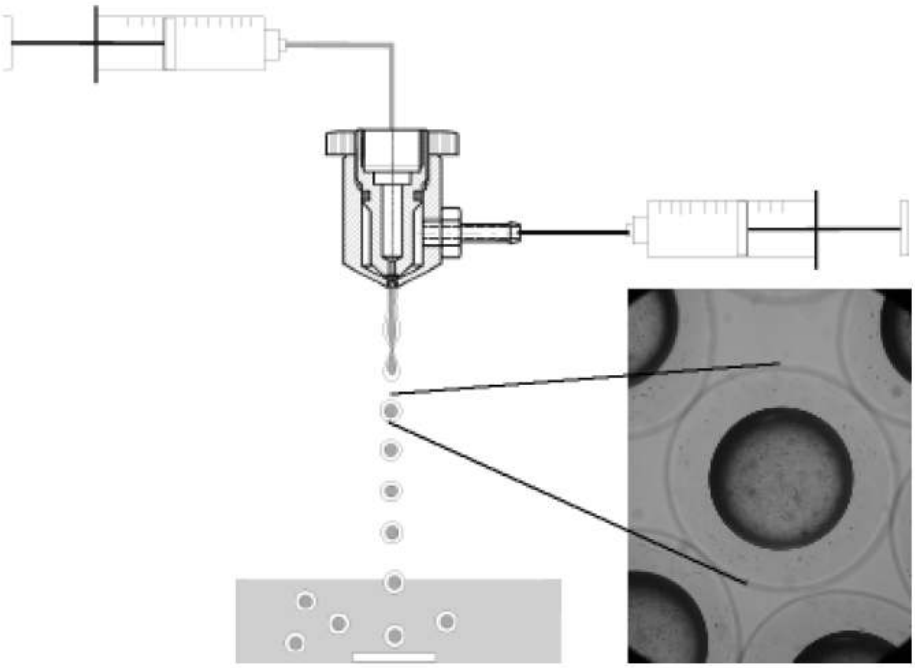

Figure 6. Coextrusion process (Capsulae © adapted from Inotech ©).

mask antigens from host antibodies [45]. PEG is fixed by direct covalent binding onto the cell surface amino groups or by insertion of a PEG-lipid or PEG-carbohydrate into the cell membrane [46].

An alternative is to absorb onto the islet surface a catalyst (i.e. eosin Y) and to suspend the islets in a monomer solution (i.e. PEG-diacrylate), then initiating photo polymerization of the monomer solution by light illumination [47]. Despite the need of a very careful optimization of the process, it is reported that cell viability can be greater than 90 % [48] and this technology is currently being evaluated for phase I/II clinical trial for human islet encapsulation (Novocell, Inc.).

Islets may alternatively be suspended in the top of a centrifugation tube filled with an alginate solution that is layered on top of a dextran calcium solution. During centrifugation, islets cross the tube while dragging a thin layer of alginate around them, which jellifies while entering in contact with the calcium solution [9]. By such technology, a coating layer as thin as 5 to 10 micrometers may be formed [10].

Conclusions

There are other alternatives to islet or cell encapsulation than the above-described methods. Many of them have been tested by a limited number of authors, while other will likely never be developed. However, it is important that researchers do not focus on too limited a number of methods. Considering the complexity of fulfilling all criteria necessary to succeed (biocompatibility, islet viability, vascularization of the capsule surrounding, mass transfer…), no one to this date could pretend to hold the optimum technology. Similarly, no one could pretend that a unique technology will be adequate for

all cases. Technologies may even need to be alternated over the period of therapy in order to avoid long-term body reaction.

The path has been long since 1980 when the first publication on islet encapsulation was published and, most probably, will continue to be long, in terms of the scale of the challenge before us, and the medical and social impact of diabetes. Treating this illness with a cell encapsulation system will open the door to many other medical applications. The main risk is that funding associations (public or private) will limit their effort to support this research in regard to the length of time required for its development.

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APPENDIX IV

BOOK CHAPTER

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7 Physical and chemical analysis of the microcapsule surface

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Abstract

The surface properties of microcapsules that are employed for the protection of transplanted islet cells are believed to play a vital role in the overall success of the microencapsulated graft system. The chemical, physical and structural nature of the surface at the microscopic and nanoscopic scale can have a significant influence on key characteristics of the microcapsules, including their biocompatibility, stability, and membrane transport properties. Surface studies of the microcapsules are also expected to play an important role in the development and standardization of the microcapsule design, as well as the eventual clinical and commercial developments of the devices. In this chapter, we discuss techniques that have been

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applied for the characterization of microcapsules designed for islet encapsulation. In particular, we describe four such techniques in detail: atomic force microscopy (AFM), attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), x-ray photoelectron spectroscopy (XPS), and time-of-flight secondary ion mass spectrometry (ToF-SIMS). For each, we explain the principles of the technique, discuss practical matters that must be considered when using the technique to analyse polymeric or hydrogel-based microcapsules, and describe studies in which the technique has been applied to analyse microcapsules (as well as certain materials and macrodevices) that are suitable for islet encapsulation.

Introduction

The definition of success

A popular and promising concept for a bio-artificial pancreas is based on microencapsulated islet transplantation [1, 2]. The most basic requirement for the successful implementation of the microencapsulated islet system is that the islet cells are both viable and functioning efficiently for several months or years after they have been transplanted into their new host environment. Just as importantly, this level of performance should be achieved reproducibly.

In the research lab, a great variety of factors has been shown to, or is hypothesized to, influence the viability and function of microencapsulated islet cells. A number of these factors are directly related to the biological state of the islet cells, including the isolation and handling procedure of the cells (as described in chapter 21 of this book), or the availability of growth factors, nutrients and oxygen supply to the islet cells once they are implanted (as described in chapters 9 and 10). Also identified to influence the success of the implant are several properties of the actual *microcapsules* that contain the transplanted islets.

A good microcapsule design

A successful microcapsule design must be capable of immuno-protecting the enclosed islets without interfering with their normal function. Once achieved, this level of performance must be reproducible and standardized before the microcapsule system can be used regularly in human patients. This demands that we define relationships between specific properties of the microcapsules and their final bioperformance. This will lead to the careful selection and quality control of key properties in order to achieve the desired performance of the microcapsules. Some of the most important characteristics of immuno-protecting microcapsules that have been associated with encapsulated islet performance include :

Specific transmembrane transport properties: The molecular weight cut-off (MWCO) of the semi-permeable membrane should ensure that larger antibodies and immune cells of the host immune system (>150 kDa) are prevented from entering the microcapsule. Simultaneously, adequate membrane permeability must allow smaller molecules such as oxygen, cell nutrients, metabolic waste, glucose, and insulin to diffuse freely across the membrane. This is the basis of the concept of immuno-protection by a semi-permeable membrane [3].

Chemical and mechanical stability: Broken or defective microcapsules may not provide adequate immuno-protection, put the islet cells at risk for mechanical injury, allow graft antigen shedding, or may provoke an immune response, for example, due to the exposure of immunogenic components at the microcapsule surface [4]. Thus, microcapsules should be able to withstand their fabrication process as well as long implantation periods (months or years) without significant loss of integrity or stability.

Short-term & long-term biocompatibility with the host: Ideally, there is no adverse reaction (acute/chronic inflammation or immune response) to the microcapsules. An immune response provoked by the microcapsules generally leads to reduced effectiveness, or failure, of the implant. An immune response against even a small portion of the implanted microcapsules can compromise the performance of the whole transplant [5]. Inflammation can be accompanied by a lowering of pH [6], thus may reduce microcapsule integrity or alter the membrane MWCO. Cytokines (e.g. IL-1β of 17.5 kDa) and nitric oxide (30 Da), which are toxic to islet cells, are secreted by nearby activated macrophages and are small enough to possibly penetrate the microcapsule [7, 8]. The adhesion and overgrowth of immune cells to the microcapsule surface, and the eventual development of fibrotic tissue around the implants, can obstruct the free diffusion of smaller molecules across the microcapsule membrane, thus compromising graft viability and effectiveness. A number of studies have associated the development of fibrotic overgrowth with transplant failure [9-11].

Controlled size: Smaller microcapsules (< 500 μm diameter) have been favoured over larger microcapsules because they allow easier diffusion of oxygen and nutrients into the microcapsules, and can be implanted into a greater range of physiological sites [12]. Smaller microcapsules may also have a greater mechanical stability. Too small of a size, however, may contribute to islet protrusion and thus surface defects in the microcapsules [13].

Although there may be other microcapsule properties that can influence the viability and function of the enclosed islets, they have not been mentioned above. Here, we simply seek to emphasize the importance of understanding and controlling such properties of the device in order to achieve optimal bioperformance of the implant. More detailed descriptions of relevant microcapsule characteristics can be found in various other chapters of this book.

In this chapter …

With the objective of optimizing the performance and design of immuno-protecting microcapsules, a wide range of methods for the assessment of microcapsule properties have been, or are continuing to be, developed. Several of the more popular methods have been reviewed by others [14, 15] (see also chapter 5) and will not be further elaborated here. Rather, the focus of this chapter is on investigations of the *surface properties* of the microcapsules, a topic that has only recently attracted the interest of most researchers in cell microencapsulation.

To open this chapter, we answer the question "*why study the microcapsule surface?*". Most researchers agree that the main purpose of studying the surface characteristics of the microcapsules is to understand and optimize their biocompatibility, an issue that has a long history in the field of cell encapsulation yet has not been satisfactorily resolved [16].

In the meanwhile, surface properties also influence several other pertinent aspects of microcapsule bioperformance and development that should not be neglected and are thus described in the following section.

Next, we present currently employed *approaches to characterizing the microcapsule surface*, particularly that of polymeric or hydrogel-based microcapsules. An overview of relevant techniques for surface analysis is presented, along with common technical challenges associated with the application of these techniques for microcapsule characterization.

In the bulk of this chapter, we present in detail four techniques that have been applied for the surface analysis of microcapsules designed for islet encapsulation. These are *atomic force microscopy* (AFM), *attenuated total reflectance Fourier transform infrared spectroscopy* (ATR-FTIR), *x-ray photoelectron spectroscopy* (XPS), and *time-of-flight secondary ion spectrometry* (ToF-SIMS). The basic principles of each of these techniques, as well as their applications for the characterization of microcapsules for islet encapsulation (and related encapsulation devices or materials) are described in detail.

Table 1. List and description of surface properties that are relevant to the bioperformance of polymeric microcapsules for cell transplantation, along with techniques that have been applied for the investigation of each surface property.

surface property of interest	**techniques for analysis**
surface chemistry : elemental composition molecular composition functional groups contaminants	XPS [18-20] ATR-FTIR [20-22] ToF-SIMS [20] SEM-EDX [23] SPR [24]
surface energy : wettability hydrophilicity/hydrophobicity	contact angle technique [25]
surface charges : type (positive/negative/neutral) density local distribution	zeta potential [26]
surface structure : topography/roughness morphology defects	AFM [23, 27-31] SEM [21, 32-35] CLSM [29, 31, 36]

Abbreviations : XPS = x-ray photoelectron spectroscopy ; ATR = attenuated total reflectance ; FTIR = Fourier transform infrared spectroscopy ; ToF-SIMS = time-of-flight secondary ion mass spectrometry ; SEM = scanning electron microscopy ; EDX = energy dispersive x-ray spectrometry ; SPR = surface plasmon resonance ; AFM = atomic force microscopy ; CLSM = confocal laser scanning microscopy.

Finally, for completeness, we include a brief description of *other techniques for the analysis of the microcapsule surface* before closing the chapter with a few *concluding remarks* about using physical and chemical analyses of the microcapsule surface as an approach to contribute to the development of the bio-artificial pancreas.

Why study the microcapsule surface ?

Relevant surface properties

Certain key surface properties are believed to be relevant to the bioperformance of polymeric microcapsules for cell encapsulation (or to the bioperformance of most biomaterials, for that matter [17]). These properties are described in the first column of **Table 1**. It is important to understand that these surface properties are not independent, but interrelated. For instance, the functional groups that are exposed at a biomaterial surface (*surface chemistry*) can influence its wettability (*surface energy*). As an example, the carboxylate group ($-COO^-$) attracts water and, if exposed, can render a surface hydrophilic. It is also important to note that, despite the recognized importance of surface properties for microcapsule performance and development, some of the properties mentioned in **Table 1** have not yet been extensively analysed in the case of microcapsules for islet transplantation.

The importance of surface studies

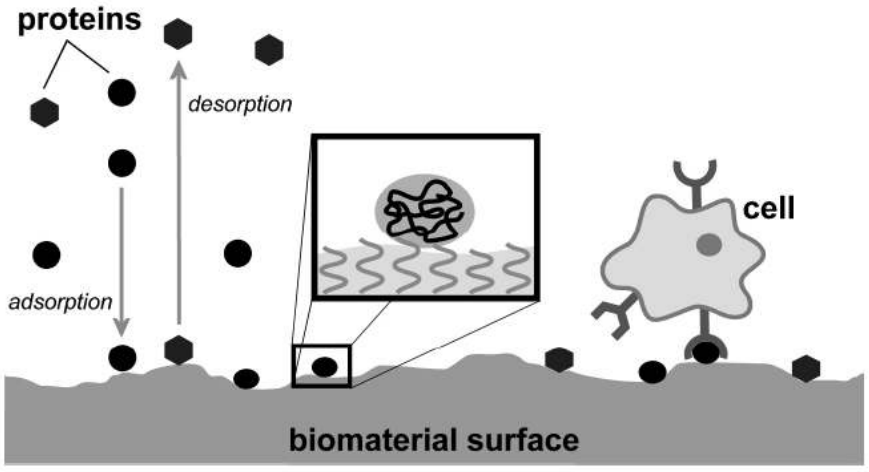

Figure 1. Schematic illustration of the relationship between a biomaterial surface, protein adsorption, and cellular response.

There are several valid reasons to investigate the surface properties of microcapsules for islet transplantation. In terms of *research development*, the purpose of these investigations is to optimize several of the characteristics of a successful microcapsule design that were presented in the previous section:

Biocompatibility with the host: It has been observed that certain properties of a microcapsule surface, including smoothness and chemical composition, influence its

biocompatibility [27, 37]. Yet, the mechanistic details explaining this relationship remain vague, demonstrating the need for more extensive analyses. There is ample evidence that, in general, the cellular response (including the foreign body response) to a biomaterial is governed by the manner in which proteins adsorb to that biomaterial, and thus by the biomaterial's surface features [38]. A growing body of evidence indicates that this concept, which is illustrated in **Figure 1**, applies to microcapsules as well. In fact, we and others have shown that biological proteins, including antibodies, do indeed adsorb to the microcapsule surface upon implantation or exposure to biological fluids [37, 39-41].

Chemical and mechanical stability: The chemical and physical structure of the microcapsule surface can both influence and provide important information about the stability of the microcapsule. Alterations of the capsule surface state have already been measured as a method to monitor capsule degradation during implantation or storage [42]. Moreover, the manner in which the polymeric components of a microcapsule are complexed together at, or near, the surface influences the strength of the capsule membrane [43]. Finally, small local defects at the surface may also lead to capsule breakage.

Transmembrane transport: In addition to porosity, pore size and distribution, the surface hydrophilicity/hydrophobicity and surface charge of a membrane will influence the passage of certain molecules across the a membrane, particularly non-neutral molecules [44]. Although this concept has not yet been explored experimentally in the case of microcapsules for cell transplantation, at least one group has investigated this relationship for macrodevices that are candidates for a bio-artificial pancreas [45, 46].

In addition to contributing to the optimization of the above-mentioned properties of immuno-protecting microcapsules, surface investigations are expected to play a significant role in several other important stages of development of the microcapsule system:

Design development: It may be necessary to design a surface modification of the microcapsules in order to obtain optimal surface properties and a higher level of bioperformance. In fact, some researchers have already proposed to graft poly(ethylene glycol) molecules, which are resistant to protein and cell adhesion, to the microcapsule surface [32, 47, 48]. When an encapsulation device undergoes any form of surface treatment, the effects on the various surface properties must necessarily be monitored using a combination of analytical techniques.

Clinical development: As clinical trials of the microencapsulated islet system become more regular, we should already be establishing the proper tools to efficiently pre-screen and evaluate the *in vivo* bioperformance of the microcapsules. Since it is becoming increasingly evident that the surface properties of the microcapsules are related to their bioperformance, we see the necessity to continue current efforts in improving and optimizing a number of techniques for the analysis of the microcapsule surface.

Commercial development: Microcapsules for islet transplantation are already being commercially developed (www.novocell.com, www.amcyte.com, www.lct.com.au, www.microislet.com, www.islet.com). Preparing these implantable devices for the market requires that they meet regulatory standards, such as those established by the American Society for Testing and Materials (ASTM) and the U.S. Food and Drug Administration (FDA), to ensure that they are safe and effective. Meeting these standards

demands a wealth of experimental results that should involve a number of surface analytical techniques.

Approaches to characterizing the microcapsule surface

Techniques for the surface analysis of microcapsules

The second column of **Table 1** lists analytical techniques that have actually been used to investigate the surface properties of microcapsules for islet encapsulation. There also exists a number of other techniques that can, in principle, yield interesting information about the surface of islet-encapsulating microcapsules, but (to our awareness) they have not yet been used for this purpose. For an excellent overview of surface analytical techniques in general, we suggest that the reader visit www.uksaf.org/tech/list.html.

In this chapter, we selected four currently available surface characterization techniques (listed in **Table 1**) to describe in detail: atomic force microscopy (AFM), attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), x-ray photoelectron spectroscopy (XPS), and time-of-flight secondary ion mass spectrometry (ToF-SIMS). Each of these techniques has been used in our laboratory to emphasize the physicochemical features of microcapsules at the micro- and nano-scale, as an approach that is necessary for elucidating the biological interactions with the microcapsules. AFM, which focuses mainly on surface morphology and topography, was among the first analytical techniques to be applied to characterize the microcapsule surface. The other three techniques focus more on the chemical or molecular aspects of a sample surface. In comparison to ToF-SIMS, the techniques XPS and ATR-FTIR are currently more popular as methods to analyse the surface of cell encapsulation devices, although all three have contributed to the advancement the encapsulated islet system. Moreover, these techniques are often used in combination with one another owing to their complementary features. The principles of each technique, as well as their applications for the characterization of microcapsules for islet transplantation, are presented. In certain instances, we also present some relevant cases of their use for the analysis of the material components of the microcapsule, and/or of the surface of macrodevices that may also serve as a bio-artificial pancreas.

For completeness, the other techniques that have been applied for the study of microcapsule surfaces (**Table 1**) are also briefly described at the end of this chapter.

General challenges of microcapsule surface analysis

The task of applying surface analytical techniques for the study of polymeric microcapsules can be quite challenging. In most cases, these techniques were originally developed for the analysis of metals, non-organic samples, or flat surfaces. Consequently, new experimental protocols must often be developed in order to adapt these techniques for the analysis of soft, hydrated polymeric spheres.

Almost all microcapsules designed for islet transplantation are hydrogel-based, which means that they contain up to 99% water [49]. In particular, cross-linked alginate gels are the most popular choice of material for microcapsule formation. The molecular structures of alginate and its corresponding gel are described in **Figure 2**. Unfortunately, the high water content of the hydrogels can make them difficult to analyse. That is, a number

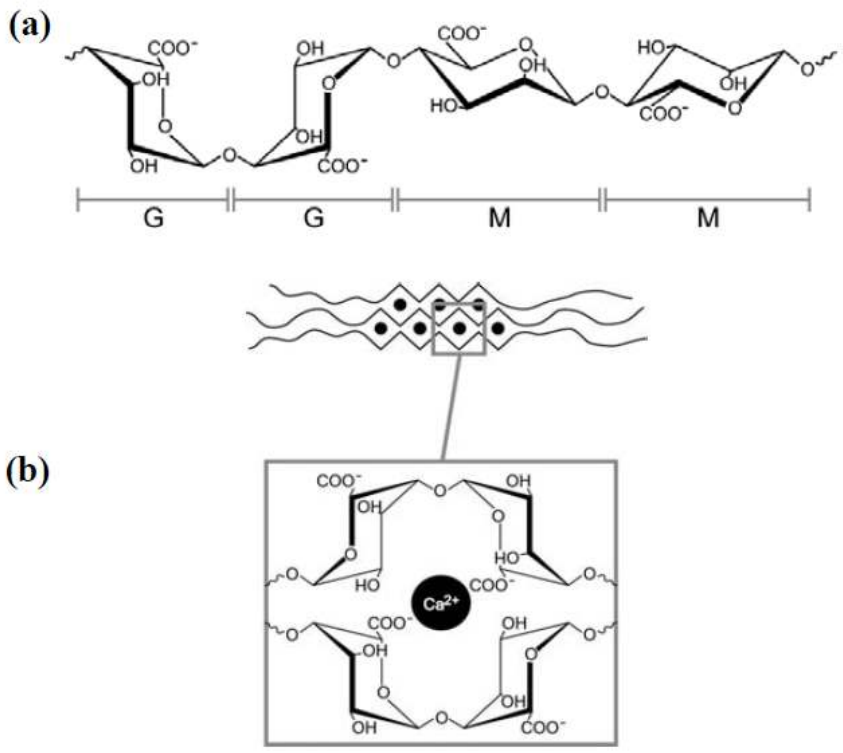

Figure 2. (a) The molecular structure of alginate (with deprotonated carboxyl groups). Alginate is composed guluronic acid (G) and mannuronic acid (M) residues that are segmented as G-blocks (…GGG…), M-blocks (…MMM…), or MG-blocks (…MGMG…). (b) Alginate gelation is induced by cross-linking the polymeric chains using divalent cations. Here, we show cross-linking by Ca^{2+} ions that tend to bind to G-blocks of the alginate.

of the analytical techniques require a vacuum environment in order to maintain instrument sensitivity, yet a sample cannot remain wet under a vacuum due to quick evaporation of the water. As a consequence, the microcapsules must be dehydrated before they can be analysed, for instance by slow air-drying, quick freeze-drying, or successive alcohol baths. Precautions must be made to ensure and verify that the microcapsules keep their structural and chemical integrity as much as possible during the dehydration process. Even with these precautions, however, dehydrated microcapsules cannot pretend to be accurately representative of the hydrated version of the microcapsules.

Another main challenge associated with the surface analysis of microcapsules comes from the fact that the microcapsules are three-dimensional (3D) spheres. More often than not, flat, two-dimensional (2D) surfaces are much easier to analyse. To overcome this challenge, researchers have recreated the microcapsule structure and composition by producing films or multilayers on flat substrates. Again, caution must be made when drawing conclusions from such studies as the properties of the actual 3D structure of the capsules may differ from 2D representation.

Given that microcapsules for islet transplantation are polymer-based, they are sensitive to degradation or destruction by the high-energy beams (x-rays, electrons, etc.) that bombard the samples during analysis. One strategy to overcome this problem is to

minimize exposure of the microcapsules to the energy beams by reducing scan times or beam intensity.

Finally, polymeric microcapsules are not electrically conductive. This can present a problem for techniques that utilize electrons or ions to irradiate the sample surface. As a consequence, there may be an accumulation of charge where the sample is irradiated, which in turn can alter the surface state of the sample or lower the quality of the analytical spectrum.

Specific challenges associated with the surface analysis of polymeric microcapsules using the techniques AFM, ATR-FTIR, XPS, and ToF-SIMS are presented in more detail in their respective sections, under the subtitle *practical considerations*.

Atomic force microscopy (AFM)

Principles of the technique

The technical details of the workings of atomic force microscopy (AFM) are described in Chapter 8 by Lacík and Chorvát, and will not be repeated here. However, to allow the reader to compare the features of AFM with the other surface analytical techniques presented in this chapter, we present a summary of the key characteristics of AFM in **Table 2**.

Analyses of microcapsules using AFM

A presentation of techniques for the surface analysis of microcapsules for islet encapsulation would simply not be complete without mentioning AFM. Since its first application for the analysis of such microcapsules in the late 1990's, AFM has become a popular choice for characterizing their *surface morphology and roughness (smoothness)*, and more recently their elastic or *surface mechanical properties*. This is owing to the fact that hydrogel-based microcapsules can be analysed by AFM while they are in their hydrated state and within a liquid of choice (including physiological fluids). Furthermore, the samples do not have to be submitted to any special kind of sample preparation. This allows researchers the opportunity to characterize the true surface state of the hydrated,

Table 2. Summary of AFM key characteristics.

key information	• 2D or 3D mapping of surface topography and morphology • quantification of surface roughness • with force curves, evaluation of local elastic properties • under appropriate conditions, mapping of electrical or magnetic forces • with modified tips, evaluation of molecule-sample interactions
detection limits	• lateral (x-y) resolution 0.5 - 10 nm • depth (z) resolution 0.05 - 1 nm
sample requirements	• all forms of solids, in air or immersed in a liquid • generally no thicker than ~ 8.5 mm, and not too rough
cautions	• sample must be properly fixed in place onto substrate • soft samples can easily be damaged (or damage the tip)

3D microcapsules in a simulated *in vivo* environment. Nevertheless, despite the ever-increasing use AFM for microcapsule characterization, a number of interesting features of this technique, including the ability to measure the force of molecule-surface interactions or to map local surface charges, have not yet been exploited for the investigation of microcapsules for cell encapsulation.

Practical considerations

Overall, a basic topographical analysis or phase imaging of the microcapsule surface by AFM is relatively straightforward and imposes very few constraints in terms of sample preparation. There are, however, a few points to be kept in mind when using this technique to analyse hydrogel-based, three-dimensional microspheres.

In order to avoid distortions of the sample image, the microcapsules must not move during AFM data acquisition. In practice, properly fixing the microcapsules in place is not always straightforward. It is preferable to hold the microcapsules in place mechanically rather than chemically to avoid contamination of the samples.

The softness and pliability of hydrogel-based microcapsules also presents a technical problem since the samples can be easily deformed when in contact with the cantilever tip. That is, dragging or catching the tip across the microcapsule surface can destroy image resolution as well as damage both the sample and tip. Also, the compressibility of the microcapsules can make it difficult to interpret phase images (i.e. maps of material surface properties including stiffness and visco-elasticity). Such problems may be minimized by limiting the cantilever force and contact between the tip and sample surface.

AFM to investigate capsule surface smoothness

AFM is among the earliest techniques applied for the direct surface analysis of microcapsules for islet encapsulation. In 1998, Xu et al. were the first to use AFM to investigate the surface of such microcapsules, with the recognition that smooth biomaterial surfaces tend to discourage cell attachment and fibrotic overgrowth [30]. They fixed microcapsules composed of sodium alginate, cellulose sulfate and poly(methylene-coguanidine) (SA-CS/PMCG) onto a mica surface using a touch of vacuum grease, placed them within a liquid bridge of a phosphate-buffered saline solution (PBS), and analysed them in contact mode. They observed qualitative changes in surface morphology and measured quantitative changes in roughness as they altered the rate of polymer complexation, which was controlled by the ratio of Na^{+}/Ca^{2+} in the cation solution for capsule formation.

Shortly afterwards, Zimmermann et al. used AFM on several occasions to analyse gel beads of barium alginate for islet transplantation. The beads were analysed in non-contact mode within an aqueous environment, generally a saline solution. In one study, by combining AFM with other imaging techniques (confocal laser scanning microscopy and nuclear magnetic resonance), they too correlated gelation dynamics (controlled by the fabrication protocol) with surface topographical features, as well as with encapsulated cell viability and secretory functions [31]. In another study, they used AFM to examine barium alginate beads before and after their implantation in rats, and observed that the surface roughness increased after an implantation period of several weeks [50]. In parallel, they used AFM on flat alginate films to demonstrate that cells migrating over the

film can deposit fibrils, rendering the surface rougher and more susceptible to further cellular attachment. These combined results served to support their hypothesis that the foreign body response to the alginate gel beads was initiated by surface inhomogeneities and roughness [51].

Bünger et al. came to a similar conclusion in the case of alginate-poly-L-lysine microcapsules having either an alginate, heparin, or polyacrylic acid coating [27]. They used AFM in tapping mode to examine their capsules, which were mechanically fixed by a plastic ring in a Petri dish and immersed in a Krebs-Ringer-Hepes (KRH) buffer solution. They associated the chemical composition of the microcapsules with their surface topography and roughness, as well as with their *in vivo* biocompatibility, and observed that capsules with smoother surfaces tended to be more biocompatible.

Among other techniques, Shen et al. [23] used AFM to evaluate the morphology of microcapsules that were produced using an iron-containing alginate, or "ferrofluid". They proposed to visualize these novel microcapsules *in vivo*, in a non-invasive manner, using magnetic resonance imaging (MRI). They immobilized monolayers of the capsules within a layer of agarose gel in a Petri dish, and performed contact AFM on samples immersed in a calcium chloride solution. They observed that incorporation of ferrofluid within the microcapsule core led to a less smooth surface. It is interesting to note that these nanoscale differences in surface morphology were observable using AFM, yet not discernable using environmental electron scanning microscopy. The biological consequences of altered surface morphology due to the ferrofluid were not evaluated.

Lekka et al. [28] used AFM in contact mode to evaluate the surface roughness and morphology of nine types of alginate-based microcapsules, which they mechanically fixed in a saline solution using a custom-made polycarbonate/poly(ethylene terephthalate) sample holder. In addition to correlating microcapsule chemistry with surface topography, they also measured the surface roughness profiles for several microcapsules within the same batch in order to evaluate batch heterogeneity.

Most recently, Podskočová et al. used semi-contact AFM to evaluate the surface topography and roughness of SA-CS/PMCG microcapsules in a liquid environment [29]. The results are discussed in more detail by the authors of this work in Chapter 8.

AFM to investigate surface mechanical properties

In the same study mentioned above, Shen et al [23] extended the use of AFM beyond morphological analysis to provide information about the surface mechanical properties of their magnetic alginate-based microcapsules. To do this, they measured the cantilever deflection to acquire force curves (i.e. plots of the force felt by the cantilever vs. the distance between the tip and sample). In the case of microcapsules with ferrofluid incorporated into their surfaces, a steeper force curve with minimal hysteresis suggested a stronger surface stability and greater elasticity than samples with either no ferrofluid or with ferrofluid integrated into the capsule core.

Similarly, in their study, Lekka et al. used AFM for more than examining topography in order to quantify local elastic properties along the surface of alginate-based microcapsules of various chemical composition [28]. In their case, they used the measured force curves to calculate the Young's modulus for each microcapsule type. The group correlated these surface properties with the bulk mechanical properties of the capsules, including their resistance to compression, in recognition that each of these

factors can play a significant role in the biocompatibility and design evolution of the microcapsules. They also used the calculated values of Young's modulus to evaluate batch heterogeneity as well as surface homogeneity on single samples.

Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR)

Principles of the technique

ATR-FTIR is a mode of infrared (IR) spectroscopy. IR spectroscopy provides information about the molecular structure of organic (and some inorganic) samples. Fourier transform refers to a method to maximize the efficiency of IR spectroscopy analysis, while the use of an ATR accessory renders the FTIR surface sensitive. In actuality, there are many more FTIR sampling techniques and accessories available than are mentioned here, as we have selected to describe just a few that are of interest for the characterization of the microcapsule system. For more detailed descriptions of the ATR-FTIR and infrared spectroscopy techniques, the reader is referred to references [52-57].

In *infrared (IR) spectroscopy*, the sample is radiated by a beam of infrared light. The IR radiation is absorbed by the sample at specific frequencies (or wavenumbers) that are characteristic of the molecular structure of the sample. IR light in the mid-infrared range (i.e. having a wavenumber of 4000 - 400 cm^{-1}) is most commonly used, as frequencies in this range correspond to changes in vibrational energy within molecules.

Molecules at temperatures above absolute zero are not static. They experience several different types of motion, including vibrations (**Figure 3**). Based on the types of elements and chemical bonds involved, a molecule can vibrate only at specific frequencies that correspond to a set vibrational energy states. A molecule can be "excited" into a higher vibrational energy state (i.e. vibrate at a higher frequency) when it absorbs IR radiation of an appropriate frequency. That is, in order for a molecule to absorb IR light, the energy (or frequency) of the IR light must be equal to the energy required to excite the molecule to its next vibrational state. If this vibrational transition is accompanied by a net change in the dipole moment (i.e. charge distribution) of the molecule, then the molecule or bond is "infrared active" and there is a strong absorption of the IR light.

To create an IR spectrum, also called an absorbance spectrum, one must measure intensity losses in the IR beam due its absorption by the sample at specific frequencies (for examples of IR spectra, see **Figure 8**). The IR spectrum is typically presented as a plot

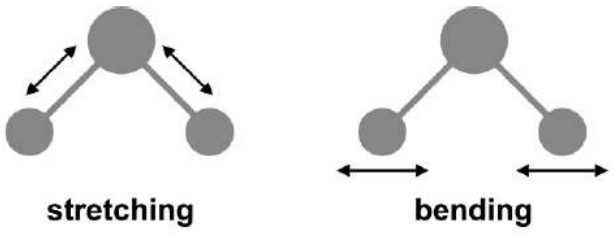

Figure 3. Schematic illustration of two examples of vibrational movements that can occur within molecules.

of measured percent transmittance or absorbance (y-axis) as a function of wavenumber or wavelength of the IR radiation (x-axis). Percent transmittance (%T) is defined by the ratio of the intensity of the IR beam after it has passed through the sample (I) to the intensity of the incident IR beam (I_0), and is related to absorbance (A) by :

$$A = -\log_{10}(T) = -\log_{10}\left(\frac{I}{I_0}\right) \qquad \text{(Eq. 1)}$$

Furthermore, the wavenumber ($\tilde{\upsilon}$), the wavelength (λ) and the frequency (υ) of the IR beam are mathematically related by :

$$\tilde{\upsilon} = \frac{1}{\lambda} = \frac{\upsilon}{c} \qquad \text{(Eq. 2)}$$

where c is the speed of light. The relationships described in **Equations 1 and 2** make it simple to convert between units for the axes of the IR spectrum.

As explained earlier, very specific conditions are required for IR radiation to be absorbed by a molecular bond. Because of this particularity, peaks in the IR spectrum (also called "absorption bands") represent the presence of specific chemical bonds or functional groups within the sample. Unknown materials can be identified by the presence of a set of characteristic peaks in their IR spectrum.

The intensities of the adsorption bands are, in theory, linearly proportional to the concentration of each component in a homogeneous mixture or solution. In practice, however, this relationship is not so linear due to instrumental and sample effects. As a result, quantification of sample components by IR spectroscopy can be demanding. Proper quantification requires empirically determined calibration curves, careful peak integration and the use of appropriate software. Semi-quantitative analysis of IR spectra is also an option, and is often sufficient for most research purposes. This can involve, for example, comparing relative peak heights or overlaying the spectra of related samples.

In *Fourier transform infrared spectrometry (FTIR)*, an FTIR spectrometer is used to measure all of the frequencies within an IR beam simultaneously. FTIR spectrometers are much more efficient and precise than dispersive spectrometers that separate and measure the IR beam frequencies individually. The layout of the FTIR spectrometer is illustrated in **Figure 4**. An interferometer uses moving mirrors to split the source IR beam into two, then recombines the split beams after they have travelled different optical distances. The recombined beams interfere with one another to create a signal called an interferogram. Each point in the interferogram has information at every frequency of the IR beam "encoded" into it. The interferogram enters the sample where some of the radiation is absorbed and some is transmitted towards the detector. To create a comprehensible infrared spectrum (e.g. a plot of %T vs $\tilde{\upsilon}$), the detected signal must then be "decoded". To do this, a computer must be used to perform an algorithm called a *Fourier transform* on the transmitted interferogram.

Attenuated total reflectance (ATR) refers to a sampling technique that can be used to analyse surfaces, as well as opaque or thick samples that are difficult to analyse using transmission FTIR (where the IR beam passes straight through the sample).

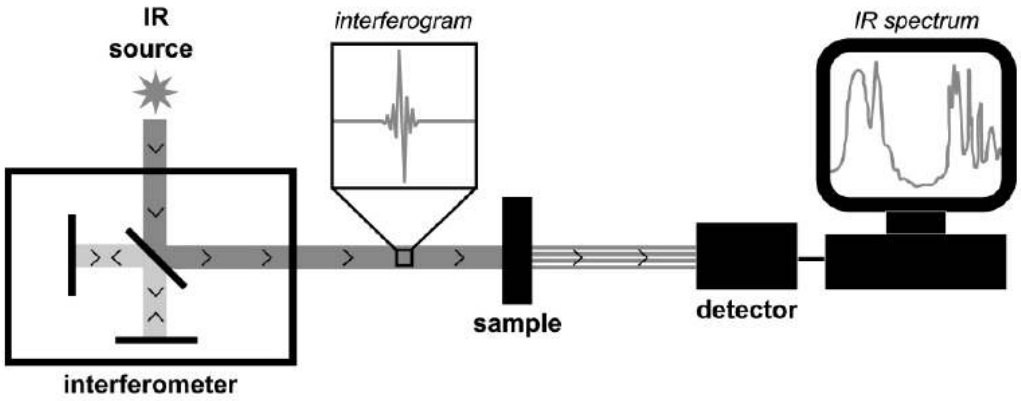

Figure 4. Simplified layout of the Fourier transform infrared spectrometer.

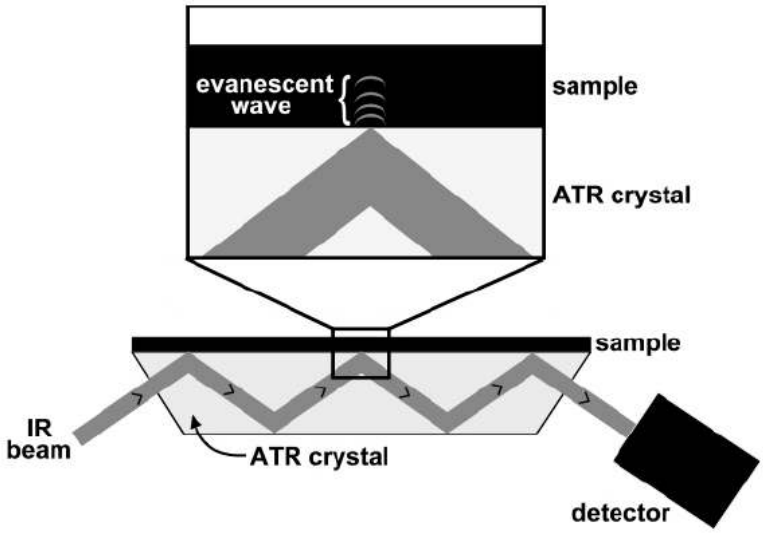

Figure 5. Schematic illustration of the use of a (horizontal) ATR crystal to penetrate a sample surface with an evanescent wave of IR radiation.

In ATR-FTIR (see **Figure 5**), the surface of the sample is pressed against a crystal having a high refractive index, such as germanium (Ge) or zinc selenide (ZnSe). The IR beam is then passed into the crystal towards the sample. If the incident angle of the IR beam exceeds a certain "critical angle", the beam reflects internally within the crystal. As the IR radiation reflects against the side in contact with the sample, a small amount of the radiation (in the form of an evanescent wave) travels beyond the crystal surface and enters the sample. The sample will absorb portions of this evanescent wave at frequencies which match the vibrational frequencies of the sample molecules. The beam is then directed out of the crystal and back towards the spectrometer, where the signal is processed as described above.

The ATR-FTIR technique is surface sensitive because the evanescent wave decays exponentially as it travels into the sample, getting no further than a few micrometers. The

penetration depth of the evanescent wave, d_p (which is defined by the distance from the crystal/sample interface at which the signal decays to $1/e$ of its initial value), is actually dependent on a number of factors, including the angle of incidence (θ), the refractive index of each the crystal (n_1) and the sample (n_2), as well as the wavelength (λ) of the IR beam. Theoretically, we can calculate that :

$$d_p = \frac{\lambda}{2\pi . n_1 \sqrt{\sin^2 \theta - \left(\frac{n_2}{n_1}\right)^2}} \qquad \text{(Eq. 3)}$$

Equation 3 is graphically shown in **Figure 6** for two ATR crystal types, germanium and diamond, and a sample with a refractive index of 1.5 (most organic samples and polymers have refractive indices that are close to this value). To compensate for the variation of penetration depth with IR wavenumber, one should re-scale the absorbance values in the measured IR spectrum by using appropriate software to perform an "ATR correction".

In our lab, we have used *micro-ATR* to analyse polymeric microcapsules. In this version of ATR-FTIR, a microscope accessory is employed and a smaller crystal is pressed into the sample. This allows the user to visualize the sample as well as target a small surface area for IR spectral analysis. Furthermore, micro-ATR requires low contact pressure and thus induces less sample damage. To increase the sensitivity, spatial resolution and speed of this technique, a liquid nitrogen-cooled detector should be used. Also, when integrated with an appropriate software package and sampling stage, micro-ATR can be used for chemical mapping, which basically involves recording IR absorbencies at several points within a defined area of the sample surface.

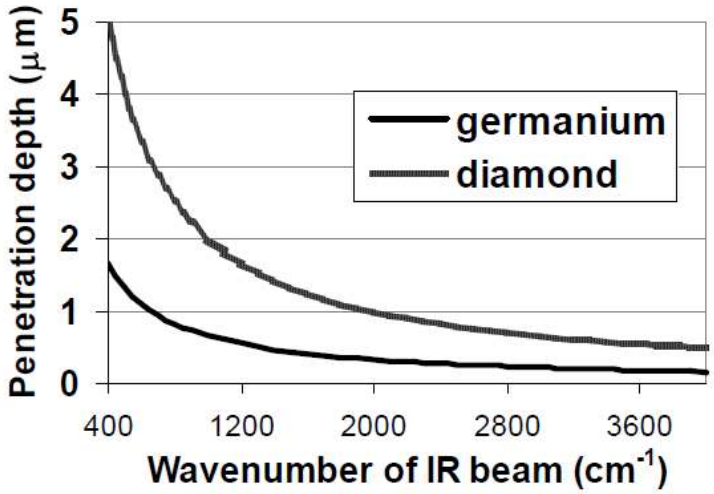

Figure 6. Theoretical penetration depth, d_p, of an evanescent wave of IR radiation into a sample, as calculated using Equation 3. Values are shown for each a germanium ($n_1 = 4.0$) and a diamond ($n_1 = 2.4$) ATR crystal, for a sample having a refractive index of $n_2 = 1.5$ and the IR beam angle of incidence being $\theta = 45°$.

Another very interesting sampling technique, particularly for the evaluation of biomaterials, is *in situ ATR-FTIR*. In this technique, a special trough or chamber surrounding the ATR crystal and sample allows the user to pass a liquid or gas across the surface of the crystal or a thin sample layer while recording the IR spectrum in real-time. This type of accessory is suitable for evaluating, for instance, the adsorption of proteins from a physiological fluid onto a thin film.

The key characteristics of the ATR-FTIR technique are summarized in **Table 3**.

Table 3. Summary of ATR-FTIR key characteristics.

key information	• identification of molecular structures and chemical bonds • measures relative quantities of detected components • imaging, depth profiling, in situ and real-time options available
detection limits	• analytical depth < 5 μm • spatial resolution (the ability to distinguish small, adjacent sample features) 10 - 25 μm for micro-ATR • position accuracy of 10 μm for micro-ATR • practical 'analysis spot' size of 50-100 μm for micro-ATR • detection limit in the ppm range or 0.1wt%
sample requirements	• all forms of solids and liquids • intimate contact with the ATR crystal • size from 10 μm diameter (micro-ATR) to several mm × mm (HATR)
cautions	• water produces a strong signal (is a strong absorber of IR radiation) and may mask other peaks of interest • homo-nuclear diatomic molecules (such as H_2 or N_2) and mono-atomic ions (such as He and Ar) are not detectable

Analysis of microcapsules using ATR-FTIR

Because of the range of information provided, as well as the relative accessibility and low cost of this technique, ATR-FTIR has become an increasingly popular technique for the analysis of the microcapsule surface. Direct examination of intact microcapsules by ATR-FTIR can reveal important information about the manner in which molecules interact with each other near or at the capsule surface, and thus provide insights about the *formation and degradation of the capsule membrane*. These studies have been complemented by ATR-FTIR or transmission FTIR studies of samples in a film or powder form (rather than intact capsules), which can provide further details about the *molecular interactions between the material components* of the capsules. In addition, in situ ATR-FTIR has proven to be an effective way to *monitor the adsorption of proteins* to the surface of films that simulate the capsule structure.

Practical considerations

While it is possible to analyse hydrated samples using FTIR, interpretation of the spectra can be problematic since water is a strong absorber of infrared radiation thus can mask other peaks of interest in the IR spectrum. In our laboratory, we have attempted to analyse hydrated alginate-based microcapsules using ATR-FTIR with a subsequent

substraction of the water spectrum (unpublished data), but the resulting signal was too weak and altered to yield any useful information. Thus, we (and others) have selected to analyse dehydrated samples. Of course, the price for eliminating the water signal is that the molecular conformations at the surface of the dehydrated samples is not an accurate representation of the hydrated version of the capsules (readers may refer to reference [20] for a more detailed discussion on this topic in relation to alginate-poly-L-lysine microcapsules).

Another aspect that must be considered when analysing microcapsules is a requirement that there be intimate contact between the sample surface and the ATR crystal. The better contact there is, the stronger the sample signal, as more of the evanescent wave can penetrate the sample. The use of micro-ATR simplifies this task since the smaller crystal (~100 μm diameter contact area) can be quite accurately pressed onto a relatively small surface area on the sample, in addition to providing the option of targeting the area of interest with the aid of an optical microscope that is aligned with the crystal. Conversely, obtaining sufficient contact between tiny (dehydrated) capsules and a horizontal ATR crystal can be more troublesome since a larger crystal area (typically 72 mm x 10 mm) should be covered as much as possible and with minimal spaces between the capsules.

Finally, although ATR-FTIR has the advantage of not requiring a vacuum environment (as do XPS and ToF-SIMS), water vapour and carbon dioxide from the atmosphere can adsorb onto the sample surface and create interfering peaks in the IR spectrum. While these peaks may be removed by subtracting a background spectrum from the sample spectrum, it is recommended to purge the analysis chamber with dry air or nitrogen in order to minimize atmospheric contamination of the sample.

ATR-FTIR to characterize the capsule membrane structure and formation

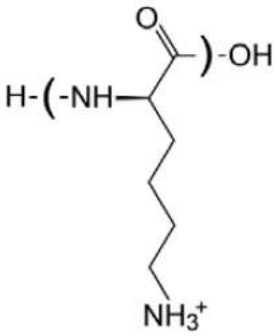

Figure 7. The molecular structure of poly-L-lysine, or PLL (with protonated amine groups).

Van Hoegmoed et al. [22] were the first to use ATR-FTIR to examine the near surface structure of intact alginate-poly-L-lysine microcapsules designed for islet encapsulation. To evaluate the adequacy of microcapsule formation, they investigated the molecular details of the capsule surfaces before and after the addition of their membranes, a process that involves the electrostatic binding of poly-L-lysine (PLL) to a "bead" of calcium alginate gel (see **Figures 2 and 7** for the molecular structures of

alginate and PLL). For the ATR-FTIR measurements, they pressed the samples (previously rinsed, lyophilized, and checked for membrane integrity by microscope) into a diamond ATR crystal and flushed the compartment with nitrogen gas during analyses. Using the relative heights of the peak at ~2928 cm^{-1}, which arises from the asymmetric stretching of the CH_2 group of the PLL, they could estimate that capsules composed of alginate having a high guluronic acid (G) content contained ~20% more PLL in their membranes than capsules composed of alginate with an intermediate-G content. Their IR spectra also allowed them to observe the "disappearance" of calcium ions from the alginate gel when the PLL membrane was added. This was evidenced by slight shifts of the peaks within the carbohydrate region at 1170-1020 cm^{-1}, including the peaks associated with C-O bond stretching, C-O-H bending, and COO^- symmetric stretching within the alginate. This effect was more pronounced for capsules of high-G alginate than intermediate-G alginate. The authors also used their IR results to explain the superior stability of high-G alginate capsules (as compared to intermediate-G capsules) by the presence of more hydrogen bonding, as evidenced by a broadening of the associated peak towards frequencies lower than 3000 cm^{-1} and an intensity increase of the O-H and N-H stretching peaks with the addition of the PLL membrane. Moreover, the presence of peak shoulders in the protein region of the IR spectra indicated that PLL existed in three different

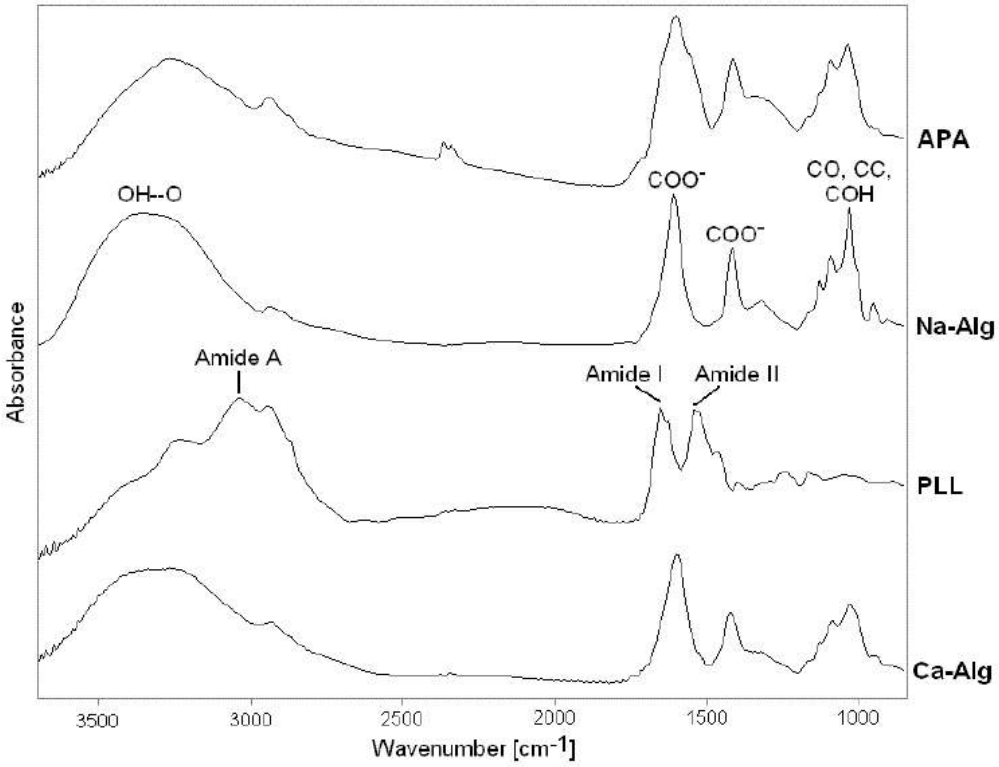

Figure 8. ATR-FTIR absorbance spectra of the surface (~ 3 μm depth) of dehydrated alginate-poly-L-lysine microcapsules (APA) and their components: sodium alginate film (Na-alg), poly-L-lysine film (PLL), and dehydrated beads of calcium alginate gel (Ca-alg). (Figure has been reproduced from ref [20] with permission from Elsevier Limited).

conformations within the capsule membrane: random coil, anti-parallel β-sheet and α-helix. The authors suggested that the first conformation indicates sufficient interactions of the PLL with the alginate, while the latter two indicate less interaction because of increased intramolecular hydrogen bonding within the PLL.

Shortly afterwards, we [20] also published a study in which we used a microscope equipped with a Ge micro-ATR crystal to examine similar alginate-PLL microcapsules, as well as calcium alginate beads and the individual alginate and PLL components as references. The IR spectra of each of these samples are shown in **Figure 8**. In our case, we prepared our samples for analysis by rinsing them with water then slowly dehydrating them onto a silicon substrate. In confirmation of the results of Van Hoegmoed et al., we also observed signs of counter-ion exchange (i.e. the NH_3^+ of the PLL replacing the Na^+ of the sodium alginate) within the capsule membrane, as the peaks near 1410 cm^{-1} and 1600 cm^{-1} arising from the COO^- stretching of the alginate were shifted between the capsule surface and the sodium alginate reference. We also measured the precise placements of the Amide I (1645 cm^{-1}) and Amide II (1540 cm^{-1}) peaks associated with the PLL to confirm that the PLL existed in at least two conformations (α-helix and random coils) within the capsule membrane. In our case, however, we interpreted the α-helix conformation to indicate a strong interaction between the PLL and alginate (as the alginate forms a super-helix around the PLL) while a random coil conformation suggests a limited interaction between the two molecules (non-interacting PLL should have converted back to a β-sheet upon dehydration).

ATR-FTIR to characterize capsule degradation

Thanos et al. used ATR-FTIR to characterize the *in vivo* stability of various types of alginate-poly-L-ornithine microcapsules that can be used for islet encapsulation [21, 42, 58]. For their studies, they selected to use horizontal ATR-FTIR equipped with a ZnSe crystal to analyse their capsules, which were first lyophilized and checked for integrity under a microscope. The microcapsules were implanted in rats, and their stability was estimated by the relative quantities of poly-L-ornithine (PLO) and alginate that were detected at the explanted capsule surfaces; an increased concentration of PLO indicated degradation or surface erosion of the samples during implantation. In this case, the authors took the necessary steps to properly quantify the amounts of PLO in their samples. That is, they recorded the IR spectra of mixtures containing known portions of pure alginate and PLO, then demonstrated a near-linear relationship between the ratio of the amide II peak (PLO) to COO^- peak (alginate) intensities and the concentration of PLO (%w/w) in the mixture. In this manner, they quantified the exposure of PLO due to surface erosion for capsules made with different alginate types [21] or different fabrication protocols [58], and for capsules implanted in different implantation sites [42]. In addition, the surface morphology of the explanted capsules (as observed by scanning electron microscopy), as well as their measured sphericity and diameter, tended to support the results drawn from the IR analyses.

FTIR to characterize molecular interactions of capsule components

Before the first published ATR-FTIR studies of intact capsules, researchers used transmission FTIR to analyse their material components. They did this in recognition of the importance of polymer interactions to the properties and overall performance of the

capsules. Although these are not direct studies of microcapsule surface, the results provide useful data than can be used for the interpretation of complete capsule spectra, as well as offer an explanation for a number of capsule properties (e.g. membrane strength) in terms of molecular interactions, and are thus worth mentioning here.

In 1994, Dupuy et al. [59] used transmission FTIR to examine the effect of an alginate's manuronic/guluronic acid (M/G) content on the strength of each the cross-linked alginate gel and the alginate-poly-L-lysine (PLL) complex. Alginate films were produced by evaporation of aqueous solutions, while alginate-PLL membranes were produced to simulate the corresponding capsule fabrication protocol (i.e. a calcium alginate gel pellet was immersed in a PLL solution, followed by solubilisation of unbound alginate by sodium citrate). Their FTIR results indicated that only slight differences exist between the structures of sodium alginate and calcium alginate, and that the mannuronic-to-guluronic acid (M/G) content of the alginates influenced these spectra very little. However, with the introduction of PLL, the appearance of new peaks in the 3400 cm^{-1} region, including the Amide A peak at ~3280 cm^{-1}, was more marked for the sample made from M-rich alginate, indicating that this alginate type formed stronger links with the PLL than the G-rich alginate. The spacing and relative intensity of two other PLL-related peaks in the sample spectra, namely the Amide I (~1645 cm^{-1}) and Amide II (~1540 cm^{-1}) peaks, indicated that the M/G content of the alginate did not influence the conformation of the PLL upon interaction, as this polycation appeared to retain a random coil conformation in both cases.

In another early study, Sartori et al. [60] performed transmission FTIR on alginate films (dried from aqueous solutions or gels) to investigate the counter-ion exchange from Na^+ to Ca^{2+} within the alginate as a function of immersion time in a $CaCl_2$ bath. First of all, they used spectral subtraction to highlight their observation that the relative intensities of numerous peaks varied with the guluronic acid (G) content of the sodium alginates. Furthermore, the IR spectra of alginates immersed for 0, 0.5, 3, 30 and 300 minutes in $CaCl_2$ revealed shifts in peak positions and intensities that were indicative of counter-ion exchange within the alginate. These effects included a narrowing and increasing intensity of the O-H stretching peak (~3380 cm^{-1}) that is characteristic of increased intramolecular bonding, a broadening and shifting of the COO^- stretching peaks (~1610 and 1420 cm^{-1}) that is indicative of a change in the ionic binding of this functional group, and shifted peak positions and relative peak heights in the 1150 to 1000 cm^{-1} region containing C-O and C-C stretching peaks that suggest a change in the alginate structure with the introduction of calcium ions. Their results were also supported by correlations between shifting peak wavenumbers and the Ca/Na ion concentrations within the samples, as measured by atomic absorption spectrophotometry. They also noted that the effects of ion exchange on the IR spectra differed slightly depending on the G-content of the alginate, where the effects were more prominent for high-G alginates, suggesting a preferential binding of Ca^{2+} to G blocks (as illustrated in **Figure 2**).

More recently, Lawrie et al. [61] used FTIR (in combination with XPS) to characterize the ionic interactions between alginate, chitosan, and certain divalent cations. This is of interest to us since cross-linked alginate gels and alginate-chitosan polyelectrolyte complexes have been investigated for cell encapsulation purposes, including islets [62]. In this study, the authors employed ATR-FTIR with a diamond

accessory, as well as external reflection FTIR, on precipitates, films, and layer-by-layer assemblies of alginates and chitosan under various conditions. As they exchanged counter-ions within the alginate, they observed only slight shifts of the COO^- stretching peaks (1596 cm^{-1} and 1412 cm^{-1}) in the IR spectra. However, when the alginate was protonated, they observed the appearance of a new peak at 1722 cm^{-1}, which they associated with the stretching of the C=O bond within the COOH group. For the chitosan, they observed that partial protonation of this polymer causes the appearance of new peaks in IR spectra at ~1530 cm^{-1} and ~1630 cm^{-1} that was associated with deformations of the NH_3^+ group. However, these new peaks overlapped closely with the Amide I and II peaks making curve fits unpractical, and the placement of these peaks did not depend on the extent of protonation. As they analysed different forms of alginate-chitosan complexes, they generally observed that the spectra of the complexes resembled an addition of the spectra of the individual components, including an observation of the peaks associated with the COO^- and NH_3^+ groups of the alginate and chitosan, respectively. However, despite varying degrees of interactions between these functional groups within differently assembled alginate-chitosan complexes (as evaluated by the stability of the samples in an ionic solution), this effect was not apparent in the IR spectra.

ATR-FTIR to monitor in situ albumin adsorption

Using a stream-coating procedure, Müller et al. [63] monitored the formation of a polyelectrolyte multilayer on a silicon crystal using *in situ* ATR-FTIR. Specifically, they monitored the consecutive deposition of a sodium alginate (SA) - cellulose sulfate (CS) mixture, poly(methylene-coguanidine) (PMCG), and simple SA onto the ATR crystal in simulation of the fabrication protocol of SA-CS/PMCG capsules that may be used for islet encapsulation. More interestingly, they passed bovine serum albumin (which was dissolved in D_2O) across the surface of the multilayer assembly and quantified the adsorption of the albumin over a time course of several hours by measuring the area of the protein-characteristic Amide I band within the IR spectra. They clearly demonstrated that the composition of the exposed "final layer" of the multilayer system influenced the extent of albumin adsorption to that surface. That is, a 4-layer system with the polyanionic SA as the last deposited layer adsorbed much smaller quantities of the protein than a 5-layer system with the polycationic PMCG as the final layer. Also, the authors demonstrated that reducing the concentration of the PMCG in the 4-layer system also reduces the extent of albumin adsorption, indicating that there is exposure of the polycation at the surface due to a certain degree of overlap between the SA and PMCG. These results can be useful for the understanding the influence of the fabrication protocol on the biocompatibility of SA-CS/PMCG capsules for cell encapsulation.

X-ray photoelectron spectroscopy (XPS)

Principles of the technique

X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis (ESCA), can be used to quantify the elemental composition of a material surface, as well as identify chemical states of these elements. Detailed reviews of the XPS technique are numerous (see references [64-67]), and the reader is directed to them for further information.

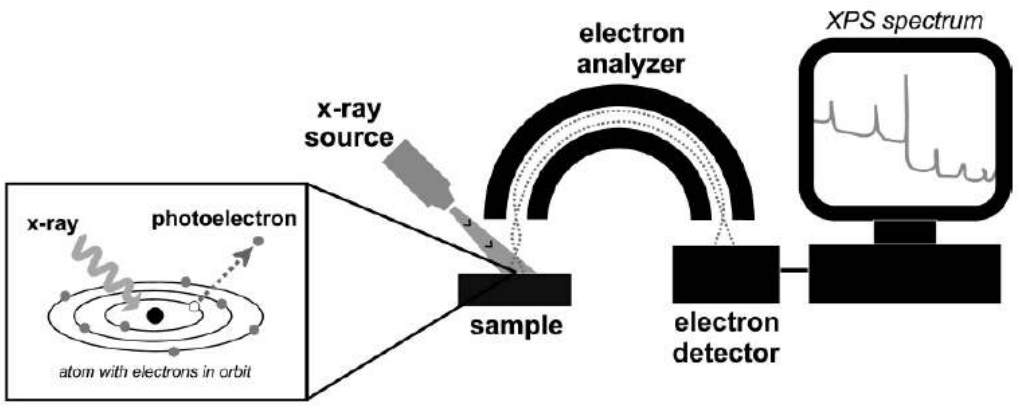

Figure 9. Illustration of the basic components and concept of the x-ray photoelectron spectroscopy (XPS) system.

In XPS, the sample is irradiated by an x-ray beam composed of high energy photons. Magnesium (Mg) or aluminum (Al) anodes are most commonly used as the x-ray source, as these metals release monochromatic x-rays (i.e. x-ray photons having a narrow energy distribution). Specifically, the Mg K_α line has an energy of 1253.6 ± 0.70 eV, and the Al K_α line has an energy of 1486.6 ± 0.85 eV. For the analysis of polymeric samples, it is advantageous to use monochromatic x-rays because they induce less degradation of the samples and provide an improved resolution of the spectral peaks than do non-monochromatic x-rays.

The basic components and concept of the XPS system are illustrated in **Figure 9**. Energy from the bombarding x-ray photons is transferred into the sample, where it excites core-level electrons in atoms located near the sample surface. If the energy of an x-ray photon exceeds the binding energy of an electron (i.e. the energy required to remove an electron from its atomic orbit), the electron is freed and any excess energy is converted into the kinetic energy of the electron. These ejected electrons (known as photoelectrons) enter an energy analyzer that uses an electrical field to filter them according to their kinetic energies. The measured kinetic energy of a photoelectron is used to calculate its binding energy, which is subsequently converted into a spectral signal.

The binding energy of an electron (E_b) that has been ejected from a sample using an x-ray photon is calculated as follows :

$$E_b = E_{hv} - E_k - \phi \qquad \text{(eq. 3)}$$

where E_{hv} is the energy of the incident x-ray photon and is known by the type of x-ray source; E_k is the kinetic energy of the ejected electron and is measured by the electron energy analyzer; and ϕ is a correction factor called the work function, and is a known property of the spectrometer.

Each time the kinetic energy of a photoelectron is measured, a data point is created. All of the data points together make up the basis of an XPS spectrum. XPS spectra are typically displayed as a plot of count (1 detected photoelectron = 1 data point = 1 count)

as a function of binding energy. Only electrons near the surface (down to 10 atomic layers for heavier elements) can actually escape the sample without any loss of energy and subsequently contribute to the characteristic peaks in the spectrum. Those photoelectrons that suffer an energy loss (due to their collision with other atoms) either contribute to the background of the spectrum or simply do not reach the detector.

Associated with each and every element is a unique set of binding energies that correspond to its core atomic orbitals. Because of this uniqueness, an element can be identified by the appearance of characteristic peaks at specific binding energies in the XPS spectrum. For example, a "C 1s peak" in the spectrum corresponds to the excitation of carbon 1s level electrons. Databases of characteristic binding energies are readily accessible to help XPS users identify spectral peaks (see www.lasurface.com).

Quantitative elemental analysis can be performed on a broad-range spectrum (generally 0 to 1100 eV) that contains most major photoelectron peaks. This type of spectrum is usually acquired from a low resolution scan, or *survey scan* (for an example of an XPS survey spectrum, see **Figure 10**). The areas of the peaks in the spectrum are proportional to the number of detected atoms of the corresponding element. When combined with the use of appropriate sensitivity factors and curve-fitting techniques, the measured peak areas can thus be used to calculate the relative quantities of the detected elements, expressed as relative atomic percentage (at%).

The chemical environment that surrounds an atom influences the electron binding energies associated with that atom. The involvement of an element in different chemical bonds thus has the effect of splitting its characteristic peaks into smaller "sub-peaks" whose placements along the XPS spectrum are slightly shifted. For example, upon a closer look, one might observe that a C_{1s} carbon peak (285 eV) is in fact composed of three peaks at 285.0 eV (**C**-C bond), 286.5 eV (**C**-O), and 288.9 eV (**C**=O). Because the sub-peaks often overlap one another, it is necessary to perform a *high resolution scan* whose range covers the width of the characteristic peak of the element of interest (generally a few eV). Examples of high resolution scans are shown in **Figure 11**. Quantification of

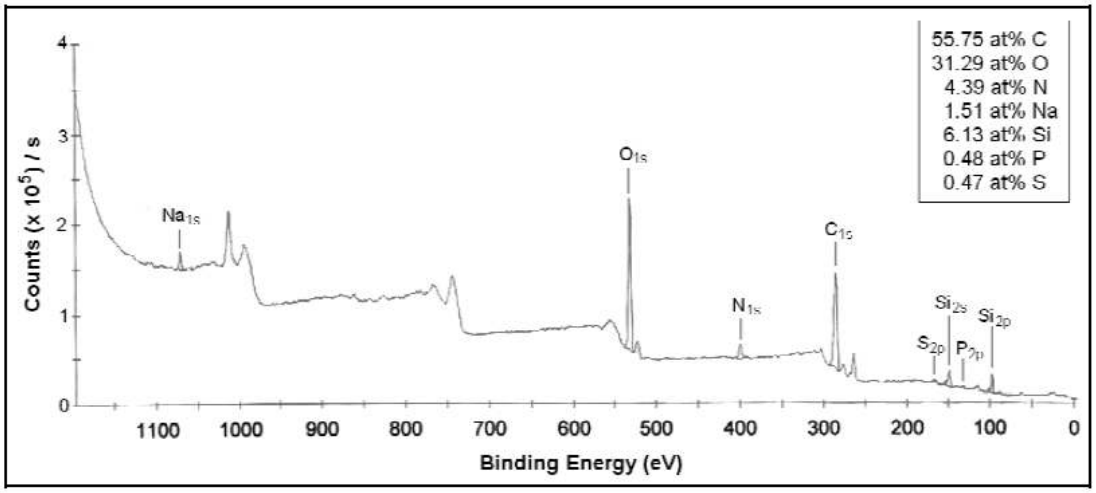

Figure 10. Example of an XPS survey scan of the surface of dehydrated alginate-poly-L-lysine microcapsules on a silicon wafer.

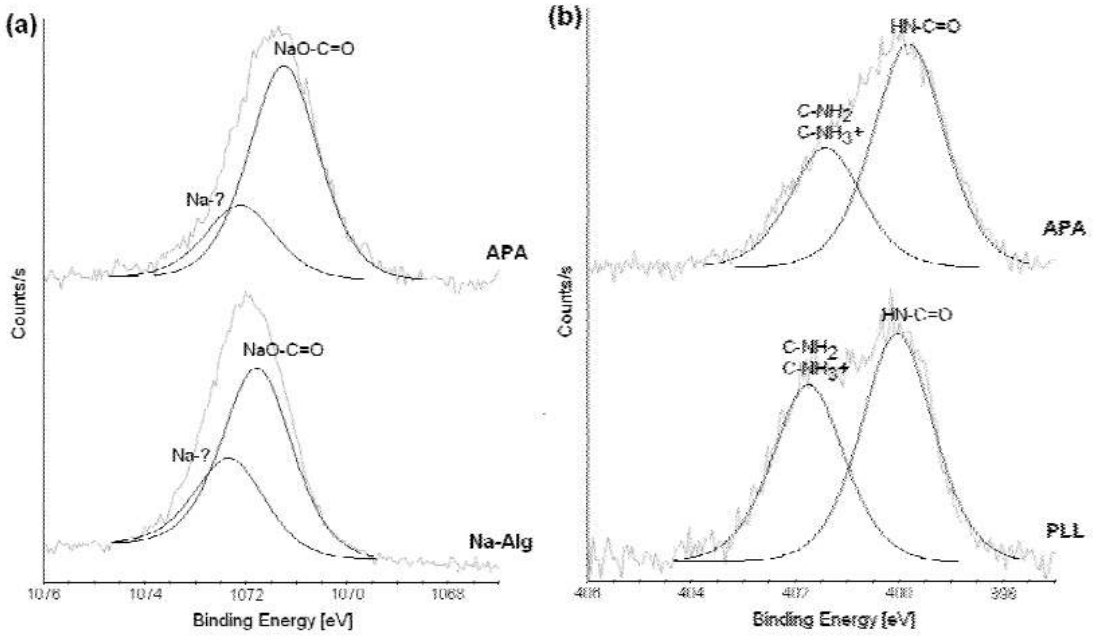

Figure 11. High resolution XPS scans of (a) the sodium Na_{1s} peak and (b) the nitrogen N_{1s} peak that were detected at the surface of dehydrated alginate-poly-L-lysine microcapsules (APA), a sodium alginate film (Na-Alg), and a poly-L-lysine film (PLL) on silicon wafers. (Figure has been reproduced from ref [20] with permission from Elsevier Limited).

Table 4. Summary of XPS key characteristics.

key information	• detection and identification of most elements (not H or He) • identification of chemical states of detected elements • relative quantity of detected elements as atomic percentage (at%) • imaging and depth profiling options available
detection limits	• concentrations as low as 0.1 at% • analytical depth 1 - 10 nm (2 - 20 atomic layers) • monochromatic beam width 10 μm - 5 mm
sample requirements	• all forms of solids, low-vapour liquids (i.e. will not evaporate in UHV) • typically 1 x 1 cm
cautions	• surface charging of insulating samples causes spectral shifts • organic samples and polymers are sensitive to degradation by x-rays

each detected chemical bond is possible by deconvoluting the sub-peaks and measuring their areas via peak integration.

XPS offers several options beyond conventional elemental analysis. For instance, it is possible to determine the elemental composition of a sample as a function of depth, either by using an ion beam to sputter layers off the surface (depth profiling), or by tilting the sample during analysis (angle-resolved XPS). Imaging or chemical mapping is another

option available with this technique. Approaches to XPS imaging include rastering a focused x-ray beam across the sample surface, limiting the area of the sample surface from which the photoelectrons are analysed using a combination of lens and apertures, or employing sophisticated array detectors and imaging optics.

The key characteristics of XPS are summarized in **Table 4**.

Analyses of microcapsules using XPS

Like ATR-FTIR, XPS is quickly becoming a standard technique for the analysis of microcapsule surfaces. However, XPS has the advantage of being a truly quantitative technique, as well as being more surface sensitive than ATR-FTIR (i.e. < 10 nm vs several microns). The most basic reason that researchers use XPS is to *determine the elemental and chemical composition* of the microcapsule surface. Yet, some have gone further by using XPS to *monitor and quantify protein adsorption* to the microcapsule surfaces, both *in vitro* and after *in vivo* implantation and recovery, as a method to evaluate the capsule biocompatibilities. Similarly to the situation with FTIR, XPS has also played a role in the *characterization of the material components* of microcapsules. Finally, XPS has also been used to *characterize newly developed macrodevices*. While these are not microcapsules as such, they employ the same concept of cell immuno-protection by a semi-permeable membrane, and are also candidates for the bio-artificial pancreas, thus their analysis is of interest to us.

Practical considerations

One of the greatest obstacles to using XPS to analyse hydrogel-based microcapsules comes from the requirement to perform the technique under ultra-high vacuum (UHV), typically < 10^{-9} Torr. An UHV environment is necessary to minimize contamination of the sample, as well as to maximize the distance travelled by the ejected photoelectrons (photoelectrons easily get scattered by gas molecules in "air") and facilitate the use of filaments used in the x-ray source cathode. As a consequence to these conditions, the capsules must be dehydrated and/or degassed before they can be analysed since water readily evaporates under UHV conditions.

In addition, the polymeric microcapsules are sensitive to degradation under the high energy x-rays. Sample degradation has the effect of altering the spectral peak shapes, positions and intensities during scans. Degradation can be minimized by limiting the exposure of the sample to the x-rays, such as by reducing the number of scans, although this may be done at the cost of compromising spectral resolution.

Finally, because polymeric microcapsules are insulating, there may be a positive charge build-up on the sample surface during XPS analysis due to the loss of ejecting electrons. Charge build-up can cause instability of the sample surface leading to spectral noise, as well as cause energy shifts or peak splits in the spectrum. This problem may be rectified by carefully radiating the sample with a low dose electron beam during scans, mounting the sample on a conductive surface, and/or by correcting the energy shift of the spectrum using carbon-based contamination (C-C) as a reference.

Characterizing the capsule surface chemistry with XPS

To our knowledge, Babensee et al. [18] from the University of Toronto were the first to use XPS to characterize microcapsules that are candidates for islet encapsulation [68].

Specifically, they characterized hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) capsules that are formed by precipitation and can be applied as conformal coatings on islets. They freeze-dried their capsules and mounted them on an adhesive copper tape before analysing them by XPS (Al K_α and Mg K_α sources) at both low and high resolution (for the C_{1s} peak). From high-resolution scans of the C_{1s} peak obtained from freshly made capsules, they noted that more carbon was bonded as C-O than C-C, and at amounts higher than expected for pure HEMA-MMA, thus suggesting that the capsule surface was not of pure HEMA-MMA. By comparing the results with reference spectra obtained from HEMA-MMA films, they could deduce that the source of carbon in the form of C-O was the Pluronic surfactant, L101, which adsorbed from the precipitation bath during the capsule fabrication process. In this study, it was not possible to determine how much of the Pluronic surfactant was adsorbed to the capsule surface because there was no elemental or structural distinguishing features in the XPS spectra to allow for its quantification. This surfactant appeared to be stable on the capsule surface *in vitro*, but its *in vivo* stability and its effect on capsule biocompatibility was not tested.

In 2002, de Vos et al. used XPS to characterize the surface of alginate-poly-L-lysine microcapsules (and corresponding calcium alginate gel beads) prepared of alginates having either an intermediate or high guluronic acid (G) content [19]. They sought to explain why capsules made with high-G alginate were less biocompatible than capsules made with intermediate-G alginate in terms of their surface chemistry, and particularly in relation to the poly-L-lysine (PLL) at their surface since PLL is immunogenic when unbound. They gradually lyophilized their capsules and verified their intactness by light and scanning electron microscopy before analysing them using XPS (Al K_α) at both low and high resolution (for the C_{1s}, N_{1s}, and O_{1s} peaks). The authors could quantify the amounts of PLL at the surface of each capsule type by the detection of nitrogen, which is present in PLL but not alginate (see **Figures 2 and 7**). Survey scans indicated that intermediate-G alginate capsule surfaces contained 6.2 ± 0.5 %N while high-G capsule surfaces contained 7.6 ± 0.5 %N. These values were used to estimate that the intermediate-G and high-G capsule surfaces were composed of ~66% and ~79% PLL, respectively. In the same study, the authors also examined the high-resolution scan of the C_{1s} peak in order to estimate the relative quantities of the negatively charged functional groups exposed at the calcium alginate gel bead surfaces, namely O=C−O and O=C−OH, that are available to bind the positively charged PLL. They saw that, relative to the quantity of C-C and C-O bonds present, beads of high-G alginate had a smaller portion of potential binding sites for the PLL than beads of intermediate-G alginate. Thus, the chemical features of microcapsules as revealed by XPS analyses allowed the authors to explain the immunogenicity of high-G alginate microcapsules by two factors: an excessive amount of PLL and a lack of binding sites to adequately bind the PLL.

In the discussion section of a later publication [69], de Vos et al. proposed a new model of the membrane structure of alginate-PLL capsules based on the same XPS results [19]. From the XPS survey scan, they detected traces of sodium (2.7 ± 0.4 %Na) at the capsule surfaces, but not calcium. This suggested to them that, despite the fact that they coated the capsules with calcium alginate during their fabrication, calcium alginate gel does not exist on the surface of the capsules in their final form. Instead, they propose that the capsule membrane is composed of a single layer of PLL complexed with alginate, where the latter polymer has retained some Na^+ ions.

Shortly afterwards, we [20] also published a study in which we used XPS (in combination with ATR-FTIR and ToF-SIMS, see other sections of this chapter for more details) to investigate the exposure of potentially immunogenic PLL at the surface of alginate-PLL-alginate microcapsules (of high-G alginate). We slowly air-dried our microcapsules (as well as calcium alginate gel beads and solutions of pure alginate and PLL) on a silicon substrate before analysing them at both low and high resolution using a Mg K_α source. Despite the fact that our research group employed a slightly different protocol for capsule fabrication than Dr de Vos's group, the XPS survey spectra (as shown in **Figure 10**) indicated that the resulting capsules from both labs had similar elemental compositions of their surfaces. Naturally, our conclusions were also similar : From the detected amounts of sodium (1.8 ± 0.3 %Na) and of nitrogen (4.54 ± 0.07 %N), which are the distinguishing elements in the alginate and PLL, respectively (see **Figures 2 and 7**), we could estimate that ~16% of the carbon atoms detected at the microcapsule surface originated from the alginate, while ~81% of the carbon originated from the PLL. Furthermore, in our case, we did not use calcium when coating our microcapsules, thus the lack of detected calcium at the capsule surface indicated that the membrane was intact because the inner calcium alginate gel core was not exposed. We also present the high-resolution spectra of the C_{1s}, O_{1s}, Na_{1s} and N_{1s} peaks (**Figure 11** shows the results for the Na_{1s} and N_{1s} peaks) to confirm that the origin of these detected elements were indeed alginate and PLL.

XPS to monitor protein adsorption to the surface

In the same study mentioned earlier, Babensee et al. [18] also used XPS to evaluate the *in vitro* adsorption of serum proteins to the surfaces of their HEMA-MMA capsules. The proteins were identified by the appearance of a nitrogen signal after the capsules were incubated in a serum-containing tissue culture medium (TCM) for 1 week (cell-containing capsules are sometimes conditioned before their implantation by pre-incubation in TCM). Quantitative analysis of the survey spectra allowed them to observe that a large portion of the adsorbed proteins could be removed by washing the capsules with PBS, as nitrogen content was reduced from 6.2 ± 0.7 %N to 1.2 ± 0.2 %N. It has been observed that pre-incubating these capsules in serum-containing TCM leads to more severe tissue reactions *in vivo*.

Using a similar approach, de Vos et al. also applied XPS to monitor the *in vivo* adsorption of proteins to the surface of their alginate-PLL microcapsules [41]. They measured an increase in the nitrogen signal on the capsule surfaces after 1 week implantation in the peritoneal cavity of rats, with 5.4 ± 1.0 %N at day 1 yet 8.6 ± 1.0 %N at day 7. This corresponded with an increase in the portion of retrieved capsules that were overgrown with cells, which was 0.5 ± 0.3 % at day 1 and 3.3 ± 1.6 % at day 7. This suggested that protein adsorption is essential for cellular overgrowth of implanted capsules. This same group later applied the same analysis to compare the bioperformance of alginate-polycation microcapsules of varying chemical composition, i.e. using alginates of intermediate or high G content, as well as different polycations (poly-L-lysine, poly-D-lysine and poly-L-ornithine) [37]. Again, they detected small increases in the amounts of detected nitrogen (approximately 2-10 %N increase) on the capsule surfaces after 1 month implantation in the peritoneal cavity of rats, which was attributed to protein adsorption.

XPS to characterize the microcapsule components

XPS has also been employed to analyse the materials components of microcapsules designed for islet cell encapsulation. While these are not direct investigations of the microcapsule surface, the results of these studies are useful as reference data for other XPS studies, and they provide detailed information about the material characteristics.

In one study, we evaluated the residual contamination of purified alginates that are frequently used for islet cell encapsulation [70]. XPS was selected to identify trace contamination of the alginates because it is a quantitative and non-biased technique that can detect very small concentrations of all elements (except H and He). Even after their purification, we detected traces of sulphur (0.28-0.78 %S), phosphorus (0.02-0.12 %P), and nitrogen (0.16-1.17 %N) in the samples, thus proving the presence of contaminants that may compromise the functionality and biocompatibility of the alginates.

In a recent publication, Lawrie et al. [61] used XPS to complement their FTIR results (see the previous section of this chapter for more details) to examine the various ionic interactions between alginate, chitosan, and divalent cations in recognition of the importance of alginate gels and alginate-chitosan complexes for cell encapsulation purposes. They used XPS to examine such details as the counter-ion exchange during alginate cross-linking (characterized by the substitution of Na^{+} by Ca^{2+} or Ba^{2+}), or the quantification of the protanation of amine groups within the chitosan via the analysis of the N_{1s} peak at high resolution.

XPS to characterize the surface of macrodevices for islet encapsulation

XPS has also been used as a tool to examine the surface of macrodevices for islet cell encapsulation. While not microcapsules as such, these devices are valid candidates for the bioartificial pancreas, and should thus be of interest to the readers.

Beginning in the late 1990's, one research group used XPS, in combination with a number of other techniques for surface analysis, to investigate the surface chemistry of membranes designed for islet isolation. In a 1997 publication, they evaluated the effects of a corona surface treatment on the surface chemistry (by XPS), energy (by contact angle), and morphology (by SEM) of AN69 membranes, and correlated these alterations with the resulting permeability and biocompatibility of the membranes [46]. They used XPS to quantify the relative amounts of carbon, oxygen, nitrogen and sulfur on the membranes, then compared these results with the theoretical elemental compositions of the membranes. This allowed them to deduce that, despite washing, some glycerol molecules had remained adsorbed onto the membrane surfaces (glycerol was used in the sterilization process of the samples) and that the glycerol content seemed to diminish after the corona treatment. In more recent studies, the same researchers favoured a polycarbonate membrane for islet immuno-protection. They used XPS extensively in order to support their ToF-SIMS analyses (see the following section for more details) for the evaluation of the surface chemistry of the membranes under different conditions. In each case, the membranes were treated by cold plasma in an argon atmosphere then coated with a hydrophilic polymer (usually polyvinylpyrrolidone (PVP) though, in one study, they also tried hydroxypropylmethylcellulose) in order to improve the hydraulic permeability and glucose diffusion of the membranes without compromising their biocompatibility. Low and high resolution XPS scans of the sample surfaces confirmed that the PVP coating was strongly adhered to the membranes only after the plasma treatment [45]. Furthermore, when

the membranes were implanted in mini-pigs, the measured ratio of N_{1s}/C_{1s} allowed the researchers to quantify the *in vivo* adsorption of biological molecules (mainly proteins) to the the membranes [71]. Most recently, the N_{1s}/C_{1s} and S_{2p}/C_{1s} ratios that were taken from the XPS spectra were used to quantify the competitive, *in vitro* adsorption of human insulin and serum albumin to the membranes [72].

La Flamme et al. also recently used XPS to characterize a newly proposed macrodevice for islet immuno-isolation based on alumina [73]. They modified the device surface using poly(ethylene glycol), or PEG, in order to improve its biocompatibility. Their XPS results confirmed that PEG molecules did indeed coat the device surface after treatment, as they saw the appearance of new carbon-based species, a decrease in aluminium, and detected the presence of silicon originating from the PEG-saline couple that was used to immobilize the PEG to the device surface.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

Principles of the technique

TOF-SIMS refers to the analytical technique secondary ion mass spectrometry (SIMS), in combined use with the time-of-flight (ToF) method for mass analysis. ToF-SIMS is a very sensitive technique that can be used to obtain detailed composition, distribution, and molecular information of a sample's surface constituents. In comparison to other analytical techniques such as XPS, ToF-SIMS is much more surface sensitive, as it can be used to analyse only the top 1 or 2 monolayers of the sample, and is capable of detecting smaller amounts of trace contaminants (in the ppb range). Readers are referred to the following publications for further details about ToF-SIMS : [74-77], as well as www.iontof.com.

In *secondary ion mass spectrometry (SIMS)*, the sample surface is bombarded with high energy ions (1-50 keV), using a focused, pulsed primary ion beam (e.g. gallium, Ga^+ or bismuth, Bi^+). This results in the desorption or sputtering of atoms, molecules, and molecular fragments from the sample surface, as illustrated in **Figure 12**. While most of

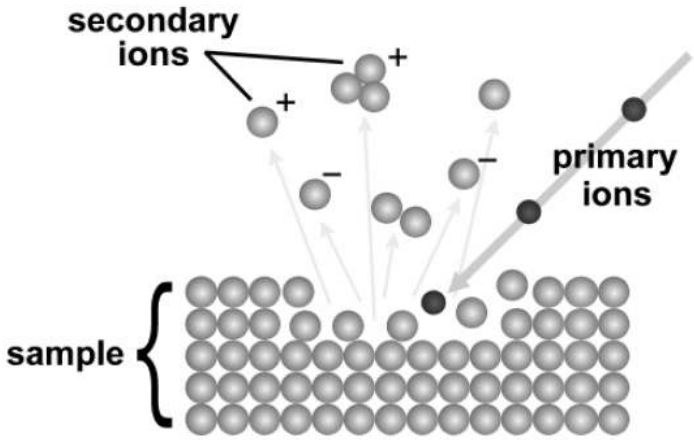

Figure 12. Schematic illustration of the sputtering process that is the basis of secondary ion mass spectrometry (SIMS). The bombardment of a sample by primary ions causes the emission of particles from the outermost atomic layers, including charged particles called secondary ions.

the emitted species are neutral, a small portion is positively or negatively charged and can be subsequently analysed. These are referred to as the "secondary ions". The structure and composition of the secondary ions are directly related to the molecular structure of the surface they were emitted from.

For the analysis of delicate organic materials, it is recommended to use *static SIMS*. In this mode of operation, the primary ion beam is maintained at a low fluence ($< 10^{12}$ ions / cm^2) so that damage to the sample is minimal. Moreover, with static SIMS, there is a very low probability that a primary ion will strike the same area twice. As a consequence, the emission of intact molecular ions and high molecular weight fragment ions is favoured, and the chemical or molecular information yielded is well-representative of the original, unperturbed surface. The sampling depth of static SIMS is in the range of 10 to 20 Å (1-2 atomic layers), since only the particles in the outermost region of a sample have sufficient energy to overcome the surface binding energy and leave the sample. Under certain conditions, it is possible to sputter up as little as ~0.1% of the top monolayer.

In ToF-SIMS, the structure and composition of the secondary ions are identified by their mass, which is precisely measured using the *"time-of-flight" technique*. The ejected ions are accelerated through an analyzer with a common kinetic energy, yet different velocities that depend on the mass-to-charge ratio (m/z). This means that the smaller ions

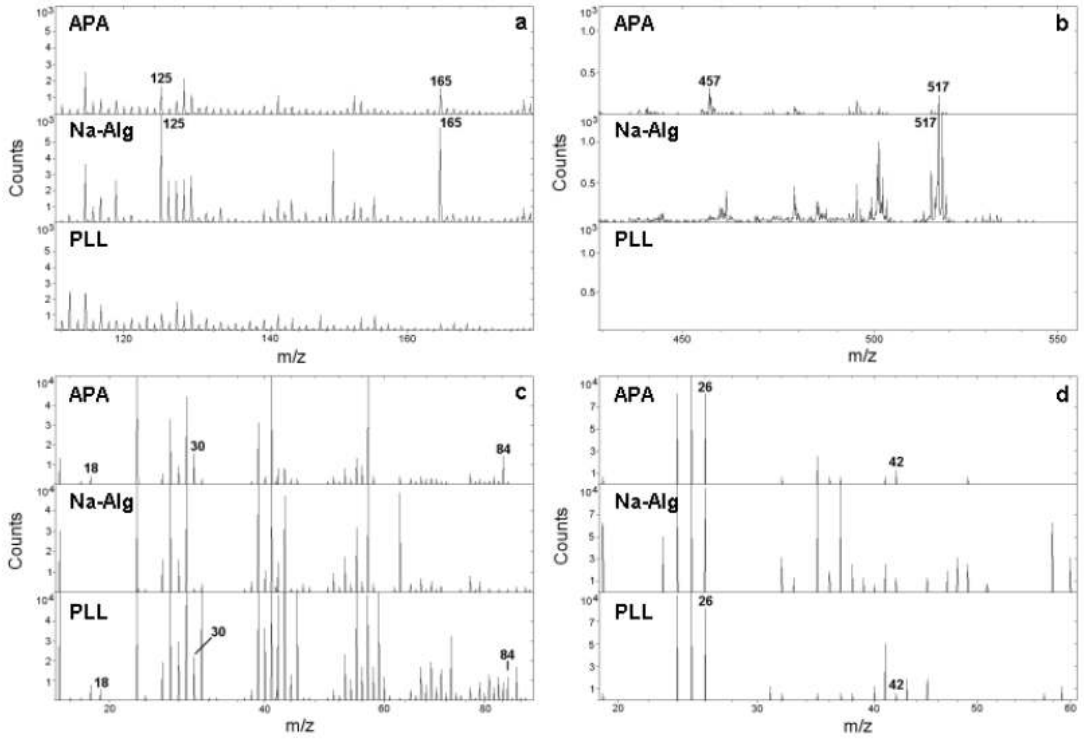

Figure 13. Mass spectra of the outermost atomic layers of a dehydrated alginate-poly-L-lysine microcapsule (APA), a sodium alginate film (Na-Alg), and a poly-L-lysine hydrobromide film (PLL), as measured by static ToF-SIMS and for spectral regions of interest. Characteristic peaks for each component are labelled by the value of their mass. (Figure has been reproduced from ref [20] with permission from Elsevier Limited).

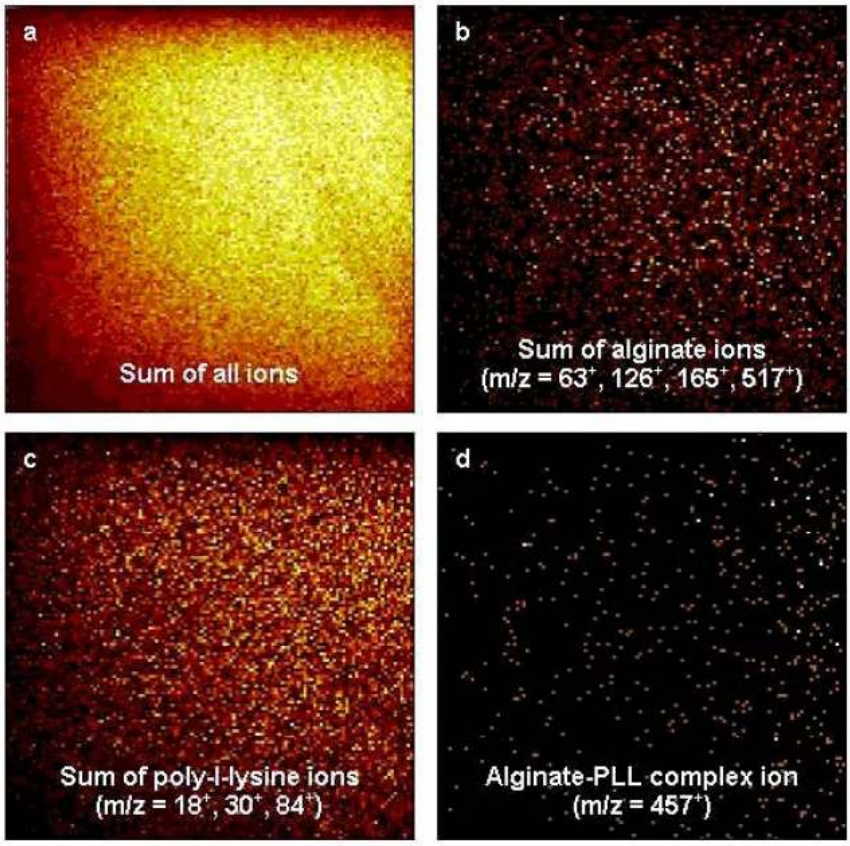

Figure 14. Distribution of select positive ions, as detected by static ToF-SIMS, within a 50 μm x μ50 μm surface area of a dehydrated alginate-poly-L-lysine microcapsule. Shown here is the distribution of (a) all detected positive ions, (b) positive ions characteristic of sodium alginate, (b) positive ions characteristic of poly-L-lysine (PLL), and (d) a positive ion characteristic of an alginate-PLL complex. Yellow represents a higher concentration, while black represents a lower concentration of the selected ions. (Figure has been reproduced from ref [20] with permission from Elsevier Limited).

move through the analyzer more quickly than the larger ions, providing mass separation of the ions. The time it takes an ion to travel through the analyzer (their "time-of-flight"), from the sample to the detector, is thus measured and used to determine its mass. Because it is possible to measure their time-of-flight on a scale of nano-seconds, mass resolution can be as fine as 0.00X atomic mass units (amu). Thus, particles with the same nominal mass (e.g. Si and C_2H_4 both have an amu = 28) are easily distinguished from one another because there is a slight mass shift as atoms enter a bound state. The ToF analyzer is also capable of high sensitivities, with detection limits in the part per billion (ppb) range, due to its high transmission and parallel detection of all masses.

A basic ToF-SIMS mass spectrum is a plot of the quantity of ions that are detected (count or intensity) as a function of ion mass or m/z (amu or daltons). The mass spectra can be recorded in either positive or negative mode. Examples of ToF-SIMS spectra are shown in **Figure 13**. In practice, a single spectrum can contain hundreds of peaks that

arise from the fragmentation of the molecules at the sample surface. A breakdown of the fragmentation pattern can allow the identification of materials at a surface. Fortunately, it is usually not necessary to identify all the peaks in the spectrum in order to identify a substance of interest, as a few characteristic peaks will suffice. More details about the interpretation of ToF-SIMS mass spectra are described in the section "practical considerations" below.

With *ToF-SIMS imaging*, it is possible to generate a two-dimensional (2D) map of the surface chemistry at a specific spot on the sample, such as illustrated in **Figure 14**. To do this, a highly focused primary ion beam (~1 μm in diameter) is rastered across the surface of the sample, and a full ToF-SIMS spectrum is collected at specific points to create the image pixels. Certain ion masses that are characteristic for a specific molecule type can be selected to reconstruct the image. The result is a visual representation of the distribution of that molecule along the analysed surface.

Dynamic SIMS (as opposed to static SIMS) refers to a high sputtering rate during measurement. This mode allows a profiling of the sample composition as a function of depth. *Depth profiles* are produced by removing surface layers as little as ~1 nm at a time during analysis. This is commonly achieved by using a second ion beam to sputter a crater in the sample while the primary ion beam analyses the crater bottom. Because dynamic SIMS is quite damaging to the sample, however, this mode is more suitable for elemental analysis than molecular analysis.

Three-dimensional (3D) analysis is also possible by combining 2D imaging and depth-profiling. That is, an X-Y image is saved at each analysed layer of a depth profile, and the images are subsequently used to reconstruct a 3D representation of the distribution of detected ions.

The key characteristics of ToF-SIMS are summarized below, in **Table 5**.

Table 5. Summary of ToF-SIMS key characteristics.

key information	• detection and identification of all elements and isotopes • identification of molecular compounds • 2D imaging, depth profiling and 3D analysis options available
detection limits	• analytical depth < 1nm (1-2 atomic layers) under static conditions • detection of trace elements in ppm/ppb range • measures ion masses 0 - 10 000 amu* • mass resolution m/Δm >10 000, i.e. differentiates 0.00X amu • lateral resolution < 60 nm (imaging) • depth resolution < 1nm (depth profiling)
sample requirements	• all forms of solids, low-vapour liquids • dimensions up to several cm x cm • ideally a flat surface
cautions	• results are, at best, semi-quantitative • analysis very complicated and difficult • expensive to operate, requires highly qualified personnel • results sensitive to topographical changes

* 1 atomic mass unit (amu) = 1 dalton (Da)

ToF-SIMS for the analysis of microcapsules

Because of the high cost of operation and the complexity of the spectral analysis, ToF-SIMS is currently less popular than XPS and ATR-FTIR as an approach to characterize the microcapsule surface. However, the extreme surface sensitivity (< 1 nm or the outer 1-2 atomic layers), high resolution, and wide range of analytical possibilities associated with ToF-SIMS make it a valuable tool for microcapsule analysis that other techniques simply cannot match. To our knowledge, ToF-SIMS has only been used once to analyse the surface composition and homogeneity of intact microcapsules designed for islet transplantation [20]. However, ToF-SIMS has been applied for the *surface analysis of macrodevices* for islet encapsulation, as explained below.

Practical considerations

As for XPS, ToF-SIMS analyses also require an ultra-high vacuum (UHV) environment and involve the loss of charged particles from the sample surface. Therefore, the same challenges associated with these conditions apply, including the requirement to dehydrate the samples, the necessity for caution when interpreting the results of dehydrated (vs. hydrated) samples, and loss of resolution due to the charge build-up on insulating samples (see "practical considerations" in the XPS section of this chapter for more details). Due to the sputtering process (see **Figure 12**), ToF-SIMS is also inherently a destructive technique.

In addition, ToF-SIMS can be considered, at most, to be a semi-quantitative technique. In practice, quantification of detected species is very difficult to perform because peak intensities depend not only on the concentration of the components in the sample, but also on the ion yield. The yield of a given type of secondary ion varies depending on the chemical state of the surface (a phenomenon known as the "matrix effect"), even up to several orders of magnitude. If the results are to be quantified, it is highly recommended to use standards with a similar chemical environment to the analyte of interest, as well as the selection of several characteristic peaks. For a complex system such as a polymer, quantification is even more difficult.

Apart from the difficulties associated with data quantification, TOF-SIMS analysis inherently complex. Without correctly structuring the analysis, TOF-SIMS can actually raise more questions than it can answer. Part of the reason for this is that a given mass spectrum can easily contain literally hundreds of peaks, thus providing an overwhelming amount of information. Recently, multivariate analysis methods have been applied to simplify the analysis process, and in particular a statistical method called principal component analysis (PCA) is commonly employed. PCA allows the user to find the combination of variables that describe the most important trends in the data sets, and aids to make a distinction between spectra which look very similar. Fortunately, even without the use and expertise of statistical software, useful information from ToF-SIMS spectra may still be yielded by comparing the results with appropriate reference spectra and carefully selecting distinguishing or characteristic peaks. ToF-SIMS software also has the ability to perform "retrospective" analysis. That is, during data acquisition, every single ion detected by the system is stored as a function of its mass and its point of origin. This allows the user to analyse the spectra or obtain chemical maps after the original data has been collected. In all cases, the combination of ToF-SIMS analysis with XPS analysis is highly recommended in order to provide complementary data that can aid in the interpretation of the results.

ToF-SIMS to characterize the capsule surface composition and homogeneity

To our knowledge, we are the only group to date that has applied ToF-SIMS (integrated with XPS and ATR-FTIR) for the analysis of microcapsules for islet encapsulation. Rinsed alginate-poly-L-lysine capsules, as well as pure sodium alginate and poly-L-lysine (PLL) hydrobromide solutions, were slowly dehydrated on squares of silicon wafer before they were analysed. Static ToF-SIMS was carried out using a Ga^+ ion source to obtain high resolution mass spectra. When comparing the reference spectra, we observed peaks that were prominent in the alginate spectrum but not present in the PLL spectrum (or vice versa), and these were selected as the characteristic peaks for each component. For the alginate, the selected peaks represented fragments of higher mass (m/z = 63 to 399) that contained sodium (for e.g., a positively charged alginate fragment with a mass of 125 amu was identified as having a composition of $C_3H_2O_4Na$). Conversely, each of the selected characteristic peaks for the PLL represented smaller nitrogen-containing fragments (e.g. CN^-). The characteristic peaks of both the alginate and the PLL appeared in the spectrum of the microcapsule surface, indicating the presence of each individual polymer at the outermost 1-2 atomic layers of the samples. Moreover, we observed a high-mass peak in the capsule spectrum (m/z = 457) that we associated with a large fragment composed of two sodium alginate units and one PLL unit (i.e. having a composition of $C_{17}H_{26}N_2O_{11}Na$). The presence of this fragment indicated to us that the alginate and PLL formed a strongly bound complex at the microcapsule surface.

In the same study, we also used the selected characteristic peaks for the alginate and PLL, as well as the peak associated with the alginate-PLL complex, to recreate 2D images that map the distribution of each of these molecules within a 50 × 50 μm area on the microcapsule surface. From these images (**Figure 14**), we could observe that the alginate, PLL, and alginate-PLL complex were distributed fairly homogeneously along the capsule surface. This indicated to us that exposure of the PLL was not due to physical defects in the capsule membrane, but rather the alginate-PLL complex was a general feature of the capsule surface. These results confirmed those of the XPS and FTIR analyses (as described in the previous sections of this chapter), which indicated that both PLL and alginate are present and co-interacting at the surface of alginate-PLL capsules. However, in contrary to the XPS and FTIR studies, the results of our ToF-SIMS analyses demonstrated that this molecular interaction occurs at the outermost, and thus exposed monolayer of the capsule membrane. Given that unbound PLL is known to be immunogenic, these results can provide insights into the biocompatibility of alginate-PLL microcapsules for islet encapsulation.

ToF-SIMS for the investigation of macrodevices for islet encapsulation

On several occasions, one group of researchers used static and imaging ToF-SIMS (integrated with XPS, see the previous section of this chapter) to study the surface chemistry of a macrodevice for islet encapsulation that incorporated a polycarbonate membrane for immuno-protection [45, 71, 72]. In their earliest study employing ToF-SIMS [45], they sought to improve the hydraulic permeability and glucose diffusion properties of the membrane by increasing its hydrophilicity. To do this, they treated the membrane with a cold plasma in an argon atmosphere (to activate the surface), then coated it with hydrophilic polyvinylpyrrolidone (PVP). Subsequently, using ToF-SIMS,

they clearly detected the presence of the PVP coating on the membrane surface, while only a weak signal was emitted from the underlying polycarbonate. This indicated that the membrane was homogeneously coated with PVP after the surface treatment, and that this coating stayed adhered to the surface after the membrane was rinsed with water. In another study [71], they used ToF-SIMS to characterize the *in vivo* adsorption of biological molecules (BM) to the surfaces of polycarbonate membranes during their implantation in mini-pigs for 11 to 92 days. As before, the membrane surfaces were treated by argon plasma, then coated with a hydrophilic polymer, either PVP or hydroxypropylmethylcellulose (HPMC). They detected a number of nitrogen-containing fragments that originated from BM (mainly proteins) that adsorbed to the membranes. They summed up the peak intensities associated with these BM, and used the ratio of this summed intensity to the total ion intensity of the mass spectrum to estimate the quantities of BM that adsorbed to the membranes under different circumstances. They then applied principal component analysis (PCA) to highlight trends in the spectral results. This allowed them to determine that the compositions of the adsorbed BM differed for each surface treatment (PVP vs. HPMC) and for each side of the membrane (in contact with tissue vs. in contact with the silicon device onto which the membrane was fixed), as well as identify a number of amino acids that made up the BM. Moreover, they used ToF-SIMS imaging to illustrate a non-homogeneous distribution of the BM on the surfaces. In their most recent study [72], these researchers used ToF-SIMS to examine the *in vitro* adsorption of human serum albumin and human insulin onto argon-PVP treated membranes. In a similar approach as before, they used the ratio of the summed intensities of characteristic peaks to total the ion intensity to estimate the extent of adsorption of each protein from solutions of known protein concentrations. The application of PCA allowed them to identify protein patterns that were most characteristic of each albumin and insulin. Finally, they used these fingerprint patterns to characterize the competitive adsorption between insulin and albumin on the membranes.

Other techniques for surface analysis

As indicated in **Table 1**, in addition to the four techniques described in this chapter, other analytical techniques have been employed to (directly or indirectly) characterize the surface of microcapsules for islet encapsulation. These are briefly discussed here.

Scanning electron microscopy (SEM) uses a high-energy beam of electrons to provide a highly magnified, high-resolution image of a sample surface [78]. Since the mid-1980's, SEM has regularly been used to examine the surface topography or morphology of various microcapsule types for cell encapsulation [21, 32-35]. Due to the analytical conditions of SEM, the microcapsules must be carefully dehydrated then sputter-coated with a thin layer of gold (or some other conductive material) before they can be analysed. **Environmental SEM**, however, can be applied to avoid such extensive sample preparation, as these analyses can be performed in a low vacuum and at cool temperatures. These conditions can slow the rate of water evaporation, thus providing the possibility of analysing humid or frozen samples. We (unpublished results) and at least one other group [23] have attempted to use environmental SEM on microcapsules for cell encapsulation, but total dehydration of the capsules during analysis has not been avoided. **Energy-dispersive x-ray (EDX)** analyses can also be performed in conjunction with SEM to provide the elemental composition of the area being imaged [23].

Confocal laser scanning microscopy (CLSM) uses a precisely focused laser beam to excite fluorescent samples and thereby provide a high-resolution image of the sample, including its surface topography. CLSM has been successfully employed by a few research groups to examine the distribution of polymers inside and at the surface of microcapsules designed for cell transplantation. The principles of the CLSM technique, along with a description of its use for the analysis of microcapsules, are described in detail in Chapter 8 and will not be repeated here.

Surface plasmon resonance (SPR) uses polarized light under conditions of total internal reflection to measure the refractive index of thin layers of molecules adsorbing from a fluid onto a sensor surface. By immobilizing one molecule of interest onto the sensor surface, one can pass another molecular species across the sensor and measure, in real-time, the interactions between the two molecules [79]. Due to sample shape restrictions, SPR cannot be used to directly analyse the surface of microcapsules. However, we have used this technique to evaluate intermolecular interactions related to the microcapsule surface. Specifically, we characterized the binding affinity between different types of alginate and poly-L-lysine (PLL), as well as explored the adsorption of serum proteins onto a coupled alginate-PLL system [24]. These results are directly related to the membrane formation and protein adsorption properties of alginate-PLL microcapsules for islet encapsulation.

Zeta potential measurements refer to measurements of the electric potential that exists a short distance (a few nm) from a solid surface in a fluid [80]. This potential arises from the attraction of ions in the fluid to that surface, and its value depends on (among other factors) the surface charge density. De Vos et al. employed the streaming potential technique to measure the zeta potentials of alginate-PLL microcapsules and calcium alginate beads in an electrolyte solution, as a method to evaluate the overall surface charge density of the capsules [26]. By varying the alginate compositions and solution pH, the authors could associate a larger zeta potential with an inferior *in vivo* biocompatibility of the microcapsules.

The **contact angle technique** is a popular method to evaluate the surface energy or wettability (hydrophilicity) of a solid surface [81]. A special camera and software can be used to measure the shape of a liquid droplet as it interacts with a sample surface, and thus to evaluate the affinity of the surface for that liquid. While the contact angle technique has not yet been used directly on microcapsule surfaces (due to sample shape requirements), we have used this method to measure the hydrophilicity of sodium alginates that are used to coat our microcapsules [70]. We found that alginate hydrophilicity is a fiable (and simple) measure of their purity. We also applied the contact angle technique to films of alginate gels and alginate-PLL complexes so as to investigate the relationship between the film hydrophilicity and the *in vivo* biocompatibility of corresponding alginate-PLL microcapsules [25].

Concluding remarks

Based on studies that have already been published, it is clear that there exist important relationships between the surface properties of microcapsules for islet encapsulation and their overall quality or bioperformance. However, these relationships are not well defined since the mechanisms that can explain how, and to what relative

extent, each surface property influences the performance of the microcapsules are not yet known in detail. There are a number of reasons for this. First of all, while the surface morphology and chemistry of the microcapsules have been investigated in several cases, there is currently a lack of published works that acknowledge the surface energy and charge. Furthermore, these surface properties are closely interrelated and can influence different aspects of the microcapsule performance to different degrees. Thus, identifying which are the most important variables to control in order to achieve a specific result is a complicated task. Moreover, there currently exists no official standards that describe, in technical terms, how to characterize the microcapsule surface, thus each research group uses slightly different protocols (as well as different microcapsule designs) in their studies. Therefore, at this point, we should hesitate to rely on a comparison of study results to draw any specific conclusions about the influence of a microcapsule's surface properties on its performance. Despite all of these challenges, however, we are confident that this situation will quickly improve as various techniques for surface analysis continue to evolve. That is, as we continue to adapt these techniques for the analysis of polymeric, hydrogel-based samples, investigations of the microcapsule surface will become more accessible, practical and efficient. Over the next few years, it is expected that analyses of the microcapsule surface will play a crucial role in the development and standardization of the microcapsule design, the processes of pre-screening and evaluating the microcapsules during clinical trials, and the commercial development of these devices.

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