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CHARACTERIZATION OF THERMOREVERSIBLE PH-SENSITIVE PHYSICAL GELS OF CHITOSAN

DONG WANG
DÉPARTEMENT DE GÉNIE CHIMIQUE
ÉCOLE POLYTECHNIQUE DE MONTRÉAL

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DU DIPLÔME DE MAÎTRISE ÈS SCIENCES APPLIQUÉES (M.Sc.A)
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CHARACTERIZATION OF THERMOREVERSIBLE PH-SENSITIVE PHYSICAL GELS OF CHITOSAN

présenté par: WANG Dong

en vue de l'obtention du diplôme de: Maîtrise ès sciences appliquées

a été dûment accepté par jury d'examen constitué de:

M. FAVIS Basil, Ph.D., président

M. BUSCHMANN Michael, Ph.D., membre et directeur de recherche

M. CHENITE Abdallatif, Ph.D., membre et codirecteur de recherche

M. AIT-KADI Abdellatif, Ph.D., membre, Université Laval

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RÉSUMÉ

Chitosan est un amino-polysaccharide naturel qui ne cesse d'attirer beaucoup d'attention, particulièrement grâce à ses caractéristiques de biocompatibilité, biodégrabilité et bioadhésion en plus de son abondance. Il a été étudié aussi bien du point de vue scientifique fondamental que pour son intérêt dans les applications pratiques.

Dans cette étude, nous nous sommes concentrés sur la transformation de solutions de chitosan en solutions aqueuses auto-gellifiantes sensibles à la température et au pH. Des efforts particuliers ont été orientés vers des solutions aqueuses de chitosan dans lesquelles des sels de polyols portant une seule tête anionique, comme le sel disodique de β-glycérophosphate, ont été incorporés. Ceci avait pour but de tirer avantage à la fois des interactions électrostatiques entre les têtes anioniques et les chaînes de chitosan, et de l'effet de déshydratation induit par le polyol sur les chaines de chitosan connu pour augmenter les interactions hydrophobes. De telles solutions aqueuses chitosan/ β-glycérophosphate avec un certain pH (neutre ou presque neutre) vont gélifier rapidement lorsque chauffées à la température appropriée.

La présente étude a établi que les solutions de chitosan neutralisées avec le sel disodique de β-glycérophosphate, à un pH entre 6.5 et 7.2, peuvent être transformées en un gel par chauffage à 37°C et plus. On a montré que les gels résultants sont totalement thermo-réversibles pour les systèmes C/β-GP dont le pH se situe entre 6.5 et 6.9, alors que les systèmes dont le pH est supérieur à 6.9 montrent seulement une thermo-réversibilité partielle. Ceci suggère que la formation des gels C/β-GP est dominée par des forces hydrophobes, les seuls forces capables de se renforcer avec l'élévation de la température.

La déacétylation du chitosan a aussi été effectuée de manière à obtenir du

chitosan possédant différents degrés de déacétylation (DDA). Le procédé typique de la déacétylation implique une traitement alcalin à ~130°C, avec une solution de NaOH 50% masse/masse et à un rapport de 10/1 en volume de solution de NaOH/masse de chitosan. Du chitosan hautement déacétylé a été obtenu. Il a été montré que le degré de déacétylation du chitosan dépend de la température et du temps de traitement. Le DDA a été facilement déterminé par un titrage conductimétrique par HCl.

Dans cette étude, des mesures rhéologiques ont été effectuées pour étudier la solution, le gel et la transition sol-gel. L'évolution de la viscoélasticité linéaire pendant la gélation thermo-réversible des systèmes aqueux C/β-GP a été mesurée par cisaillement en mode oscillatoire. Le point de gélation a été déterminé par l'observation de la valeur de tangente(δ) indépendante de la fréquence. Au point de gélation, point où le module élastique ($G' \sim \omega^{n'}$) et le module visqueux ($G' \sim \omega^{n''}$) sont indépendants de la fréquence, a été consistant avec $n' \approx n'' \approx n$, avec $n = 0.435$. La température de gélation ainsi que la force du gel ont été déterminées en fonction du DDA, de la concentration de chitosan et de la concentration du sel (β-GP). On a trouvé que la température de gélation diminue avec l'augmentation de la concentration de chitosan, l'augmentation de la concentration de β-GP et l'augmentation du degré de déacétylation du chitosan. Quant à la force de ces gels, elle dépend du temps et augmente avec l'augmentation de la température, la concentration de chitosan et la concentration de β-GP.

Ce sont les interactions hydrophobes ou les interactions micellaires qui sont soupçonnées d'être à l'origine de la gélation des solution C/β-GP. L'impact majeur de cette gélation thermique consiste en l'obtention d'hydrogels injectables se formant *in-situ*, de sorte qu'on puisse envisager des formulations liquides pouvant être chargées avec des matériaux thérapeutiques (médicament, protéines ou cellules) et injectées dans le corps pour former des implants de gel *in-situ*.

ABSTRACT

Chitosan, a natural amino-polysaccharide, has been shown to have attractive properties, particularly due to its biocompatibility, biodegradability and bioadhesive characteristics in addition to its abundance. It has been investigated from a basic scientific point of view as well as for its practical applications.

In this study, we focused on the transformation of chitosan solutions to thermally-sensitive and pH-dependent gel forming aqueous solutions. Particular efforts have been oriented toward chitosan aqueous solutions where polyol salts bearing one single anionic head such as β-Glycerophosphate disodium salt were incorporated. This was expected to take advantage of the synergistic interactions between anionic heads and chitosan chains, as well as the polyol-induced dehydration effect on chitosan chains which is known to increase the hydrophobic interactions. Such chitosan/β-Glycerophosphate aqueous solutions will gel rapidly when heated up to an appropriate temperature at certain pH.

The present study established that chitosan solutions neutralized with β-glycerophosphate disodium salt, to a pH between 6.5 and 7.2, can be transformed to a gel upon heating to 37°C and above. The resulting gels are completely thermo-reversible for C/β-GP systems with pH in the range of 6.5~6.9, while systems with pH above 6.9 show only a partial thermo-reversibility. This suggests the domination of hydrophobic forces, able to reverse with temperature, in the formation of C/β-GP gels.

The deacetylation of chitosan has also been performed in order to provide chitosan having various degree of deacetylation (DDA). The typical deacetylation process involves treatment at ~130°C, with 50% w/w NaOH solution at a ratio of 10:1 in volume of NaOH solution/weight of chitosan. Highly deacetylated chitosan was obtained. It was found that the degree of deacetylation of chitosan depends on the

temperature and time of treatment. DDA was easily determined by acidic titration and conductivity measurements.

In this study, rheological measurements were performed to study the solution, the gel and the sol-gel transition. The evolution of linear viscoelasticity during the thermoreversible gelation of the aqueous C/β-GP systems was measured by oscillatory shear. The gelling point was determined by the observation of a frequency independent loss tangent. At the gelling point, a power law frequency dependence of the dynamic storage modulus ($G' \sim \omega^{n'}$) and loss modulus ($G'' \sim \omega^{n''}$) was consistantly observed with $n' \approx n'' \approx n$. The gelation temperature and gel strength was determined as a function of DDA, the concentrations of chitosan and β-GP. The gelation temperature decreases with increasing the concentration of chitosan, the concentration of β-GP or the degree of deacetylation of chitosan. The strength of these gels is time dependent, increases with increasing temperature, the concentration of chitosan or the concentration of β-GP.

Hydrophobic interactions or micellar interactions are postulated to be behind the gelation of C/β-GP solutions. The major impact of this thermal gelation consists in providing injectable in-situ forming hydrogels. Liquid formulations can be loaded with therapeutic materials (drugs, proteins or cells) and injected into the body to form a gel implants *in-situ*.

CONDENSÉ EN FRANÇAIS

La chitine ou poly(β-(1-4)-2-acetamido-2-deoxy-D-glucose) est un poly(N-acetyl-D-glucosamine) qui appartient à la famille des polysaccharides. C'est le polymère naturel le plus abondant après la cellulose, avec laquelle il présente d'ailleurs des ressemblances structurales. Le chitosane est le dérivé déacétylé de la chitine, obtenu par un traitement alcalin. C'est un électrolyte biodégradable et non toxique.

En plus de son abondance, le chitosane présente des propriétés intéressantes dues à sa biocompatibilité, sa biodégradabilité et ses propriétés bioadhésives. Il a été largement étudié autant d'un point de vue purement scientifique que pour ses applications pratiques.

Par rapport à la cellulose, la structure du chitosane comporte plus de centres réactifs. En fonction des conditions de réaction, on peut effectuer une substitution soit sur l'atome d'oxygène et/ou sur l'atome d'azote (O- et/ou N-substitution) : en milieu acide ou en l'absence de catalyseur, c'est le groupe azoté qui réagi, (N-substitution), tandis qu'en milieu alcalin, l'anion alcoolate est plus réactif, on a alors une O-substitution.

Le chitosane et ses dérivés sont des composés commercialisés et relativement peu chers. Ils constituent donc des matériaux biocompatibles et biodégradables intéressants. En modifiant leur composition chimique et/ou leurs caractéristiques physico-chimiques, ces composés peuvent acquérir les propriétés soit d'un solide soit d'une solution. Grâce à ses fonctions alcool et amine, de nombreux dérivés du chitosane ont été synthétisés ces dernières années. Plusieurs d'entre eux ont été même développés au niveau industriel. Récemment, le chitosane a également été largement étudié pour des applications dans le domaine pharmaceutique : comme matrice pour la diffusion de

médicaments, comme produit cicatrisant...Ses aptitudes à former des gels ou des films ainsi que son caractère de polymère cationique, sont à l'origine de ses applications.

Parmi les gels formés à partir du chitosane, la plupart sont non-réversibles thermiquement, et seulement quelques-uns sont des gels réversibles. Différentes techniques permettent de gélifier physiquement le chitosane et ses dérivés : par neutralisation à l'aide d'une base (NaOH, KOH ou NH_4OH), par complexation ionique (avec du borate, molybdate, polyphosphate, sels de sulfate ou macromolécules sulfatées), par réticulation chimique (avec de l'anhydride glutaraldéhyde ou du glutamate succinimide-PEG). Les hydrogels à base de chitosane présentent un intérêt dans de nombreuses applications, particulièrement dans les domaines pharmaceutique et biomédical, de par ses propriétés biologiques de non toxicité, biocompatibilité et biodégradabilité.

Récemment, nous avons mis au point un nouveau gel réticulé physiquement et sensible à la température, à partir d'une solution de chitosane contenant un sel dibasique de β-glycerophosphate. Cette solution qui se trouve à pH neutre est capable de se transformer en un hydrogel tridimensionnel stable à la température physiologique, soit 37°C, qui est la température du corps humain et de la plupart de mammifères, ce qui explique tout l'intérêt biomédicale qu'on accorde à tel système. On comprend davantage un tel intérêt si on sait que des substances biologiques (protéines, cellules, etc...) peuvent être encapsuler dans ce gel, qui en plus d'offrir un environnement compatible avec le milieu physiologique, ses deux composés sont bien connus par leur biocompatiblité avec les organismes vivants et permettent la préservation de la viabilité des cellules. Ce système offre également de bonnes propriétés mécaniques et ce, pendant un temps relativement long, dans des conditions physiologiques ($T \approx 37°C$ et dans un milieu aqueux contenant des acides aminés, des ions ou des protéines).

Dans cette étude, nous avons mis au point la transformation de solutions aqueuses de chitosane, en un gel sensible au pH et à la température. Nous nous sommes particulièrement intéressés aux solutions auxquelles on a ajouté des sels polyoliques ayant une tête anionique, comme par exemple le sel dibasique de β–glycérophosphate. Le principal avantage de telles solutions réside dans la présence et la modification d'interactions entre les chaînes de chitosane, dues à l'effet des parties polyols. Tel effet est susceptible d'augmenter les interactions hydrophobes entres les chaînes de chitosane. Nous avons trouvés que des solutions aqueuses à base de chitosane et de β-GP forment des gels rapidement, dans des conditions de température et de pH bien définies.

Cette étude montre aussi que les solutions de chitosane amenées à un pH compris entre 6.5 et 7.2, à l'aide des β-GP peuvent former un gel à partir de 37°C. Pour un pH compris entre 6.5 et 6.9, le gel ainsi obtenu est entièrement thermoréversible, tandis que si le pH de la solution est supérieur à 6.9, le système n'est que partiellement reversible. Ceci laisse supposer que les interactions hydrophobes, qui sont également thermoréversibles, jouent un rôle non négligeables dans la formation de gel de chitosane/β-GP et que dans certaines conditions de pH elles sont même prépondérantes. Il faut noter que dans ce travail la rhéologie a été largement utilisée aussi bien pour la détermination des caractéristiques des systèmes à étudier que pour mettre en évidence les transitions sol-gel.

En accord avec la littérature, nous avons trouvés que la température de transition sol-gel dépend de la nature du solvant utilisé. Des expériences en rhéologie nous ont permis de confirmer ce résultat et de vérifier que la nature du solvant n'avait aucune influence sur les autres caractéristiques du gel. On pense que la taille des contre-ions du solvant (Cl^-, $CH3COO^-$, $gluconic^-$) intervient dans l'effet protecteur sur les groupes aminés protonés. Cet effet entraîne une diminution des forces électrostatiques répulsives entre 2 segments adjacents chargés.

La déacétylation du chitosane a permis d'obtenir différents degrés de déacétylation (DDA). Nous avons suivi le procédé classique de déacétylation qui consiste en un traitement à 130°C avec une solution basique très concentrée (50%NaOH), utilisée à un rapport de 10/1, en volume de solution NaOH/masse de chitosane. Des DDA très élevés ont pu être ainsi obtenus. Ensuite, nous avons montré que le DDA du chitosane dépend de la température et du temps de traitement. Les DDA de ces nouveaux chitosanes ont été déterminés à l'aide d'une nouvelle méthode conductimétrique qui consiste à doser le polymère neutre par un acide fort, une solution d'HCl (0.1M) par exemple. Cette technique simple et rapide s'est avérée très efficace car elle nous a permis d'obtenir des résultats qui se comparent avantageusement à ceux déduites de l'analyse des spectres de ^{13}C-RMN.

Le mécanisme qui se trouve à l'origine de la formation du gel est très complexe vue le nombre d'interactions mises en jeu. Cependant, à la lumière de certains travaux revendiquant le rôles des polyols et des sucres dans la stabilisation du collagène, des protéines et des polysaccharides, nous avons essayer de proposer un mécanisme qui nous semble plausible. L'addition de sels de β-GP au chitosane modifie les interactions électrostatiques, hydrophobes, ainsi que les liaisons H entre les chaînes de chitosane, lesquelles interactions sont impliquées dans la formation du gel.

La transition sol-gel est donc due essentiellement à la modifications des ces principales interactions:

1) l'augmentation du nombre de liaisons hydrogène entre les chaînes de chitosane, en raison de la réduction de répulsion électrostatique, elle-même provoquée par l'action basique des sels.

2) les attractions électrostatiques entre le chitosane et le glycérophosphate, via les groupements ammonium et phosphate respectivement.

3) les interactions hydrophobes chitosane-chitosane qui sont augmentées par l'action structurante du glycérol sur l'eau, d'après les travaux rapportant sur la stabilisation des polypeptides et des polysaccharides par le glycérol.

L'aspect complexe de ce processus de gélification, vient probablement de sa dépendance avec la température. La transition sol-gel qui se produit dans ce système avec l'élévation de la température rend compte de la présence et de l'importance des interactions hydrophobes entre les chaînes du chitosane. En effet, ces interactions sont les seules susceptibles de se renforcer avec l'augmentation de la température. Nous avons attribué ce renforcement à la présence de la moitié glycérol du glycérophosphate.

A basse température, grâce à de fortes interactions chitosane-eau, les molécules d'eau forment une couche protectrice autour des chaînes de chitosane (hydratation) et les empêchent de s'associer en aggrégats. En chauffant, cet enveloppe que constituent les molécules d'eau est fragilisée ou détruite (déshydratation), favorisant ainsi l'association des chaînes de chitosane. Cette déshydratation est probablement aidée par la présence de la moitié glycérol du glycérophosphate. Ainsi, même si les forces électrostatiques sont modifiées par la température, ou bien par un couplage conformation-charge, les interactions hydrophobes sont supposées jouer un rôle majeur dans la gélification de solutions de chitosane/β-GP. Cependant, on peut remarquer qu'une telle gélification ne pourrait avoir lieu, sans l'augmentation des interactions de type 1) et 2). Ceci explique le rôle du pH dans le processus de gélification de systèmes aqueux chitosane/β-GP.

Les modifications hydrophobicité/hydrophilicité peuvent aussi être expliquées par des changements de polarité du chitosane et des molécules d'eau. La présence de β-GP modifie la polarité soit du polymère (chitosane) soit du solvant (eau). Ceci se traduit par des changements dans les interactions chitosane-chitosane, chitosane-eau et eau-eau. La gélification du chitosane/β-GP qui a lieu avec l'augmentation de température, laisse supposer que la polarité des macromolécules de chitosane, et donc leur solubilité est

augmentée à basse température. Ce phénomène est probablement une conséquence de l'effet structurant de la partie glycérol sur les molécules d'eau.

Par exemple, on sait par expérience que les systèmes qui contiennent la séquence oxyéthylène (EO), deviennent moins solubles dans l'eau à haute température. Dans le poly(oxyéthylène), un des composés les plus étudiés, ce phénomène a été attribué à une transition de conformation. La diminution ou la perte de solubilité à haute température dans l'eau résulte d'un équilibre entre des conformations plus ou moins polaires de chaînes d'EO. Les polysaccharides sont également considérés comme des polymères contenant de l'EO, et certains d'entre eux, comme les dérivés de la cellulose, [méthyl cellulose (MC) et hydroxypropyl méthyl cellulose (HPMC) par exemple], montrent une diminution de solubilité en milieu aqueux, lorsqu'ils sont chauffés.

Une étude rhéologique a été menée afin d'étudier la solution, le gel et la transition entre ces deux états. Le mélange chitosane/β-GP a été soumis à une contrainte de cisaillement oscillatoire, et la viscoélasticité linéaire a été mesurée aucours de la gélification du système aqueux. Le point de gel a été défini et déterminé expérimentalement comme étant le point pour lequel tanδ est indépendant de la fréquence. A partir du point de gel, on observe un comportement loi-puissance des modules dynamiques G' et G'' ($G' \sim \omega^{n'}$ et $G'' \sim \omega^{n''}$) avec une valeur du coefficient telle que $n' \approx n'' \approx n \approx 0.435$. La température de gélification et la consistance ou fermeté du gel ont été déterminées en fonction du DDA, des concentrations en chitosane et en β-GP.

En effet, on a trouvé que la température de gélification diminue avec une augmentation du DDA ou en β-GP. Mais par contre, la diminution de la concentration en chitosane fait diminuer la température de gélaification. La consistance de ces gels dépend du temps, elle augmente avec la température, ainsi qu'avec la concentration en chitosane ou en β-GP.

La transition sol-gel a également été suivie par des mesures de DSC (Differential Scanning Calorimetry). Le résultat obtenu confirme le caractère endothermique exceptionnel de la gélification des systèmes chitosane/β-G. En général, dans cette analyse, on sait qu'un pic endothermique correspond au passage d'un système ordonné à un système désordonné (par exemple, la fusion d'un cristal ou la transition d'un gel à une solution), et inversement pour un pic exothermique (cas d'une cristalisation ou de la formation d'un gel).

En conclusion nous avons mis au point un système basé sur une combinaison du chitosan et le β-GP, dans lequel les interactions hydrophobes ou micellaires sont supposées être impliquées dans le processus de gélification. Le résultat est que le système chitosane/β-GP est rendu sensible à la température. Le fait qu'on peut préparer des solutions neutres capable de gélifier à 37°C est d'un intérêt capital pour les applications biomédicales, puisqu'on peut envisager des formulations injectables qui se transforment en gels sur place une fois injectées dans le corps des mammifères.

Dans des travaux futurs, nous chercherons à mieux connaître le mécanisme de gélification, l'influence du solvant et d'autres composés. Un autre aspect intéressant réside dans la recherche de substituants au chitosane et au β-GP, dans le but de développer des systèmes thermo-gélifiants analogues.

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LIST OF SYMBOLS AND NOTATIONS

a : exponent in the Mark-Houwink equation

C_p : heat capacity

C/β-GP: chitosan with β-Glycerophosphate disodium salt

DA : degree of N-acetylation

DDA : degree of de-N-acetylation

f : molarity

F_A : mole fraction of anhydro-N-acetyl-D-glucosamine

F_D : mole fraction of anhydro-D-glucosamine

G' : storage modulus (Pa)

G'' : loss modulus (Pa)

GP : gelling point

GPC : gel permeation chromatograghy

I : ionic strength

K : constant in the Mark-Houwink equation

LS : light scattering

M_n : number-average molecular weight

M_v : viscosity-average molecular weight

M_w : weight-average molecular weight

m : weight (g)

NASA : 1-naphthylamine-4-sulphonic acid

NSA : 1-naphthol-4-sulphonic acid

RG : radius of gyration

S : gel strength parameter ($Pa \cdot s^n$)

T : temperature (°C)

t : time (s)

δ : phase angle

γ : shear rate (s^{-1})

η : shear viscosity (Pa·s)

σ : shear stress (pa)

ω : frequency (rad/s)

CHAPTER 1 - INTRODUCTION

1.1 Generalities

Chitin is a fibrillar crystalline polymer of unbranched chain of β-(1-4)-2-acetamido-2-deoxy-D-glucose, N-acetyl-D-glucosamine, which belongs to the family of structural polysaccharides, as shown in Figure 1.1. After cellulose, with which it bears a structural resemblance, chitin is much more abundant natural polymer. Insect skins and the shells of anthropoids are composed of chitin. Chitosan, the N-deacetylated derivative of chitin obtained by alkaline treatment, is a biodegradable and nontoxic polyelectrolyte. For commercial applications chitosan is defined as at least 75% deacetylated at which point it is soluble in dilute acetic acid.

Chitosan chemistry has been widely investigated due to basic scientific interest as well as for practical applications, such as use in food industry, as well as biomedical and agricultural applications. In comparison with the molecule of cellulose, chitosan has more reactive centres in its structure, and depending on the reaction conditions, O- and/or N-substitution can take place. In an acidic medium or without a catalyst, reactions can take place at the nitrogen group. In an alkaline medium, the alkoholate anion is more reactive and O-substitution can occur.

Chitosan and its derivatives are relatively inexpensive and commercially available materials and represent an attractive group of biocompatible and biodegradable polymers. They have solid or solution properties, which can be modified by changing their chemical composition and/or physico-chemical characteristics. The degree of deacetylation and molecular weight has been shown to greatly influence solution properties, enzymatic degradability and biological activity (Roberts, 1995). Chemical modifications, for instance, have been proposed to neutralize or modify chitosan chains by incorporating

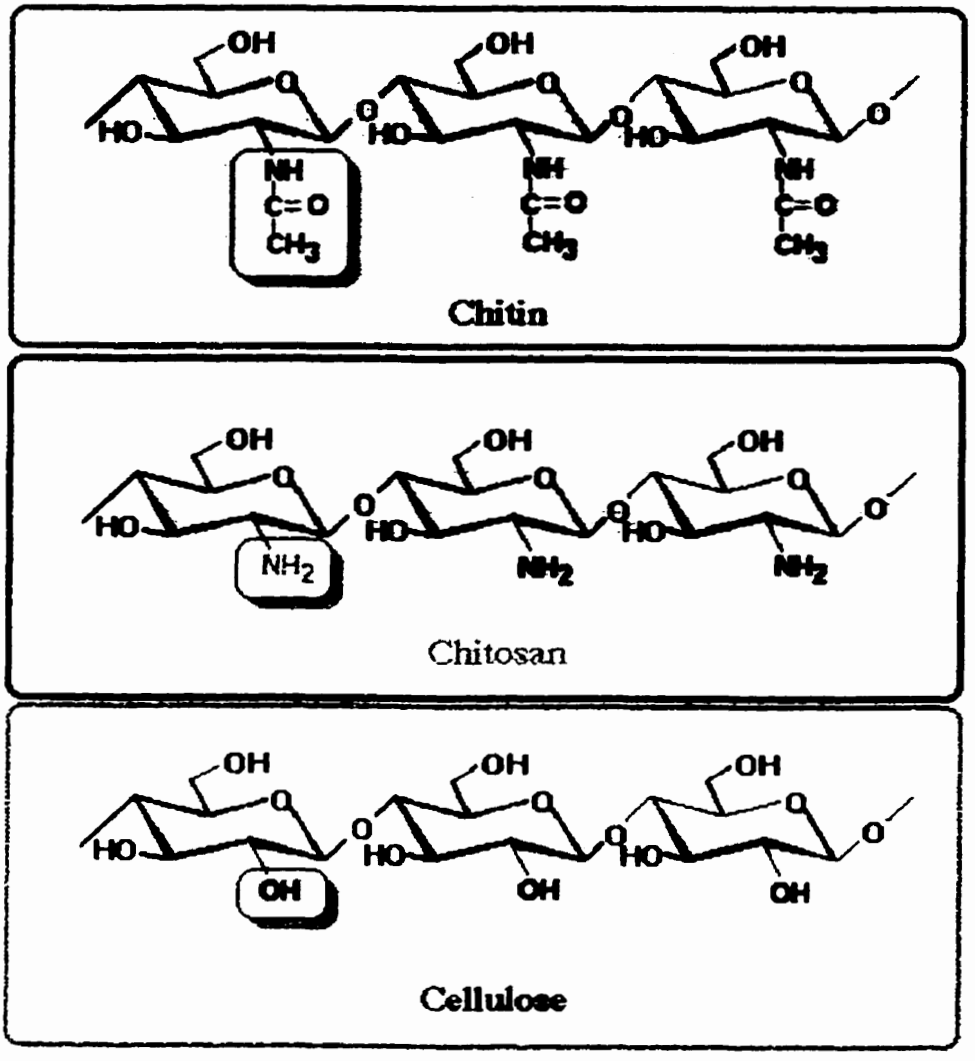


Figure 1.1: Structure of chitin, chitosan and cellulose.

carboxylic acid such as acetic acid, glutamic acid, carboxymethyl or sulphate groups. As chitosan has reactive hydroxyl and amino groups, a great number of new derivatives have been created in the last years by derivatisation of it. Many of them have been extensively used in industry, foremost as a flocculent in the clarification of waste water in Japan (Stanford et al., 1987), as a chelating agent for harmful metals in the detoxification of hazardous waste (Mitani et al., 1995), for the clarification of beverages such as fruit juices and beers, and for agricultural purposes such as a fungicide in the protection of crops and the coating of apples. It is also a constituent of many food products.

In recent years chitosan has been introduced as a material in the nutritional supplement market, especially as a weight loss aid and cholesterol lowering agent. So far as is known chitosan has been widely investigated for use as pharmaceutical products such as drug delivery products, wound healing products, etc. (Nagai et al., 1984). This is due to its unique polymeric cationic character and its gel and film forming properties. A scientific basis for the efficacy of chitosan in the promotion of wound healing was first reported in 1978 (Balassa et al., 1978). Chitosan acetate films, which were tough and protective had the advantages of good oxygen permeability, high water absorptivity and slow enzymatic (lysozyme) degradation, thereby avoiding the need for repeated application (Allan et al., 1984).

As a linear polyelectrolyte in acidic aqueous solution, chitosan salts will carry a positive charge through protonation of the free amino group of glucosamine. Its reactivity with negatively charged surfaces depends directly on the density of positive charges of macromolecular chains. This cationic nature renders the chitosan mucoadhesive and therefore gives it the ability to bind strongly to several mammalian tissues and cells, a characteristic needed in many potential applications, particularly in the medical field. Due to this mucoadhesive characteristic, chitosan has been exploited in the cosmetic industry and in hair products, in the dental industry and in ophthalmic applications, as a contact lens coating or as the contact lens material itself (Illume, 1998).

Chitosan-based gels may be broadly divided into thermally non-reversible gels and the very much smaller group of thermally reversible gels. Within the first group a further subdivision into those formed by N-acylation and those produced by Schiff's base (aldimin) formation is useful. Physical gelation of chitosan and its derivatives can be obtained through different techniques: neutralization (NaOH, KOH, NH_4OH···), ionic complexation (Borate, Molybdate, Polyphosphate, Sulphate salts and sulphated macromolecules···) or chemical cross-linking (anhydride, glutaraldehyde, glutamate succinimide-PEG···). Hydrogels of chitosan are of interest for various applications, particularly in pharmaceutical and biomedical fields, since chitosan exhibits favorable biological properties such as nontoxicity, biocompatibility and biodegradability. In most previous studies, chitosan gels were chemically cross-linked by using different cross-linking techniques.

Recently, we have successfully discovered a new thermal setting physical gel system based on a chitosan/β-Glycerophosphate solution. This three-dimensional chitosan-based hydrogel can be formed and stable maintained at physiological temperatures; which includes nontoxic biocompatible components for mammalian or human environments; with both components and processes having low toxic effects towards living biologicals and preserving high cellular viability. It also provides good mechanical/handling performances at physiological temperatures for long periods of time in physiological aqueous media containing amino acid, ions and proteins.

β-Glycerophosphate, or Glycerophosphate, is a well-studied molecule in biological sciences. β-Glycerophosphate is widely used as a cell culture medium supplement for culturing cells isolated from musculo-skeletal tissues, and has been shown to induce or maintain the synthesis of specific matrix components when delivered to bone/cartilage cells in culture (Matthew et al., 1993). Gelation of Chitosan will occur with any grade or purity of Glycerophosphate while encapsulation of living biologicals would require cell culture tested medical grade β-Glycerophosphate.

1.2 Scope of Present Work

In the present study, we focused on the modification of cationic chitosan solutions into thermal setting, pH-dependent, gel-forming aqueous solutions. Our efforts have been oriented toward chitosan aqueous solutions where we added polyol salts bearing a single anionic head, such as glycerol-, sorbitol-, fructose- or glucose-phosphate salts (polyol-phosphate or sugar-phosphate). We find that these salts form ideal agents for transforming purely pH-dependent chitosan solutions into temperature-controlled pH-dependent chitosan solutions. The combination of chitosan, a cationic polysaccharide, and polyol-phosphate salts was chosen to benefit from several synergistic forces favorable to gel formation including hydrogen bonding, electrostatic interactions and hydrophobic interactions.

Because the properties of chitosan solutions are greatly influenced by the characteristics of chitosan, such as the molecular weight and the degree of deacetylation, different samples with various molecular weight and degree of deacetylation have been used to form a C/β-GP thermogelling system at physiological pH. The influence of such parameters on the gelation properties will be discussed.

A variety of physical and chemical techniques are available for studying gels and networks. Among these techniques are rheological methods, which are particularly useful for studying slow molecular motions in networks and for monitoring changes in properties during gelation or network formation. Rheological and viscoelastic methods have been used extensively in our studies on the gelation process of Chitosan/β-Glycerophosphate solution. Certain conclusions can be reached via viscoelastic measurements such as gelation temperature, gelation time, and mechanical properties of gels, etc. Another efficient instrument (DSC) has been used on the study of determination of gelation point.

CHAPTER 2 - LITERATURE REVIEW

2.1 Historical background

Braconnot, a professor of natural history in Paris, discovered chitin when he treated mushrooms with warm alkali in 1811. In 1823 Odier isolated a KOH insoluble residue from insects which had a similar structure to that of plants and named this substance chitin from Greek term χιτων (tunic, envelope). Following Odier, Children restudied this material in1824, detected nitrogen was inside, and gave an empirical formula of $C_{11}H_{17}O_7N_2$, which was very close to that of disaccharide fragment from chitosan ($C_{12}H_{22}O_8N_2$). It was Payen who started the scientific investigation of the structural difference between cellulose and chitin in 1843. In 1878, Ledderhose indicated that chitin was composed of glucosamine and acetic acid, confirmed by Gilson (1894).

Chitosan was discovered in 1859 by Rouget when he boiled chitin using concentrated sodium hydroxide. He names it "modified chitin" In 1894; Hoppe-Seyler reinvestigated this material and renamed it chitosan.

At the beginning of this century, the study of chitin mainly focused on the occurrence and distribution of the chitinous structure in nature. The breakthrough in the investigation of the chitin structure came when X-ray diffraction and other physical techniques including infrared spectroscopy, NMR, etc. became available. X-ray is by far the most reliable method for determining the crystalline structure of chitin in cell walls. Polarized infrared analysis of the chitin structure by Marchessault et al. (1960, 1967) confirmed the chain conformation and the orientation of interchain amide H bonding .In 1930s the first patent on producing chitin and chitosan was granted to G.W.Rigby an employee of Du Pont de Nemours & Co. The first book on chitin focusing on biological aspects was published in 1950s (Richards, 1951). Around the same time, chitosan was clearly described as a polymer of glucosamine. With the exception of the 1940s, the

number of published papers on chitin or chitosan has increased each decade since the 1930s. Recently, hundreds of papers per year have been published on this topic. A significant number of papers have described the bioapplications of chitin and chitiosan, including medical, cosmetic, agricultural, and food-related uses. Industrial activities for chitin and chitosan production started in 1971, led by a Japanese company, Kyowa Oil & Fat Inc. Now more than fifteen companies in Japan are producing chitin or chitosan related materials. In North America several companies including Protan, FMC, Vanson, etc. produce chitin and chitosan. Although not all chitin is accessible, the abundance of chitin and its unusual properties are factors underlying current interests in developing its commercial potential.

2.2 Production and characteristics of chitin

The shells of crabs, shrimp, prawns and lobsters coming from the peeling machines in canning factories are used for the industrial preparation of chitin. The isolation includes two steps: demineralization with HCl and deproteination with aqueous NaOH. Lipids and pigments may also be extracted. These operations are mainly empirical and vary with the differently mineralized shells, the seasons, and the presence of different crustaceans in the catch.

Chitins are copolymers of 2-acetamido-2-deoxy-β-D-glucose and 2-amino-2-deoxy-β-D-glucose (Figure 2.1). Note that unlike other abundant polysaccharides, chitin contains nitrogen. The structural unit is chitobiose: (2-amino-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-amino-2-deoxy-D-glucose (YUI et al., 1994). Bound water is also part of the structure.

Chitin isolates differ in many respects, e.g. in degree of N-acetylation (DA), typically close to 0.9, in elemental composition, with a nitrogen content typically close to 7% and a N/C ratio of 0.146 for fully N-acetylated chitin, in molecular size, and in

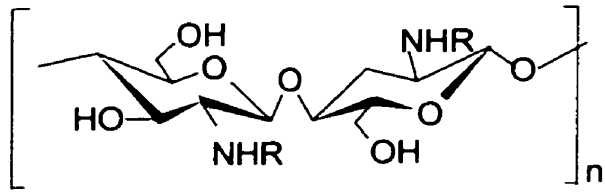


Figure 2.1: Chemical structure of chitin and chitosan. In chitin the acetylated units prevail.

polydispersity. The average molecular weight for commercial chitins is 0.5-1.0 MDa. Polydispersity depends on the treatment and possible blending of various batches. Native chitin is a highly ordered biopolymer. This becomes evident on a macroscopic scale when microfibrillar fragments of purified crustacean chitin are prepared in 3M HCl at 104 °C. After removal of the acid, sonication yields colloidal chitin suspensions that spontaneously self-assemble in a chiral nematic liquid crystalline phase, reproducing the helicoidal organization that characterizes cuticles (Revel et al., 1993). Chitin is a polymorphic polysaccharide. The adjacent chains are in successive sheets. In the β-form all chains are aligned in a parallel manner; in α-chitin they are positioned antiparallel (Figure 2.2). The high molecular order of chitin dictates the tissue characteristics. For instance, grasping spines of *Sagitta* are made of pure α-chitin, because they should be suitably hard to hold prey, whereas the pen of Cephalopoda consists of β-chitin because it has to be flexible. Also, solubility and reactivity of the polymorphic forms differ.

Chitin is easily hydrolyzed by acids, but is stable to dilute alkali; in warm concentrated alkali it undergoes oxidative degradation by air as most polysaccharides do. Chitin hydrolysates can be prepared by adding chitin to concentrated HCl at 4 °C and subsequent stirring at 40 °C. Excess acid is then removed by adding an ion-

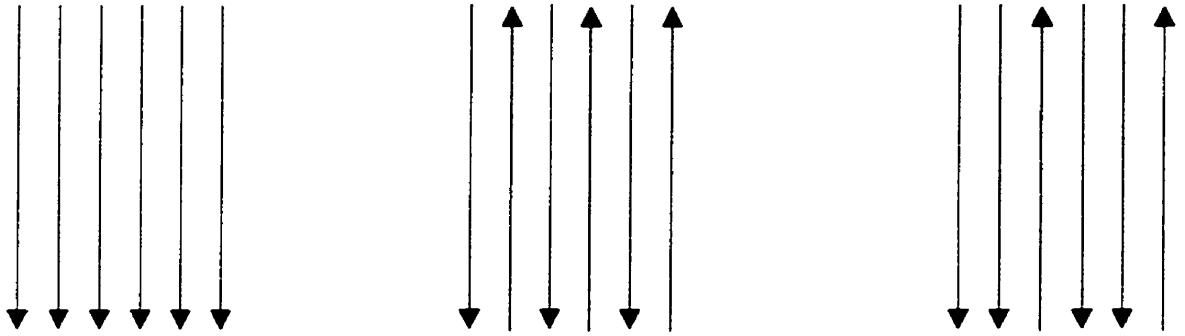


Figure 2.2: Different packing arrangements of the polymer chains of α-chitin, β-chitin, and γ-chitin.

exchange resin, and the product is resuspended to prepare the "colloidal chitin", which remains stable for several weeks when stored at 4 °C. Wet chitin is degraded by a number of microorganisms, which produce chitinolytic enzymes or other enzymes with nonspecific activity towards chitin. In the presence of chlorine bleaches, chitin gives N-chloro compounds; peroxide bleaches therefore are preferred.

The solubility of chitin is remarkably poorer than that of cellulose due to its high crystallinity, supported by hydrogen bonds mainly formed by the 2-acetamide groups. N,N-dimethylacetamide containing 5-9% LiCl (DMAc/LiCl) and N-methyl-2-pyrrolidinone/LiCl are systems wherein chitin can be dissolved up to 5%. At room temperature, the main chain of chitin is rigid, so that mesomorphic properties may be expected at a sufficiently high concentration of the polymer (Terbojevich et al., 1988). When chitin films are obtained by exposing DMAc/LiCl chitin solutions to water vapor, circular dichroism of complexes of Congo red bound to chitin films reveals a cholesteric structure having an organization similar to that occurring in the native chitin cuticle

(Bianchi et al., 1990). In contrast, mesophase formation is not observed in DMAc/LiCl solutions, irrespective of the molecular weight and the concentration of the sample (Carpaneto et al., 1994). Aggregation phenomena occurring in chitin solutions even at low concentrations can be responsible for this behavior (Terbojevich et al., 1996). Chitin gels are obtained by heating chitin solutions in DMAc/LiCl at T > 90 °C. The sol-gel transition is reversible and its critical temperature depends on the molecular weight (Bianchi et al., 1997).

Recognition of the chitin microfibrillar arrangement pointed to fiber and film-forming possibilities. Chitin fibres spun through the xanthate process led to the use of DMAc/LiCl solvent systems (Hirano et al., 1994). These solvent systems are also suitable for chitin modification and for analytical characterization of the polysaccharide in terms of polydispersity (Hasegawa et al., 1993).

2.3 Production and characteristics of chitosan

Chitosan indicates a continuum of progressively de-N-acetylated chitin. In general, chitosans have an N-content higher than 7% and a degree of N-acetylation lower than 25%. The removal of the acetyl group requires a harsh treatment, usually performed in a concentrated aqueous or alcoholic NaOH solution. Protection from oxygen, with a nitrogen purge or by addition of sodium borohydride to the alkaline solution, is necessary in order to avoid undesirable reactions such as depolymerization and generation of reactive species. However, the excess amount of NaOH represents an economic and ecological worry. Alternatives are therefore being sought. For instance, chitin is mixed with NaOH powder (weight ratio 1:5) by extrusion at 180 °C, and highly de-N-acetylated and soluble chitosan is thus obtained with just one half of the amount of NaOH needed for current methods in an aqueous system (Rogovina et al., 1994). The quantities of alkali can also be reduced by carrying out the reaction in dispersing media such as polyethylene glycol dimethylether or paraffin oil, containing small amounts of concentrated aqueous

alkali, or more dilute aqueous alkali solutions at higher-than-usual temperatures and pressures (Castelli et al., 1996).

Commercial chitosans may contain insoluble, highly N-acetylated fractions that originate from the core of the granules submitted to heterogeneous de-N-acetylation. The N-acetylated groups in the acid-soluble fractions are randomly distributed, whereas the insoluble fractions contain relatively long sequences of N-acetylated units (Aiba et al., 1991). Determination of intrinsic viscosity, weight-average molecular weight (Mw) and polydispersity indices (Mw/Mn) indicate negligible depolymerization of the acid-soluble fractions provided that de-N-acetylation is performed under nitrogen atmosphere at 75 °C (DA 0.2-0.52) (Ottoy et al., 1996).

The presence of a prevailing number of 2-amino-2-deoxyglucose units in a chitosan permits to bring the polymer into solution by salt formation. Chitosan is a primary aliphatic amine that can be protonated by certain selected acids (pKa of the chitosan amine is about 6.2). The following salts are among those that are water-soluble: formate, acetate, lactate, malate, citrate, glyoxylate, pyruvate, plycolate and ascorbate.

Chitosan chains can be chemically depolymerized. Using nitrous acid, the reaction is selective, rapid and easily controlled; stoichiometry and products are well established. Nitrosating species attack the glucosamine, but not the N-acetylglucosamine moieties, cleaving the glycosidic linkage yielding 2,5-anhydro-mannose residues at the cleaving end. The rate-limiting step in the reaction is the nitrosation of the unprotonated nitrous acid (Allan et al., 1995). Hydrogen peroxide can also conveniently be used to depolymerize chitosan. As for enzymatic depolymerization, many commercial enzyme preparations exert hydrolytic activity on chitosan. Unexpectedly, the polymer appears to be vulnerable to a range of hydrolases; several protease such as pepsin, bromelain, ficin and pancreatin display lytic activities towards chitosans that surpass those of chitosanases and lysozyme. Cellulases, hemicellulases, lipases and other enzymes are also effective

(Yalpani et al., 1994). This phenomenon is possibly a result of the simplicity of the enzymatic mechanism.

Chitosans can be obtained from fungi, easily cultured on simple nutrients, which means that the production can become independent of the seasonal shellfish industry (Muzzarelli et al., 1980). Chitosan is present in the cell wall of *Mucorales* and can be separated from the accompanying glucans by extraction with either acetic acid or alkali, the latter being preferred when the glucans are to be dissolved. The final molecular weight is in the order of 500 kDa; the degree of N-acetylation is around 0.10 (Rane et al., 1993).

2.4 Solution properties of chitosan

The solution behavior of chitosan was investigated by using a variety of techniques, including intrinsic viscosity ([η]) determination, light scattering (LS) and gel permeation chromatograhpy (GPC). Chitosan in dilute acidic aqueous solution exhibits a polyelectrolyte character at low pH, and its hydrodynamic and rheological behavior in solution is intricate. The physicochemical properties of solutions of chitosan are expected to be governed by factors, such as temperature, pH, ionic strength, surfactant concentration, and degree of de-N-acetylation (DDA). It is known that the charge density along the chain increases with an increase in the DDA, and that chain flexibility of chitosan molecules can be manipulated by changing the DDA (Nyström et al., 1999).

As reported for polyelectrolyte chains, [η] values depend on the ionic strength (I) of the medium: plots of [η] vs. $1/\sqrt{I}$ (which is proportional to debye length) are linear (Smidsrød et al., 1971). This was found to be also valid for chitosan (Rodriguez-Sanchez et al., 1982). The stiffness parameter, proposed as an empiric parameter for chain rigidity, is in the range of values found in other polysaccharides and decreases with increasing DDA, indicating that a higher N-acetyl content increases the stiffness of the chain

(Anthonsen et al., 1993). Light scattering (LS) measurement is not easy to perform because microgels are frequently present and/or multimerization processes occur. From the values of the radius of gyration (RG), a persistence length (≈200 Å) has been deduced, which is lower than that of chitin (≈350 Å) (Terbojevich, et al., 1991). GPC measurements, carried out in 0.3 M acetic acid/0.2 M sodium acetate, allow the determination of the molecular weight distribution and the viscosimetric parameters a and K of the Mark-Houwink-Sakurada equation $[\eta] = KM_v^a$ (Rinaudo et al., 1993).

Chitin and chitosan polymers differ structurally from each other in terms of chain morphology and mole fraction of anhydro-N-acetyl-D-glucosamine (F_A) or mole fraction of anhydro-D-glucosamine (F_D). The term chitin and chitosan refer to a continuous series of copolymers ranging in composition from fully N-acetylated (chitin F_A=1) to fully deacetylated (chitosan F_A=0). There have been several studies of the effects of variation in F_A on the constants K and a of the Mark-Houwink equation for determination of $<M_v>$. Rinaudo et al. (1993) examined three chitosan samples covering the range of chitosan [0.02-0.21], using gel chromatography together with viscosity measurements. They concluded that the value of a remained constant at 0.76 over this range of F_A values but that the value of K decreased from 0.82 to 0.74 with increase in F_A. However applying these values to earlier [η] values reported by this group for an extremely well characterized chitosan gives very low $<M_v>$ values.

Anthonsen et al. (1993) examined chitosan samples over the range of chitosan (F_A: 0.0-0.6), the latter produced by homogeneous deacetylation. At each F_A level nitrous acid degradation was used to produce a series of samples having different molecular weights but the same F_A value and these were used for osmotic pressure and viscosity measurements. The results indicated that K decreased and a increased with increase in F_A, the equation for measurements at 0.1 M ionic strength being:

$$\text{Log } K = -0.427 - 3.821F_A \qquad (2.1)$$

$$a = 0.6169 + 0.759F_A \quad (2.2)$$

Wang et al. (1991) using chitosan samples over the range of chitosan[0.0-0.31] obtained similar trends to those of Anthonsen et al., namely K decreases and a increases with increase in F_A values, although the actual numerical values were different. Their relationship may be given as:

$$\text{Log } K = -1.6800 - 7.25F_A \quad (2.3)$$

$$a = 0.805 + 1.01F_A \quad (2.4)$$

The explanation proposed by this group for the increase in a with increase in the mole fraction of anhydro-N-acetyl-D-glucosamine residues is that the N-acetyl group hydrogen bonds with the C(6)-OH group of the next sugar unit in the chain , thereby increasing the chain stiffness. They also considered that the bulky N-acetyl group would restrict the ease of rotation of sugar residues around the glycosidic bond. Both of these factors were also considered important by Anthonsen et al. (1993).

One possible explanation for this apparent contradiction of increasing a value and decreasing K and viscosity is that as the F_A value increases there is less electrostatic repulsion between chain segments so that the polymer chain coil becomes more compact, leading to a reduction in viscosity. At the same time the shape of the chain changes from a random coil conformation at $F_A = 0.0$ to a more extended shape at $F_A = 0.31$, leading to an increase in a (Figure 2.3) (Roberts, 1995).

2.5 Chitin and chitosan derivation

In the past, chitin has been considered an intractable biopolymer, due to the difficulties encountered in dissolving and reacting it. But, as soon as association of polymer chains is prevented or depressed, chitin lends itself to many reactions, affording

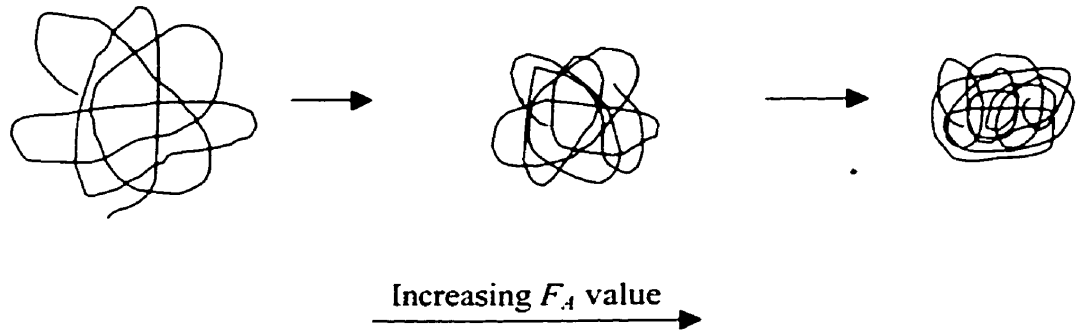


Figure 2.3: Change in chain conformation with change in F_A

a wide choice of modified chitin (Figure 2.4). During the past few years, a number of novel solvents have been proposed, and reactions in homogenous or nearly homogenous media have been carry out (Terbojevich et al., 1988). Because de-N-acetylation is also feasible in various ways, chitosans and modified chitosans can easily be produced.

Indeed, it has been shown that derivatization of the primary amino group and the primary and secondary hydroxyl groups can easily be performed. Chemical modifications of chitin and chitosan, carried out under mild conditions in order to protect glycosidic and acetamido linkages, yield more soluble polymers. These show a higher biodegradability in vivo and physical properties of interest for applications in the solid state or in solution.

Chitin treated with NaOH yields alkalichitin, which can react with 2-chloroethanol to yield 2-hydroxyethylchitin, also known as glycolchitin. This product was probably the first derivative to find practical use and to be listed in catalogs of chemical products. It is recommended as a substrate for lysozyme. The reaction of alkalichitin with sodium monochloroacetate gives carboxymethylchitin sodium salt, soluble in water.

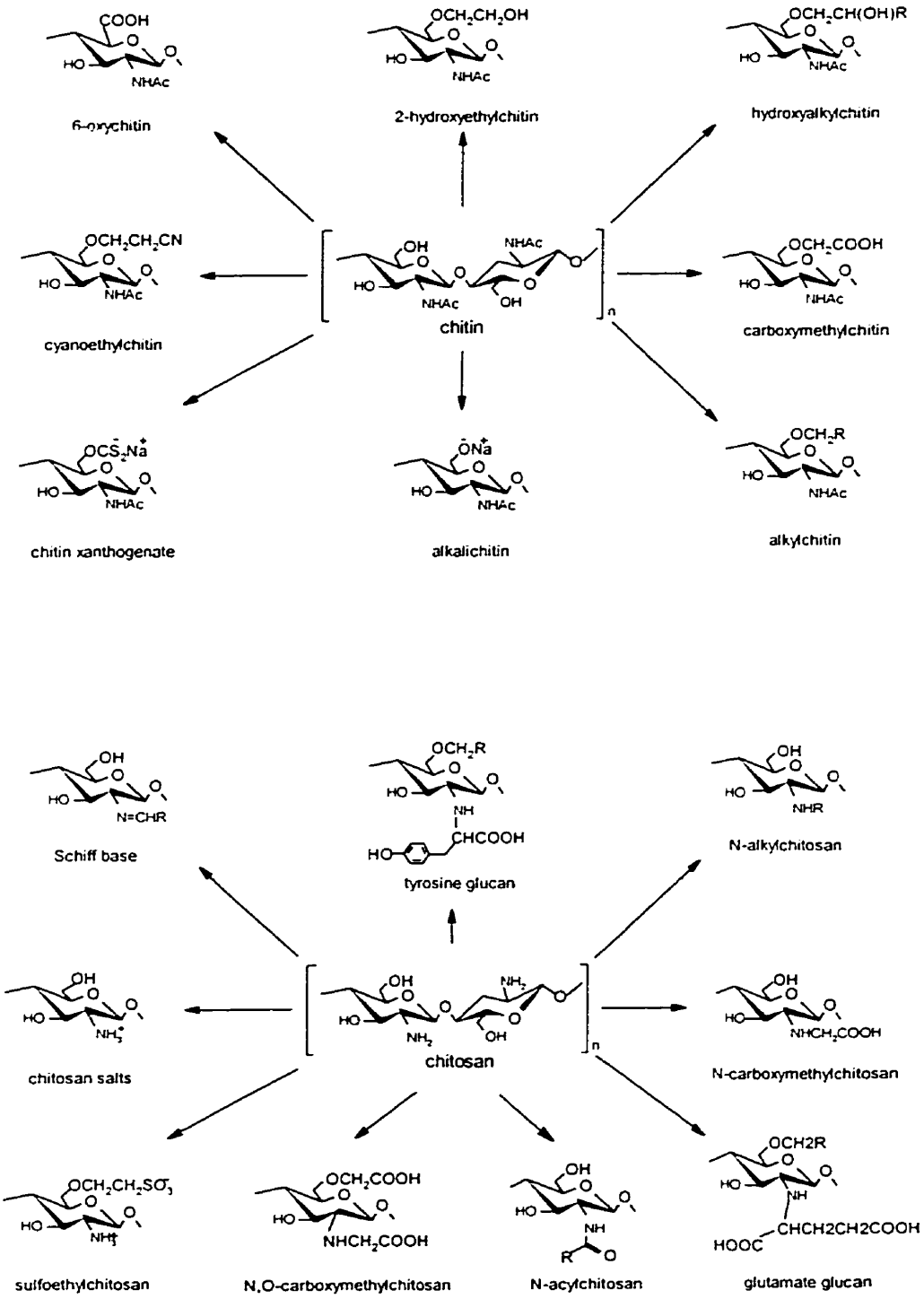


Figure 2.4: Some chemical derivatives of chitin and chitosan (Reproduced from Peter, et al., 1995).

The formation of derivatives suitable for industrial applications with good solubility in various organic solvents can be effected through the introduction of hydrophobic substituents by N-acylation with long chain fatty acyl halides or anhydrides. The simplest example of N-acylation of chitosan is the production of completely N-acylated chitin. For this and other N-acylation reactions, the major problems are achieving N-acylation with or without (as desidered) accompanying O-acylation, and realizing uniformity of reaction under heterogeneous conditions. Chitosan can also be re-N-acetylated with acetic anhydride to obtain water-soluble partially re-N-acetylated chitin (Hirano et al., 1993). The Schiff reaction between chitosan and aldehydes or ketones gives the corresponding aldimines and ketimines, which are converted to N-alkyl derivatives on hydrogenation with cyanoborohydride (Figure 2.5).

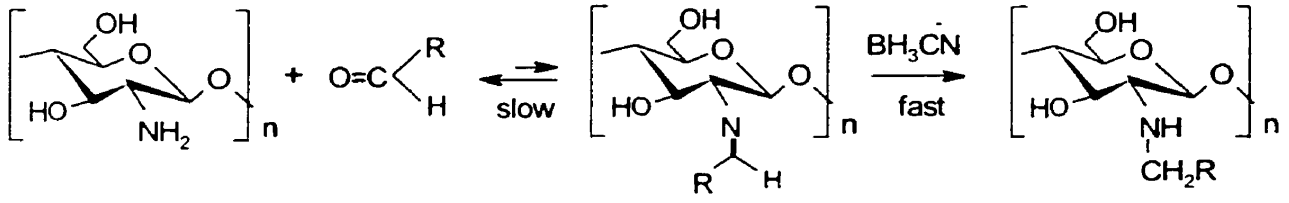


Figure 2.5: The Schiff reaction in the alkylation of chitosan with aldehydes or ketones under reducing conditions.

2.6 Chitosan Gels

Biopolymer gels differ from synthetic polymer counterparts in a number of important respects. Firstly, they contain large amounts of solvent (usually water) and are prepared by crosslinking in the presence of this solvent. Second, the crosslinks are more often physical than chemical, the point covalent crosslinks of the synthetic polymer network being replaced by weaker (and potentially more reversible) forms of chain-chain

interaction. This in turn means that the point crosslinks are not only replaced by weaker physical bonds, but that they also cease to be geometrically small in relation to the rest of the network structure. Contacts between sizeable regions of the polymer chain become involved, two such interacting regions providing the simplest situation, with associations of many such segments to form "junction zones" also being possible (Figure 2.6).

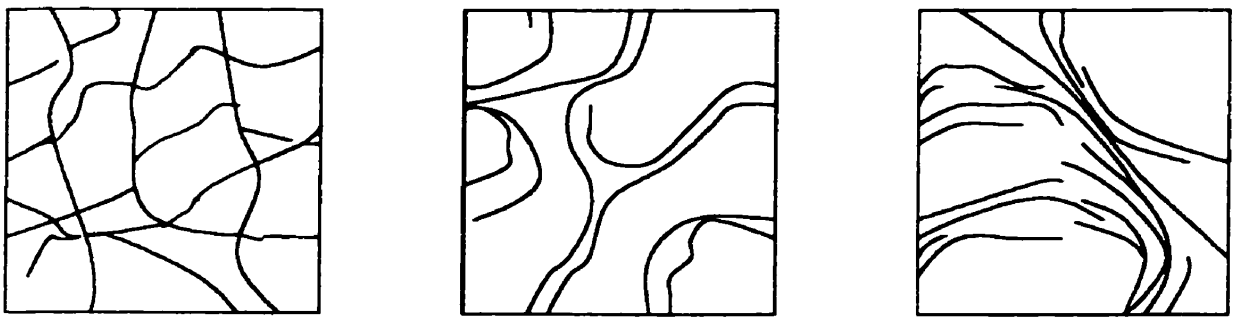


Figure 2.6: Schematic representation of polymer networks involving: (A) point (covalent) crosslinks; (B) two-chain junction zones; (C) multiple-chain junction zones (adapted from Clark, et al., 1990).

Polysaccharide gels from agars, carrageenan and alginate provide classic examples of this behavior, crosslinking being induced either by the cooling of solutions or by the introduction of specific counterions. In general, the temperature or electrolyte changes just described drive the polymer to a more ordered conformational state, and it is the formation and/or the association of such ordered sections of polymer chain that is the origin of the physical crosslinking process (Clark et al., 1990).

Gel-forming polymers undergo phase transitions from liquid to solid at a critical extent of reaction. This phenomenon is called gelation. The polymer is said to be at the

gelling point (GP) if its steady shear viscosity is infinite and its equilibrium modulus is zero (Winter et al., 1986). Several processes may contribute to this transition besides the connecting of molecular strands by chemical crosslinking including: physical entanglements between the macromolecular strands, vitrification as the glass transition temperature rises with increasing extent of reaction, phase separation of the reaction components or products, and crystallization (Winter et al., 1986).

The first report of a chitosan gel – based on a thermally reversible gel is that of Hayes and Davies who found that solutions of chitosan in 1.1M oxalic acid gradually gelled upon standing at room temperature. The length of time required for gelation was considerable and depended on the chitosan concentration, being approximately 21days for a 30g dm^{-3} solution and 24 h for a 70 g dm^{-3} one. The gels melted upon heating and gelled upon cooling, the time required to gel after melting being considerably less than that required for the initial gel formation (Hayes et al. 1977).

Hirano and co-workers subsequently reported on this type of gel and proposed that the mechanism of formation of the junction points is one of salt formation similar to that for formation of alginate gels in the presence of Ca^{2+} ions. In a study of partial *N*-succinylation of chitosan [0.0] the same authors noted that the product having 35% of the amine groups acylated gave a thermoreversible gel, but no details were given (Yamaguchi et al. 1981).

A method of preparing thermoreversible chitosan gels through the use of large organic counter ions has been described. The process involves mixing heated solutions of chitosan acetate and of the sodium salts of either 1-naphthol-4-sulphonic acid (NSA) or 1-naphthylamine-4-sulphonic acid (NASA); the mixture gels upon cooling (Roberts, 1992).

Chitosan and its derivatives have been widely explored for drug delivery system through hydrogels. Peptide delivery was proposed nasally with Chitosan while DNA delivery was obtained from Chitosan/Alginate systems. Wound healing and reconstructive devices made of Chitosan materials have been proposed for open wounds or corneal wounds as well as periodontal tissues and skin. Entrapment of living biologicals (cells, enzymes, etc.) has been investigated with different Chitosan products (Zielinski et al., 1994; Matthew et al., 1993). However, in nearly all cases, living cells have been encapsulated within Alginate/Chitosan microbeads. Encapsulation of chondrocytes (cartilage cells) and yeast cells were proposed within Calcium-Alginate/ Chitosan beads (Guo et al., 1989; Li, 1996), but hepatocyte transplantation and liver engineering have been reported with pure Chitosan (Gupta et al., 1993).

Polysaccharide capsules have been proposed for entrapping physiologically active cells such as the Langerhans Islets (US 4,391,909 patent). Chitosan/Hydrochloride Cisplatin mixture were cross-linked and proposed as drug delivery systems. Chitosan derivatives have been incorporated in numerous carrier compositions or drug formulations (e.g. drug formulation EP-00443027 patent, carrier composition *International Patent Application published under* # WO 93/24476, wound healing formulation *International Patent Application published under* # WO 96/02260, tissue stimulating agent formulation *International Patent Application published under* # WO 96/02259). Those patents show chitin or chitosan products have been disclosed as useful products for treating arthritis, giving better results than sodium hyaluronate.

Chitosan materials such as wound filling materials or contraceptive products were also proposed (US 4,956,350 and US 4,474,769 patents). Granular gels of chitosan were cross-linked through polyfunctional agents for immobilizing insolubilized active enzymes (US 4,089,746 patent). Chitosan gels were again reported as supports for immobilizing and encapsulating living biomaterials such as cells, bacteria and fungi (US 4,647,536

patent). Ophthalmic drug delivery systems made of Chitosan were also proposed for in situ gelling and forming (US 5,422,116 patent).

Chitosan gels have been prepared from glycerol/acid/water systems as biodegradable carriers for drug delivery. Jackson (US 4,659,700 patent) has reported that the resulting chitosan gels remain quite stable, keeping intact their three-dimensional shape for long periods and over a wide range of temperatures, particularly between 4°C and 40°C. Gels and gel-like materials were processed by dissolving 1 to 3% w/v chitosan within acid-water-glycerol solutions wherein acetic, formic or propionic acid and 10-90% glycerol proportions are used preferentially, and by neutralizing with liquid bases such as the sodium, ammonium and potassium hydroxides or ammonia vapors.

The pH value of the resulting chitosan-glycerol gel materials is about 7.0. After neutralization, the resultant mixtures turn into gels upon standing, such gels resulting apparently from the interaction of chitosan, glycerol and water. It must be noted, however, that such three-dimensionally shaped chitosan-glycerol gels will occur only when the solution is previously neutralised with a base. One-piece three-dimensional gels can be molded easily as well as gel-like membranes. The role of the glycerol component and chitosan-glycerol interactions is not elucidated. Composition and processing methods of chitosan-glycerol gels, as well as their uses in bandages and wound products, were claimed (David, US 4,659,700 patent).

Gelation of chitosan through polyphosphates has been promoted for encapsulating cells such as neural or musculo-squeletal tissues. Generally, chitosan in an acid/water medium was loaded with cell suspensions, and the resulting mixture was dropped in a buffered pentasodium tripolyphosphates so as to form cell-loaded chitosan beads and capsules. Entrapment of neural cells within polyphosphate-gelated chitosan beads has led to good cellular viability but low proliferation rate. No large or specific three-dimensional shaped materials were proposed.

In recent years significant advances have been achieved in developing an understanding of the underlying physics controlling the gelation process particularly in chemically gelling systems, while physical gelation is a phenomenon that is much less understood. Principally due to the transient nature of the physical network junctions, which makes it difficult to study physical gels near their gel point. Physical gels are built up by thermoreversible cross-linked networks in which the junctions are formed by secondary forces capable of forming bonds that are typically weak enough to be broken by thermal fluctuations. The principle differences between chemical and physical gels lie in the lifetime and the functionality of the network functions (Nyström et al., 1995).

Most rheological studies made on gels use the viscosity of gel as the main rheological parameter (see e.g. Lenaerts et al., 1987; Deasy and Quiley, 1991). Unfortunately this doesn't provide data which is relevant for a gel. When the viscosity is measured the gel is sheared at a high speed thus destroying the structure of the gel. By using oscillatory measurements, where the oscillating amplitude is small enough, the gel structure remains intact during measurements. Oscillatory measurements give information on the dynamic properties: the elastic modulus, G', and the viscous modulus, G'' (Carlfors et al., 1998).

Rheological techniques widely used in monitoring the process of gelation may be classified into (i) small deformation rheology where the linearity between stress and strain is satisfied, and therefore, the methods of analysis for experimental results have been well developed, and (ii) large deformation and fracture both of which are difficulty because of non-linearity and probabilistic nature. Clark and Ross-Murphy (1987), Nijenhuis (1997) have written excellent reviews on gelling polymers mainly from the viewpoint of a rheologist, while a unique monograph by Guenet (1992) treated thermoreversible gels. The applications of small deformation rheology and differential scanning calorimetry (DSC) in aqueous polymer solutions and gelation processes have been discussed in detail by K. Nishinari (Nishinari, 1997). The value of loss $\tan\delta$ tends to

infinity for a purely viscous fluid, while it tends to zero for a purely elastic solid (Nishinari, 1997).

There are two accepted methods for the rheological study of crosslinking polymers. In the first method, the polymer in its liquid state is subjected to shear flow. The measured viscosity increases with increasing extent of reaction until the stress reaches the limit of the instrument or until the material breaks. For characterisation beyond the gelling point, the material is subjected to strain and the steady state modulus is measured during its growth with increasing extent of reaction. Measurements in either the liquid state or the solid state give reliable data away from gelling point. The transition itself is defined by a singular behavior, which is not accessible to these experiments except, by extrapolation (see Figure 2.7).

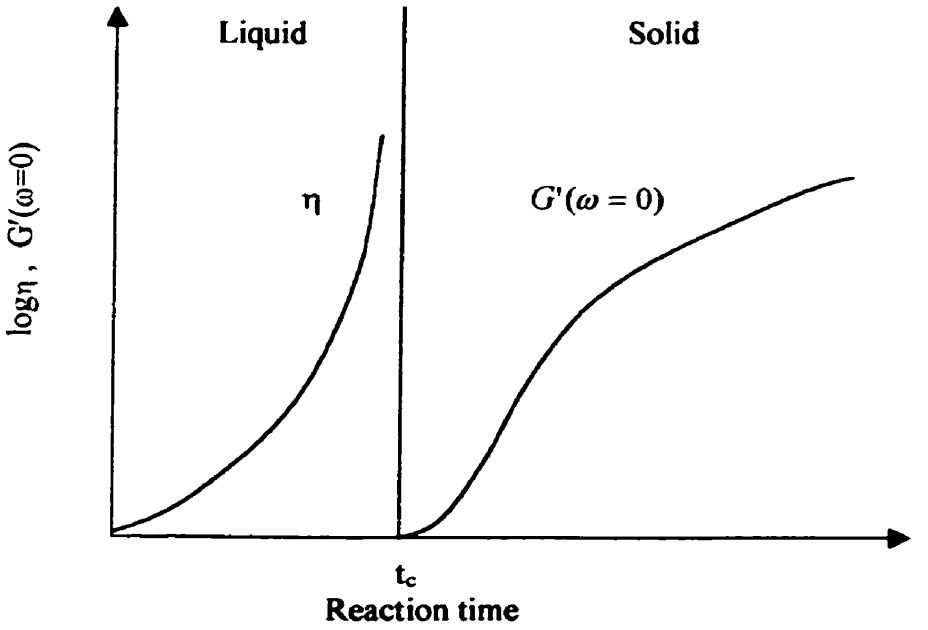


Figure 2.7: Schematic of viscosity and elastic modulus of a gelling polymer. No such experiments are possible in the close vicinity of the sol-gel transition (adapted from Winter, H.H., 1986).

In the second method, small amplitude oscillatory shear gives the components of the complex modulus during the gelation process. The loss modulus, G'', is large while the storage modulus, G' is still negligible. With forming gels, the loss modulus increases while the storage modulus rises sharply until it exceeds the loss modulus. The storage modulus keeps increasing to a high level while the loss modulus tends to a negligible value. The oscillatory shear method gives a continuous evolution of the viscoelastic properties as the polymer goes through its gelling point. The data give no indication of the specific instant at which the phase transition occurs.

Basically, measurements of the oscillatory shear moduli are frequently used to monitor continuously the viscoelastic properties of cross-linking systems from the solution state through the transition to the gel state. In the framework of the generalized rheological model of Chambon and Winter (Winter et al. 1986, Chambon et al. 1987) the gel point is described by the equation, which is a linear viscoelastic constitutive equation for the stress

$$\sigma(t) = S\int_{-\infty}(t-t')^{-n}\dot{\gamma}(t')\mathrm{d}t' \qquad (2.5)$$

where σ is the shear stress, $\dot{\gamma}(t')$ $(-\infty < t' < t)$, is the rate of deformation of the sample at gel point, t is the present time , S is the gel strength parameter depending on the cross-linking density and the molecular chain flexibility , and n is the relaxation exponent. The material parameters S (Pa·s^n) and n are characteristic parameters for each gel and n must be greater than 0 and less than 1. This equation (only valid at gel point) has been found to predict all known rheological properties of critical gels of both chemical and physical origin. It should be noted that the gel equation is restricted to small strains only. It was shown that the shear relaxation modulus $G''(t)$ is characterized by a power-law in time at gel point: $G''(t) = St^{-n}$. This situation, when an incipient gel forms, represents an intermediate state between a liquid and a solid. The same scaling behavior is also

apparent in dynamic mechanical experiments where the storage modulus, G', and the loss modulus, G'', at gel point are given by:

$$G' = G''/\tan\delta = S\omega^n \Gamma(1-n)\cos\delta \tag{2.6}$$

where $\Gamma(1-n)$ is the Legendre gamma function. The phase angle (δ) between stress and strain is independent of frequency (ω) but proportional to the relaxation exponent:

$$\delta = n\pi/2 \quad \text{or} \quad \tan\delta = G''/G' = \tan(n\pi/2) \tag{2.7}$$

Equation 2.6 suggests that gel point is characterized by following scaling relation:

$$G' \sim G'' \sim \omega^n \tag{2.8}$$

Also, based on functions of a complex variable (The Laplace variable $s = \alpha + j\omega$), $G(j\omega) = G(s)$ with $s \rightarrow j\omega$, and $G' = \mathrm{Re}[G(j\omega)]$ $G'' = \mathrm{Im}[G(j\omega)]$. With the hypothesis: $G' \sim G'' \sim \omega^n$, one requires $G(s) \propto s^n$. Thus G′ = G″ which is $\mathrm{Re}[G(j\omega)] = \mathrm{Im}[G(j\omega)]$ at gel point. This latter function require n=1/2. A number of theoretical models have been advanced to predict the numerical value of n. A simple electrical analogy and an effective medium theory predict n = 0.5, as for regular and nonfractal RC (resistor-capacitor) line response (Kirkpatrik, 1973). On the other hand, based upon a suggested isomorphism between the complex modulus and the electrical conductivity of a percolation network with randomly distributed resistors and capacitors, a value of n = 0.72 has been predicted. Computer simulations, performed in three dimensions, based on this analogy yielded a value of n = 0.7 (Nyström et al., 1995). Notably these latter models don't have $G' \sim G'' \sim \omega^n$ and $G' = G''$ as a basic hypothesis.

CHAPTER 3 - EXPERIMENTAL SECTION

The experimental section comprises five major steps:

- Preparation of chitosan: 1) Deacetylation, 2) Purification.
- Determination of the degree of deacetylation (DDA).
- Measures of the pH value of the solution and analysis of its effect on the gelation properties.
- Rheological measurements during the gelation process.
- Thermal analysis using Differential Scanning Calorimetry (DSC).

3.1 Materials

Chitosan of various purity grades, degree of deacetylation and average molecular weight is commercially available and was purchased from several suppliers (See table 3.1).

β-Glycerophosphate disodium salt, F.W.216.0, H_2O content 4.5 mol/mol (Sigma Chemical Co.. Lot 48H5400).

Hydrochloric acid, 37%, F.W.36.46, d 1.190, A.C.S. reagent (Aldrich Chemical Company, Inc.. Cat. No. 32,033-1).

Hydrochloric acid volumetric standard (0.1038M), F.W.36.46, d 1.000 (Aldrich Chemical Company, Inc.. Cat. No. 31,896-5).

Acetic acid, 99.9%, F.W.60.05 (J.T.Baker, Inc..Cat. No. 9508-03).

DL-Lactic acid, 88%, F.W.90.08 (A & C, Ltd.. Cat. No. L-101).

D-Gluconic acid, 50% (w/w) solution in water, F.W.196.2 (Sigma Chemical Co.. Lot 87H0099).

D-Glucuronic acid, 98%, F.W.194.14, m.p.159-161°, $[\alpha]^{20}$ + 36° (c=3, H_2O) (Aldrich Chemical Company, Inc.. Cat. No. 27,163-2).

Sodium hydroxide, pellets, 97 + %, F.W.40.00, m.p.318°, d 2.130 (Aldrich Chemical Company, Inc.. Cat. No. 22,146-5).

Table 3.1: Chitosan Source

No.	Name	Generic Name	Batch No.	Viscosity mPas	DDEA %	Producer
1	Chitosan HMW		41,941-9	361.2	81	Aldrich
2	Chitosan MMW		44,887-7	316.0	81	Aldrich
3	Chitosan LMW		44,886-9	76.0	81	Aldrich
4	Chitosan		777956	36.0	95	Maypro Ind. Inc.
5	Chitosan		98-ASDQ-0551	26.0	88.2	Maypro Ind. Inc.
6	Chitosan		98-ASSQ-0537		86.2	Maypro Ind. Inc.
7	Chitosan		98-ABSB-0595	651.0	83.7	Maypro Ind. Inc.
8	Chitosan		98-ASJQ-0550	23.0	81.2	Maypro Ind. Inc.
9	Chitosan		98-ASCC-0071	1278.0	80.3	Maypro Ind. Inc.
10	Chitosan SC 342		98-ABSB-0102	370.0	85.2	Natural Biopolymer, Inc.
11	Chitosan CL 213		608-783-01	145.0	77	Pronova Biopolymer, Inc.
12	Chitosan CL 214		607-783-08	171.0	89	Pronova Biopolymer, Inc.
13	Chitosan CL 311		306-492-02	247.0	63	Pronova Biopolymer, Inc.
14	Chitosan CL 311		707-771-10	202.0	60	Pronova Biopolymer, Inc.
15	Chitosan CL 312		408-782-07	228.0	73	Pronova Biopolymer, Inc.

Potassium hydroxide volumetric standard (0.1018M), F.W.56.11, d 1.000 (Aldrich Chemical Company, Inc.. Cat. No. 31,932-5).

Acetone, 99.5%, F.W.58.08 (A & C, Ltd.. Cat. No. A-145).

Ultra pure dionized water (18.2 MΩ·cm)

3.2 Preparation of chitosan

3.2.1 Alkaline deacetylation

In typical experiment, 10g of chitosan was soaked in 100ml of 50% w/v NaOH solution at room temperature, and autoclaved at a constant temperature, 121 or 133°C, for a certain time. The alkali treated sample was then washed with deionized water until pH value is below 7.5, and dried. Highly deacetylated chitosan was obtained. Different samples listed in Table 3.1 were used in this experiment.

3.2.2 Purification

10g of chitosan was dissolved in 1000ml of HCl (0.1M). After stirring at room temperature, clear solution of chitosan (1% w/v) was obtained and subsequently filtered with a sintered glass filter (40~60μm), dialyzed against pure water for 24 hours to remove microions with dialysis tubing (Fisherbrand Co.: #21-152-6; flat width: 50mm; wall thickness: 30μm; dry cylinder diameter: 31.8mm; nominal MWCO: 6000~8000), precipitated with NaOH 0.1M, filtered, washed with acetone, and dried in *vacuo*. After the first drying, the powder was rinsed with deionized water until neutral, then it was washed with acetone again and dried in *vacuo*. A pure chitosan powder was obtained.

Chitosan from Maypro: 98-ABSB-0595 (No.7 in Table 3.1) was chosen for this experiment.

3.2.3 Determination of degree of deacetylation by conductimetric titration

Method 1: Base titration

A quantity of chitosan (150mg~200mg) was dissolved in an excess of HCl solution (0.1038M) 10ml. After stirring, a clear solution was obtained. Follow by using deionized water diluted to 200ml which was contained in plastic beaker, titrated with NaOH (0.1018M) and conductivity was measured. This gives a titration curve has two inflection points; the difference between the two along the abscissa corresponding to the amount of acid required to protonate the amine groups.

Method 2: Acid titration

Chitosan (150mg~200mg) was suspended in 250ml of deionized water which was contained in a plastic beaker, titrated with HCl (0.1038M) and conductivity was measured. One inflection point was obtained from the titration curve. This gives the amount of acid required to protonate the amine groups.

3.3 Influence of pH

3.3.1 Preparation of chitosan solution

Acetic acid solution (0.1M) was prepared using acetic acid anhydride and the pH was adjusted to 4.0 by adding droplets of potassium hydroxide solution (0.1M). Chitosan powder (50mg ~ 200mg) was dissolved in 10 ml of this acetic acid solution under stirring until complete solubility was achieved. Chitosan solutions having chitosan proportions rang from 0.5 to 2.0 % w/v, then the pH values of samples were measured.

3.3.2 Gel formation

Glycerol-2-phosphate disodium salt (β-Glycerophosphate disodium salt) was added and homogeneously dissolved in chitosan solutions at a cold temperature (~4°C) so as to obtain clear chitosan/Glycerol-2-phosphate disodium salt solutions. For a chitosan/Glycerol-2-phosphate disodium salt solution, pH was measured, and then the solution was transferred to glass vials and maintained at 37°C and 60°C. Bulk and uniform gelation was noted within 24 hours at both temperatures for all samples.

Chitosan from Aldrich: 41,941-9 (No.1 in Table 3.1) was chosen for this experiment.

3.4 DSC analysis

A mother aqueous acetic acid solution (0.1M) was prepared. The pH of this solution was adjusted before hand to 4.0 by adding droplets of a 0.1M potassium hydroxide solution. High to medium molecular weight chitosan (MW 340,000-2,000,000) was added in powder form, and dissolved in a volume of the mother acidic solution so as to produce chitosan aqueous solutions with chitosan proportions ranging from 0.5 to 2.0% w/v. Fresh chitosan aqueous solutions were prepared for each experiment. β-Glycerophosphate disodium salt was added and homogeneously dissolved within the chitosan solutions at low temperature (~4°C) so as to obtain clear Chitosan/β-Glycerophosphate solutions. All Chitosan/β-Glycerophosphate solutions were maintained under stable conditions at a low temperature (~4°C). Proportions of the β-Glycerophosphate disodium salt vary from 2.0 to 8.0% w/v.

Pyris 1 DSC (produced by PERKIN ELMEER Co.) was used in this experiment. Cells were made by filling with the solutions, which were prepared as above (The cell type was Al Capsule 50UL + Cover, CTRY ORIG: Switzerland, P/T#: 87530, PART#:

B0169321). The scanning range of temperature was from 15°C to 75°C at different scanning rates. The DSC pan was hermetically sealed to avoid the leakage of water or other volatile ingredients.

Chitosan from Aldrich: 41,941-9 (No.1 in Table 3.1) was chosen for this experiment.

3.5 Rheological studies

3.5.1 Preparation of chitosan/β-GP solutions

Chitosan solutions with concentrations of 0.5~2% w/v were obtained by dissolving a quantity of chitosan into an acid (0.1M). The mixtures were stirred at room temperature for at least 12 hours to ensure complete dissolution. Different quantities of β-Glycerophosphate disodium salt were dissolved in chitosan solutions at ~4°C; homogeneous and optically clear chitosan/β-Glycerophosphate disodium salt solutions were obtained.

3.5.2 Rheological measurement

The rheological properties were studied using a Bohlin CVO rheometer (Bohlin Instruments Ltd.). The measuring system used was C25 concentric cylinders. The sample volume was about 12 ml. Silicone oil was added to surface of the sample to prevent evaporation of solvent.

Strain sweep measurements were made for all samples to determine the maximum strain amplitude for the gel. Above certain strain amplitude the three-dimensional network of the gel is destroyed. So, all measurements of rheological properties were made within the linear region, i.e. just below this maximum strain.

1 Measurement of sol-gel transition temperature

Solution samples (12 ml) prepared as mentioned above were placed between the concentric cylinders. Small deformation oscillation was chosen for this measurement. The frequency was set at 1 Hz, the temperature swept from 5 °C to 75 °C at 1 °C/min. From oscillation measurements, the elasticity modulus, G', and viscosity modulus, G'', (storage modulus and loss modulus) were determined. The sol-gel transition temperature was determined from G' and G'' measurements.

2 Measurement of gelling time

Solution samples (12 ml) prepared as mentioned above were placed between the concentric cylinders. Small deformation oscillation was chosen for this measurements. The frequency was set at 1 Hz, the temperature maintained at constant values of 37°C, 45°C, 51°C and 58°C. From oscillation measurement, the elasticity modulus, G', and viscosity modulus, G'', (storage modulus and loss modulus) were determined. The sol-gel transition time was determined from G' and G'' measurements.

3 *Measurement of gel strength*

Solution samples (12 ml) prepared as mentioned above were placed between the concentric cylinders. A perfect gel was obtained after increasing the temperature to 70 °C at 1 °C/min and keeping it at 70 °C for 30 min then the temperature was decreased at 1 °C/min. After that small deformation oscillation was taken for this measurement. The frequency swept from 0.05 Hz to 50 Hz at a constant temperature. From this measurement, the elasticity modulus, G', and viscosity modulus, G'', were determined at different conditions. Values of G' were used as a measure of gel strength.

4 Determination of gelation point

Solution samples (12 ml) prepared as mentioned above were placed between the concentric cylinders. Small deformation oscillation was chosen for this measurement. The frequency was set between 0.5 Hz to 2.5 Hz and the temperature swept from 5 °C to 75 °C at 1 °C/min. From oscillation measurements, the elasticity modulus, G', and viscosity modulus, G'', (storage modulus and loss modulus) were determined. A gelation point is defined in rheological terms as a preparation where G' and G'' are frequency dependent and loss tan δ ($\tan\delta = G''/G'$) is independent of all frequencies (Nishinari, 1997; Nyström et al., 1995).

CHAPTER 4 - RESULTS AND DISCSSION

4.1 Deacetylation of chitosan

The deacetylation reaction, as one of the main reactions performed on chitin or chitosan, consists of the substitution of acetyl group by a hydrogen atom. Complete deacetylation is rarely achieved nor is it normally necessary since solubility in dilute aqueous acids is obtained at an extent of DDA of ~60% or above.

The most commonly used method for the deacetylation of chitin or chitosan is the treatment with highly alkaline aqueous solution, but no standard conditions have yet been established. The most frequently used alkali is NaOH, but KOH has been used in some instances and LiOH, $Ca(OH)_2$ and Na_3PO_4 have also been claimed to be suitable (Rigby, US patent 2,040,879; UK patent 458,839). These two early patents established the main principles of the process. They stated that the extent of deacetylation is governed by the alkali concentration, the temperature, the time of reaction, and the particle size and density. The higher the concentration of the alkali used the lower the temperature and/or the shorter the time of treatment required.

A typical deacetylation process involves the use of 50% wt% NaOH solutions with a ratio of 10:1 in volume of NaOH solution/weight of chitosan at different temperatures. In this study, the objective of deacetylation was to provide a highly deacetylated chitosan (DDA 90 ~ 95%) by treating a moderately deacetylated chitosan (DDA $\leq$ 82%) The resulting polymer has proven to be of great interest. Table 4.1

Table 4.1: The effect of time and temperature on the deacetylation of chitosan

Temperature (°C)	122			133		
Time (min)	30	40	45	30	40	45
DDA (%)	85	86	86	90	91	91

summarizes the results obtained with chitosan (HMW, DDA 81%) from Ardrich. The higher the temperature and/or the longer the time of treatment is chosen, the higher the DDA of chitosan is obtained. In general, alkaline deacetylation of chitosan proceeds rapidly until the polymer is about 75~85% deacetylated at certain temperature, after which further treatment has only a very limited effect on the extent of deacetylation. The likely explanation states that chitosan adopts a morphology which limits the access of the base to the remaining amide groups.

4.2 Determination of DDA by conductimetric titration

Figure 4.1 shows a typical titration curve of chitosan dissolved in excess HCl (0.1038N) and then titrated with NaOH (0.1018N). This method has been used by a number of workers, but its precision has been questioned because of the tendency towards gel-like precipitation of the chitosan at pH around 6.2, close to chitosan pKa. The results are compared to NMR measurements (adapted from chitosan supplier) in Table 4.2. Determination of the degree of deacetylation by NMR spectroscopy is most accurate.

Table 4.2: Titration ressults in comparisions with NMR

Chitosan	DDA (%)		
	NMR	Base-titration	Acid-titration
Aldrich 9012-76-1	81	72.2	81.2
MayPro 98-ABSB-0595	83.7	75.9	83.5
MayPro 98-ASDQ-0551	88.2	80.3	88.8
MayPro 777956	95		95.7

In the first method, chitosan is dissolved in a known excess of acid and the solution is then titrated potentiometrically with NaOH. This gives a titration curve

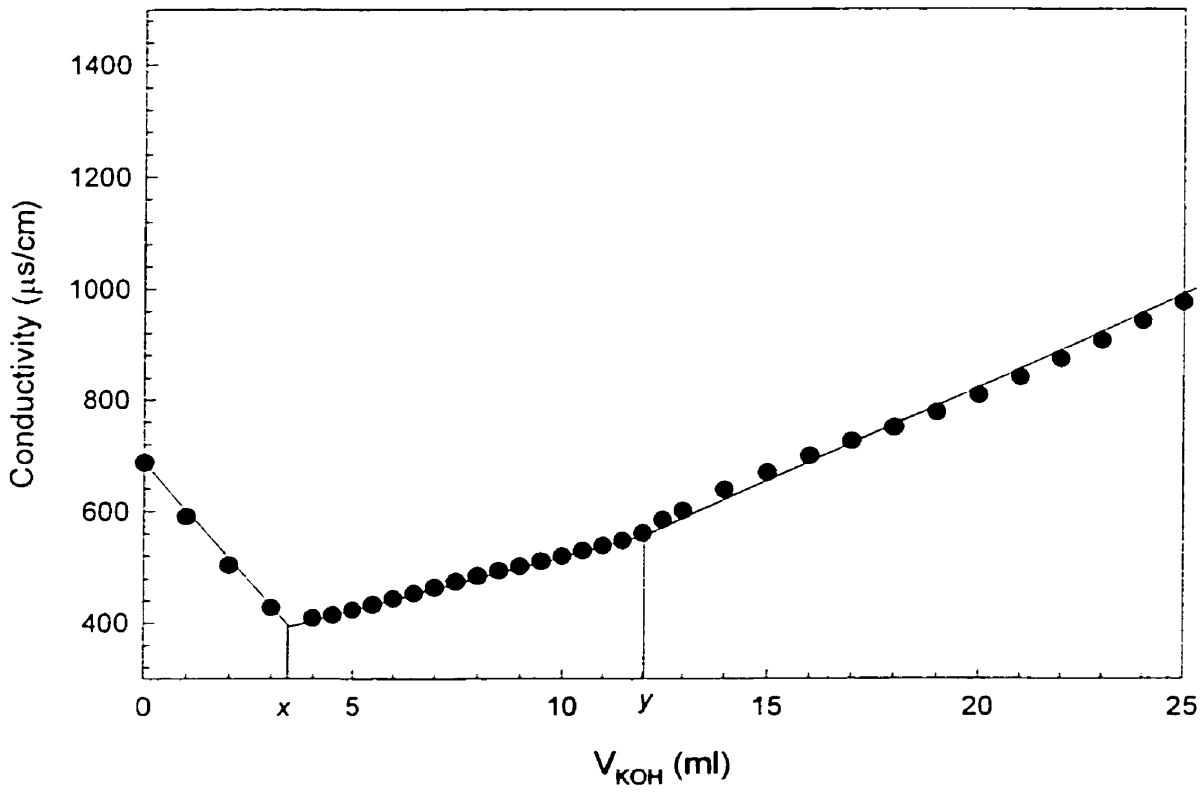


Figure 4.1: Titration curve of chitosan dissolved in excess HCl (0.1038N) and then titrated with NaOH (0.1018N)

(Figure 4.1) having two inflection points, the difference between the two along the abscissa corresponding to the amount of acid required to protonate the amine groups. The amine group concentration is determined. To make the calculation simple

$$DDA = \frac{concentration\ of\ deacetylated\ group}{concentrtion\ of\ deacetylated\ group + concentration\ of\ acetylated\ group} 100\% \tag{4.1}$$

The molecular weight of the deacetylated monomer is calculated to be 161g/mole and that of acetylated monomer to be 203g/mole. Based on this, we could calculate the molar concentration of these two components according to the following two equations.

$$\text{Mol of deacetylated group} = \frac{(y-x)f}{1000} = \Delta n_{NaOH} \tag{4.2}$$

$$\text{Mol of acetylated group} = \left[m - \frac{161(y-x)f}{1000} \right] \Big/ 203 \tag{4.3}$$

where f = molarity of the NaOH solution, m = weight, in grams, of the sample, x, y, are equivalent volumes of NaOH solution as shown in Figure 4.1, in milliliter, and Δn_{NaOH} is molar number of NaOH. So the DDA can be deduced from equation 4.4 or 4.5.

$$DDA = \frac{203(y-x)f}{1000m + 42(y-x)f} 100\% \tag{4.4}$$

$$DDA = \frac{203\Delta n_{NaOH}}{m + 42\Delta n_{NaOH}} 100\% \tag{4.5}$$

An improved method has been used in which a fine powder of chitosan was progressively dissolved with HCl while the conductivity is monitored as function of the volume HCl solution added as shown in Figure 4.2. Since the consumed amount (inflection point in Figure 4.2) of the acid corresponds to that of glucosamine unit in chitosan, thus the degree of deacetylation can be calculated by equation 4.6

$$DDA = \frac{203 n_{HCl}}{m + 42 n_{HCl}} 100\% \qquad (4.6)$$

where m = weight, in grams, of the sample and n_{HCl} is the molar number of HCl. This method was employed for the determination of the degree of deacetylation of all of the samples involved in this study. Compare these two methods (see Table 4.2), we can see that acid-titration is much more accurate than base-titration, based on comparison to NMR measurement.

4.3 Gelation properties

4.3.1 Effect of pH value

It is well known that the association behavior of semidilute solutions of chitosan can be affected by the pH. As a linear polyelectrolyte, chitosan has both reactive amino groups and hydroxyl groups that can interact with the surrounding environment. Chitosan can be dissolved in aqueous acidic solutions, where the protonation of the amino groups promotes electrostatic repulsion between charged chains. Indeed, at pH higher than about 6.2, chitosan in solution carries a small amount of positive charges along its backbone. At pH values below 4, nearly all of amino groups of chitosan are expected to be protonated, leading to enhanced solubilization. Neutralization of chitosan aqueous solutions, to a pH exceeding 6.2, which is close to the pK_a of the amino group, systematically leads to a phase separation or the formation of a hydrated gel-like precipitate. This incapacity to

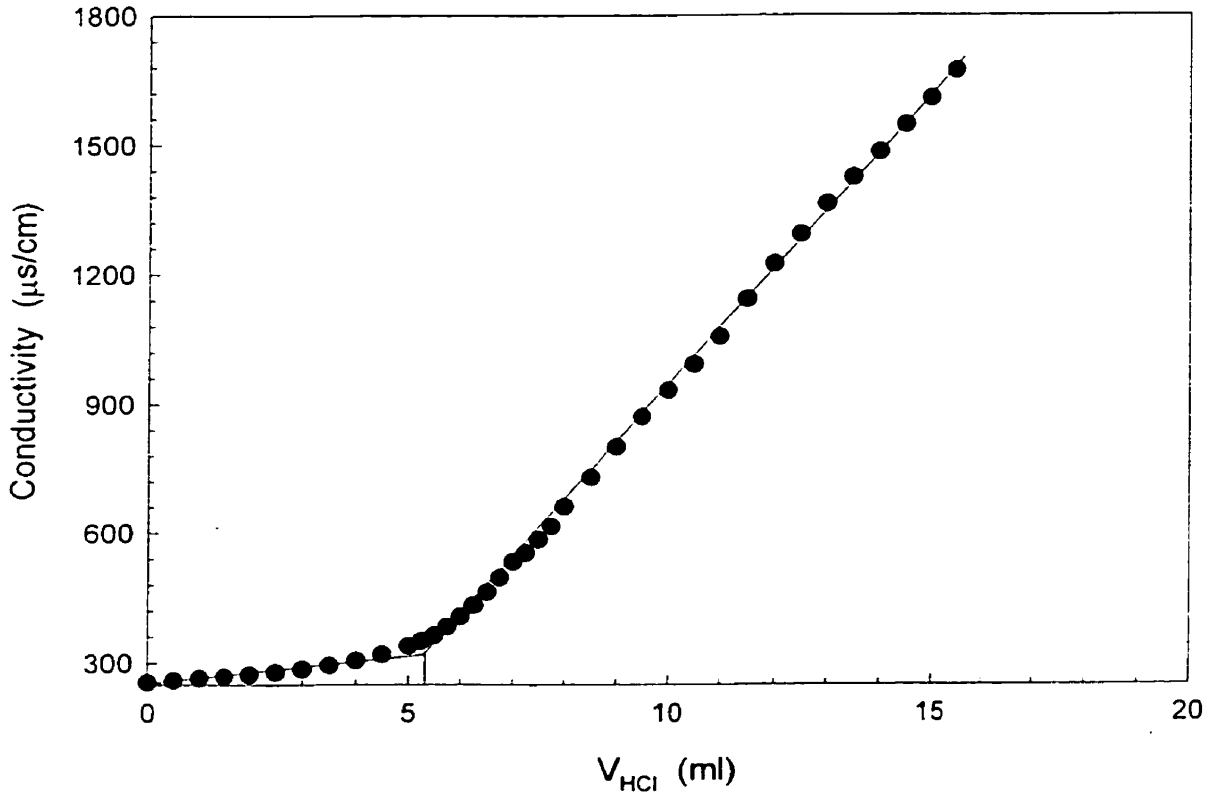


Figure 4.2: Titration curve of chitosan with HCl (0.1038N)

maintain chitosan in solution at pH above 6.2 represents the main obstacle towards chitosan's use as an encapsulating matrix for living cells and proteins. Therefore, the development of chitosan-based systems able to remain liquid at physiological pH and room temperature is of great interest for biomedical applications. Furthermore, an attractive between chitosan chains would be able to turn into a gel when warmed up to the body temperature (37°C). In order to retain the integrity of chitosan, no chemical cross-links should be involved. Thus, we have focused our effort on the development of chitosan-based systems, meeting these biomedical requirements.

This objective was attained by using β-glycerophosphate disodium salt (β-GP) as a particular additive to the chitosan solution. Addition of this salt has two main effects: 1) increase the pH to the physiological level without immediate gelation at below room temperature, and 2) induces the solution to gel when heated up to body temperature or above. In other words, β-GP as additive has the potential in transforming a pH-dependent chitosan solution into thermally-sensitive auto-gelling chitosan solution.

The gel formation of chitosan/β-glycerol-phosphate disodium salt systems is governed by a delicate interdependence between the temperature and the pH of solutions. Figure 4.3 shows the effect of pH on gelation properties of a typical chitosan (1.5%)/β-GP (8%) solution. The higher the pH of the solution is, the lower the gelation temperature is.

Admixing a glycerol-phosphate disodium salt to a chitosan aqueous solution increases the pH of the solution due to the neutralising effect of the phosphate groups (base). In the presence of this salt however, chitosan solutions remain liquid below room temperature, even with pH values within a physiologically acceptable neutral range from 6.8 to 7.2. These nearly neutral chitosan/β-glycerol-phosphate (C/β-GP) aqueous solutions will gel quickly when heated. Figure 4.4 shows the rheological behavior of a

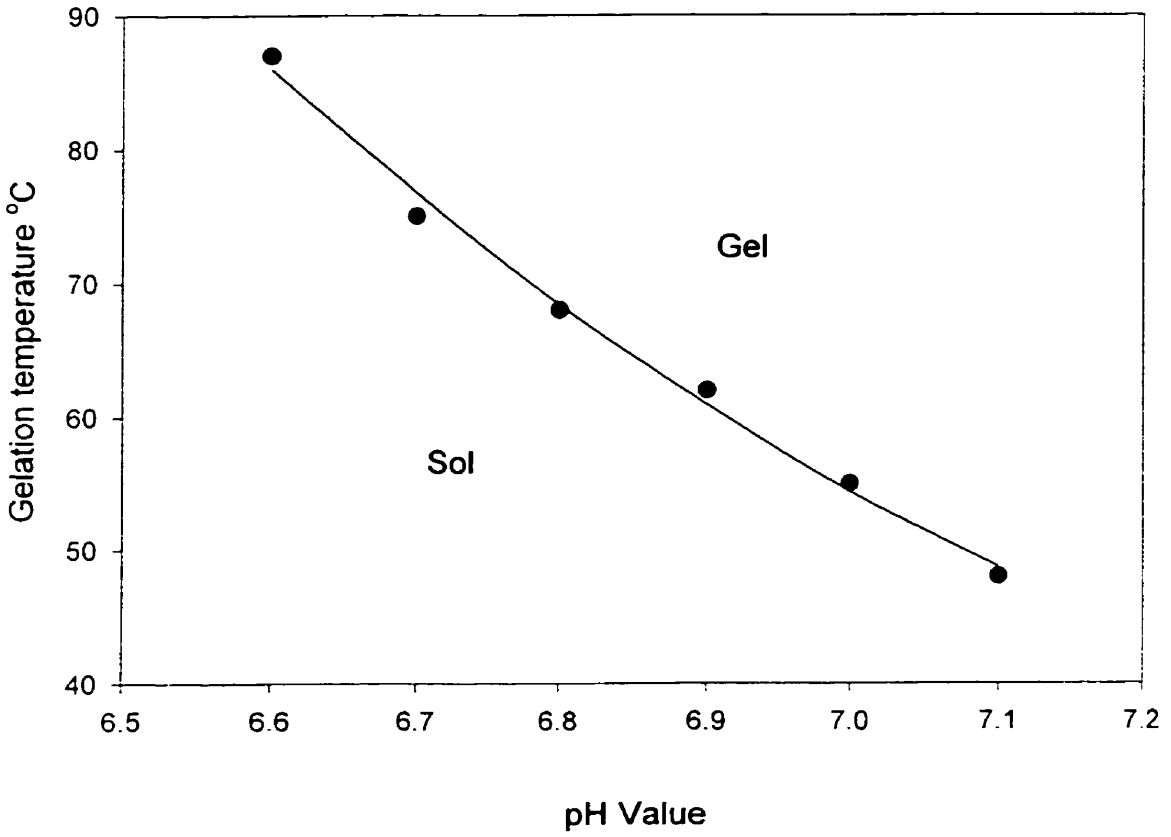


Figure 4.3: Effect of pH value on gelation temperature of chitosan (Aldrich HMW) 1.5% w/v% with β-GP 8% w/v%.

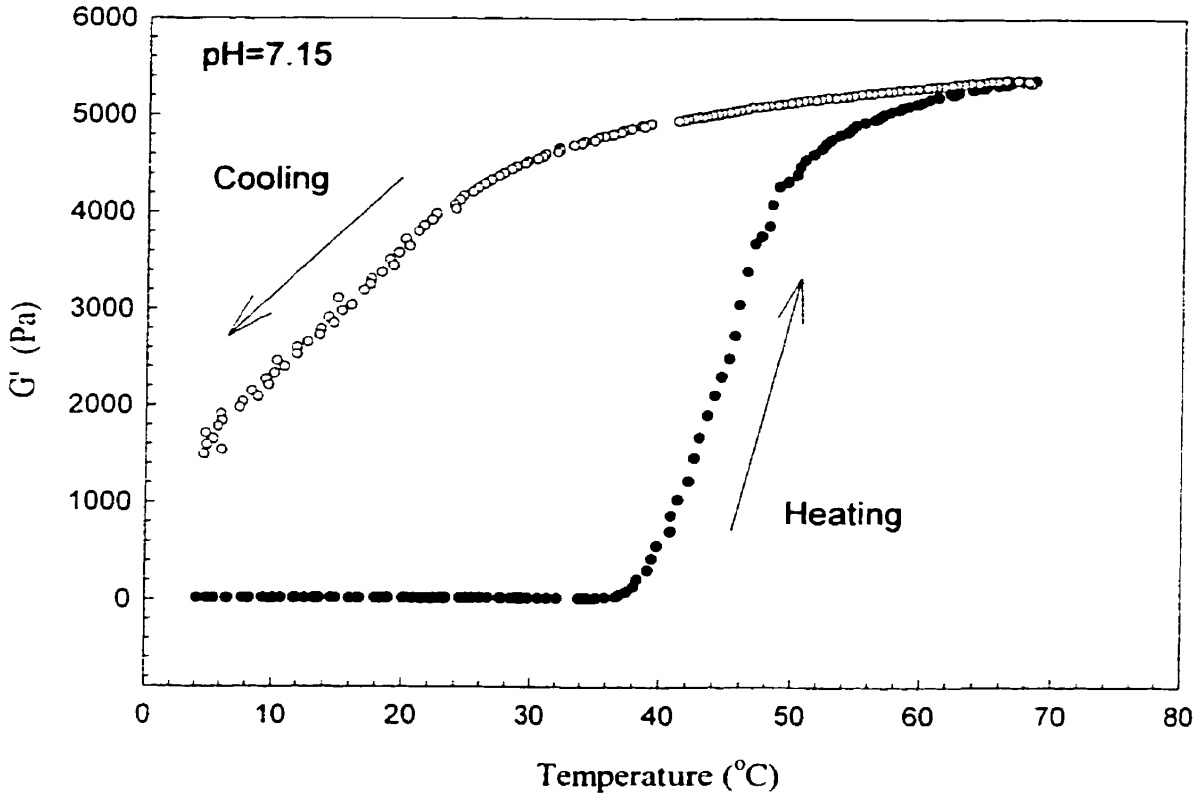


Figure 4.4: Heating and cooling of C/β-GP system at pH=7.15.

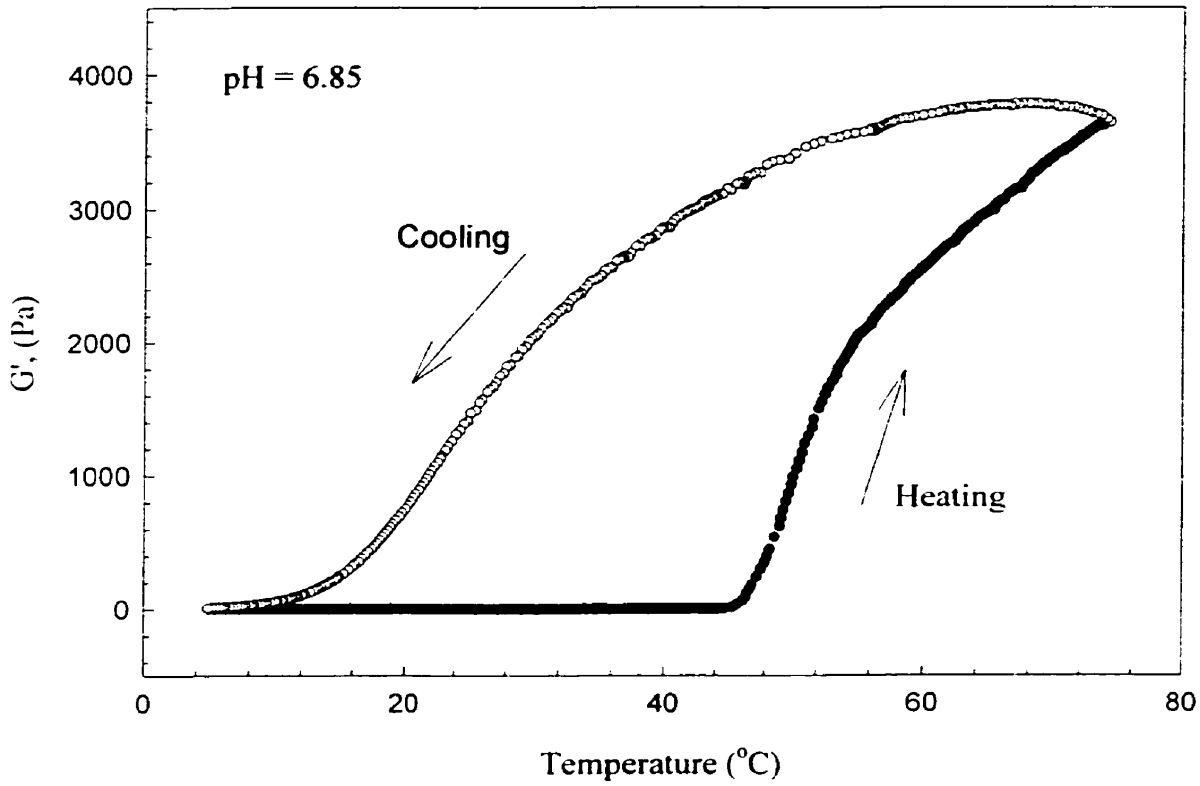


Figure 4.5: Heating and cooling of C/β-GP system at pH=6.85.

typical C/β-GP neutral solution (pH ≈ 7.15), during a heating-cooling cycle between 5°C and 70°C. The sharp rise of the elastic modulus upon heating clearly indicates that the liquid solution turned into a solid-like gel in the vicinity of 37°C, while the decrease of the same elastic modulus upon cooling reveals a tendency of the gel to return to a liquid state. Such a tendency becomes more pronounced for C/β-GP solutions having pH's within 6.5 to 6.9 range, as shown in Figure 4.5.

4.3.2 Mechanism of gelation

The addition of a glycerol-phosphate salt to chitosan aqueous solutions directly modulates electrostatic and hydrophobic interactions, and hydrogen bonding between chitosan chains, which are the main molecular forces involved in gel formation. The effective interactions thought to be responsible for the Sol/Gel transition are: 1) the increase of chitosan interchain hydrogen bonding as a consequence of the reduction of electrostatic repulsion due to the basic action of the salt, 2) the chitosan-glycerol-phosphate electrostatic attractions *via* the ammonium and the phosphate groups respectively and 3) the chitosan-chitosan hydrophobic interactions which should be enhanced by the structuring action of glycerol on water. The nontrivial aspect of such a gelation, most likely originates from the strengthening of chitosan hydrophobic attractions upon increasing the temperature, due to the presence of the glycerol moiety. At low temperatures, strong chitosan-water interactions can protect the chitosan chains against aggregation. Upon heating, sheaths of water molecules can be removed by the glycerol moiety, which in turn allows association of chitosan macromolecules. Thus although electrostatic forces may be modulated by temperature, either via conformation-charge coupling or due to pair correlation of divalent ions (Atala et al., 1993), hydrophobic interactions are expected to play a major role in the gelation of C/β-GP solutions. It should be noted that such a gelation would still not occur without increased attractions through mechanisms 1) and 2), which are present within the C/β-GP solutions, and which also explain the role of the pH in the temperature-controlled gelation of C/β-

GP aqueous systems.

The changes of hydrophilicity/hydrophobicity could also be explained in terms of changes in the polarity of chitosan and water molecules. Glycerol-phosphate induces changes in the polarity of either the polymer (chitosan) or the solvent (water), which results in changes of the effective chitosan-chitosan, chitosan-water and water-water interactions. The gelation of C/β-GP with the elevation of the temperature suggests that the polarity of chitosan macromolecules, and therefore its solubility, is increased at low temperature, probably as a consequence of the structuring effect of glycerol moiety on water molecules.

It is for example experimentally well-known that the ethylene oxide (EO) containing systems becomes less soluble in water at higher temperatures (Saeki et al., 1976). In Poly(ethylene oxide) (PEO), one of the most extensively studied compounds, this behaviour has been attributed to a conformational transition. As a consequence of the equilibrium within the EO chains between polar and less polar conformations, EO-containing compounds become less polar and lose their solubility in water at elevated temperature. Polysaccharides are also considered as EO-containing polymer and some of them, as for cellulosic derivatives, show a decreased solubility in aqueous media when heated up (Karlström et al., 1990).

Similarly, it can be believed that for C/GP systems, the temperature increase favours the less polar conformation and thus allows chitosan-chitosan associations *via* hydrophobic attractions and hydrogen bonding. In fact, the contribution of hydrogen bonding is modulated by the pH. Unlike hydrogen bonds, the strengthening and weakening of hydrophobic attractions with temperature is believed to cause the remarkable thermoreversibility found in C/GP gels.

Thermoreversibility itself of gelation is linked to the pH value attained in C/β-GP solutions before heating and to the fact that cooling after heat-induced gelation weakens hydrophobic forces but strengthens hydrogen bonding. Thus systems having pH between 6.9 and 7.2 before heating appear to be only partially thermoreversible as indicated by rheological measurements presented in Figure 4.4. However complete thermoreversibility is attained for C/GP solutions with pH values ranging from 6.5 and 6.9 (Figure 4.5), suggesting inhibition of hydrogen bond formation at lower pH due to the presence of signification interchain electrostatic repulsion. As consequence of such repulsion forces, the incipient zone of gelation is shifted to a higher temperature (~45°C).

4.4 Rheological studies

4.4.1 Determination of gelling point

The gelling point of a thermoreversible gelling system may be determined by observation of a frequency independent value of tan δ obtained from a multifrequency plot of tan δ versus temperature (Nishinari, 1997; Nyström et al., 1995) as in Figure 4.6. All C/β-GP solutions exhibit a similar pattern of behavior, showing a small decrease in tan δ with increasing temperature before gelling, with the decrease being most significant during gelling process. It turns out that the different curves across at the same point at which the gel point was determined and the gel temperature was identified at the particular temperature where the loss tangent first became frequency independent at the gel point.

In Figure 4.7 the dynamic storage and loss modulus are plotted against frequency at the gel point. At the gel point, G′ and G″ exhibit a power law behavior over the considered frequency range, and G′ > G″. The lines representing the frequency dependencies of G′ and G″ are practically parallel and the resulting values of n′ and n″ are, within experimental error, equal (n′ ≈ n″≈ 0.435).

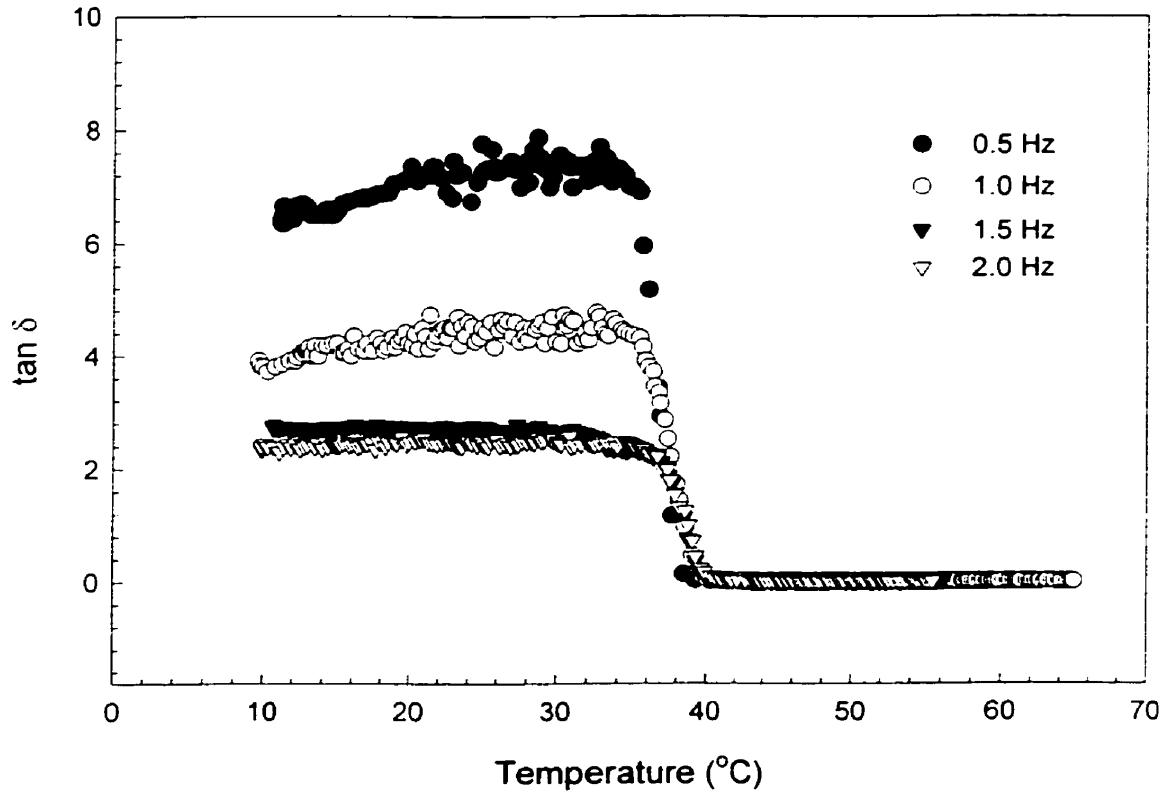


Figure 4.6: Loss tangent as a function of temperature at different frequencies. Chitosan (Maypro, DDA=90) 2%/β-GP 8%.

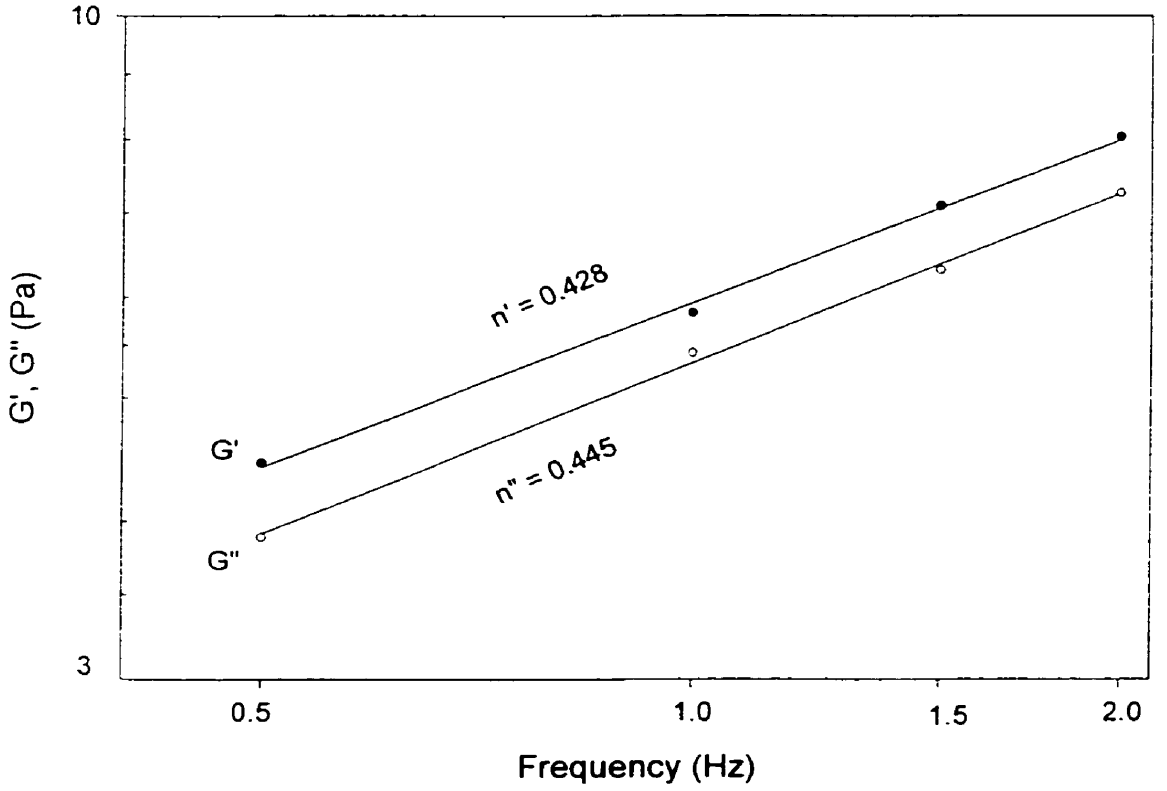


Figure 4.7: Storage and loss moduli are plotted against frequency at the gel point. Chitosan (Maypro, DDA=90) 2%/β-GP 8%.

4.4.2 Time dependence of gelation

When a solution prepared at a non-gelling temperature is kept at a certain gelling temperature, G^* begins to increase with time. The ideal condition of measurement to be adopted should be as gentle as possible that the structure being formed is not broken. A frequency of 1 Hz has been chosen in most cases. Generally, the gelation proceeds faster at higher temperature for C/β-GP system. It is easier to carry out the rheological measurement at temperatures where the gelation proceeds slowly.

In Figure 4.8, Figure 4.9 and Figure 4.10, typical time evolutions of G' and G'' for gelling process of C/β-GP systems at different temperatures and different concentrations of chitosan were shown. The gelation time (which was defined at the crossing point of G' and G'' of rheological measurement) decreased as temperature increased. Solutions at high temperature begin to form a gel at an earlier time and G' increases faster than for solutions at lower temperature. The final saturated value of storage modulus was increased with increasing concentration of chitosan at a constant temperature, after a sufficiently long time. Although the gelation mechanism of C/β-GP systems has not been clarified completely, an essential point is that this polysaccharide forms a gel upon heating by chain association induced by hydrophobic interactions. It is quite clear that hydrogen bonding is not negligible for the gelation process, which is governed mainly by hydrophobic interaction as shown in Figure 4.8. G' is lower when increasing temperature because of hydrogen bonding. The relationship between gelling time, chitosan concentration and temperature is shown in Figure 4.11.

4.4.3 Frequency dependence of spectra

Frequency dependence of G' and G'' shown in Figure 4.12 and Figure 4.13 were observed at 25°C after keeping the temperature for 60 min to reach equilibrium. A 0.2%

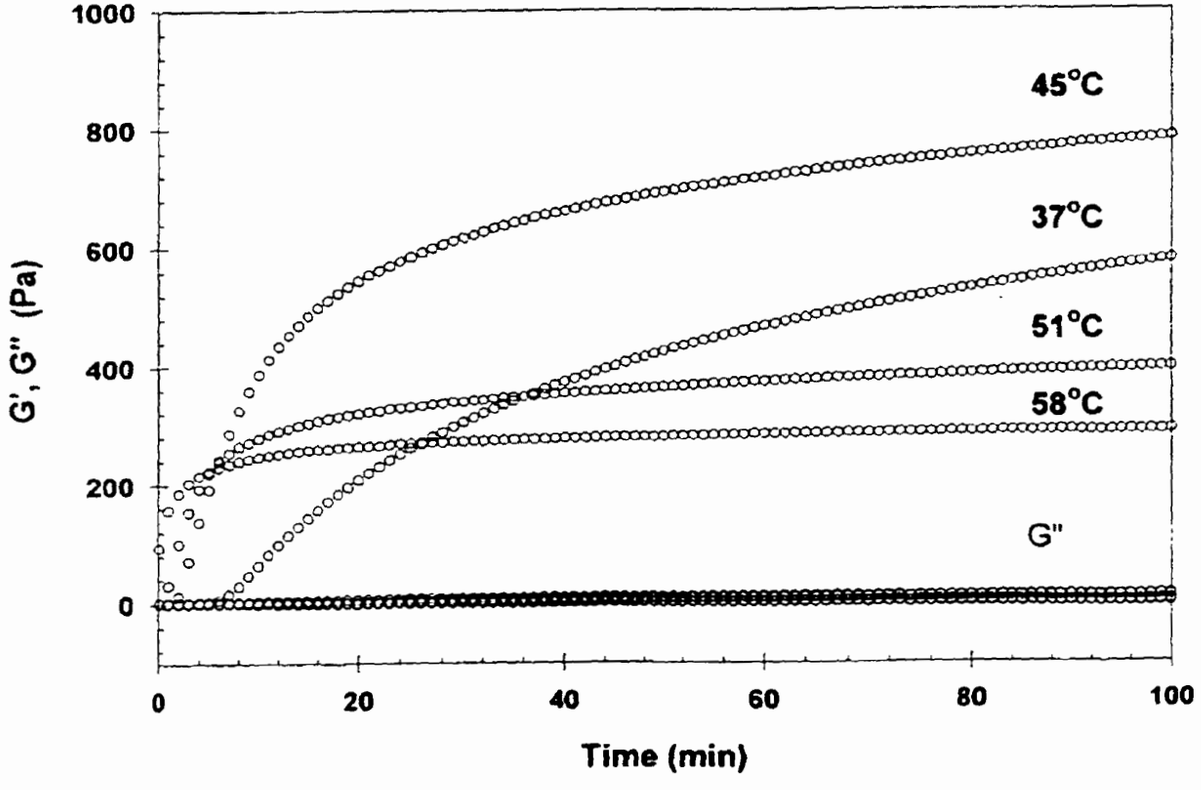


Figure 4.8: Isothermal rheological measurement of chitosan (1%)/β-GP (8%) solution for different temperatures.

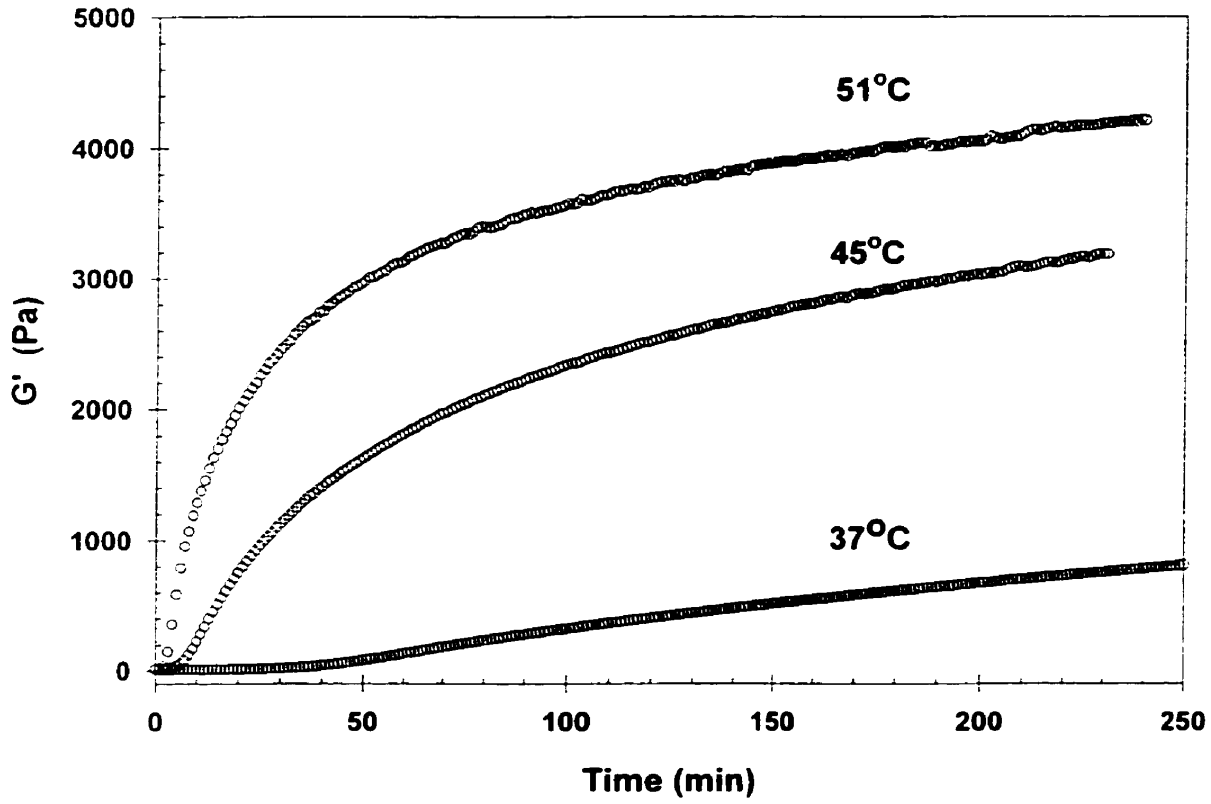


Figure 4.9: Isothermal rheological measurement of chitosan (1.5%)/β-GP (8%) solution for different temperatures.

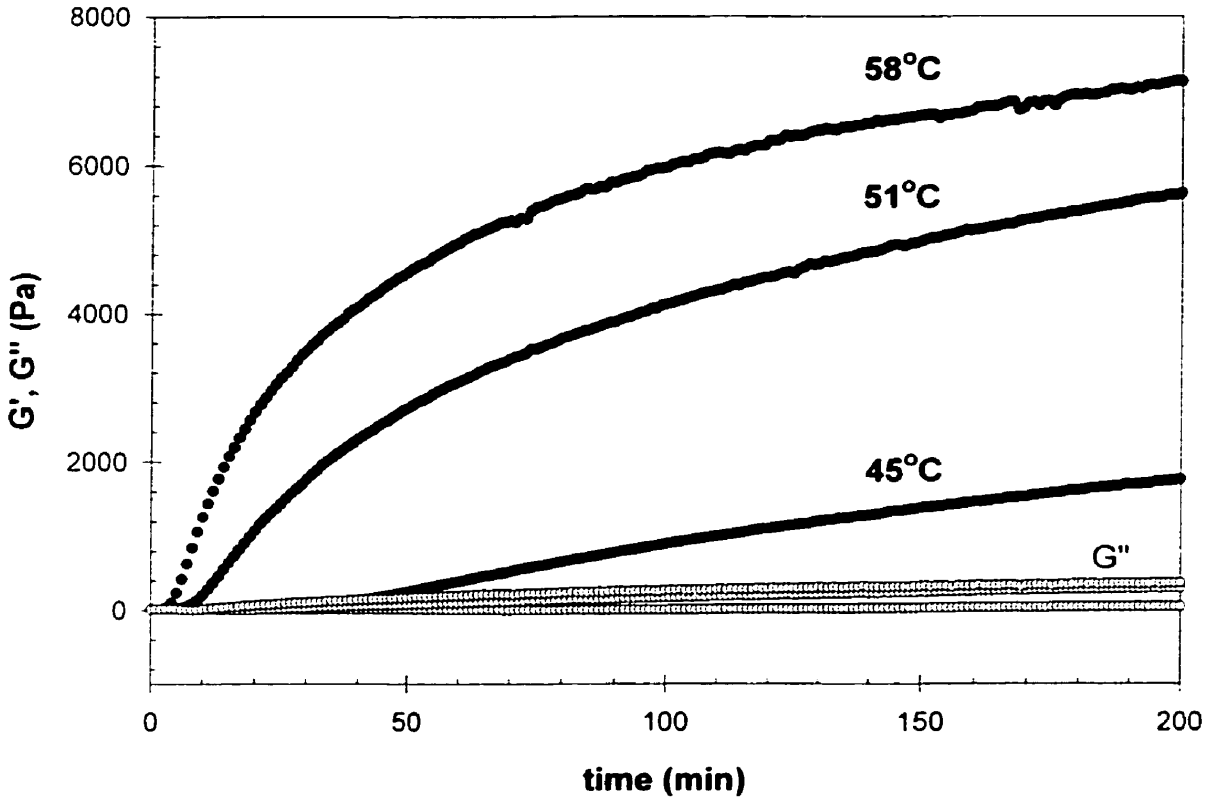


Figure 4.10: Isothermal rheological measurement of chitosan (2%)/β-GP (8%) solution for different temperatures.

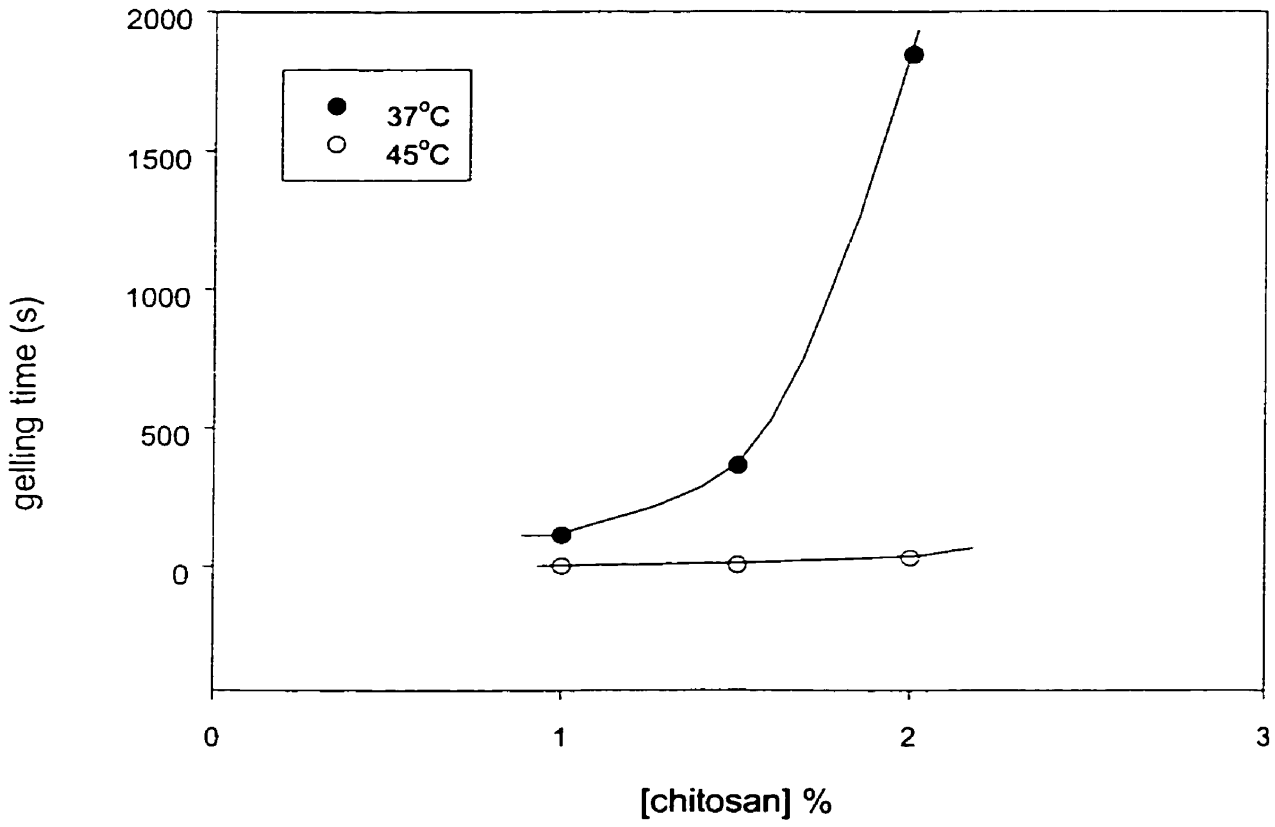


Figure 4.11: Gelling time as a function of chitosan concentration at different temperatures.

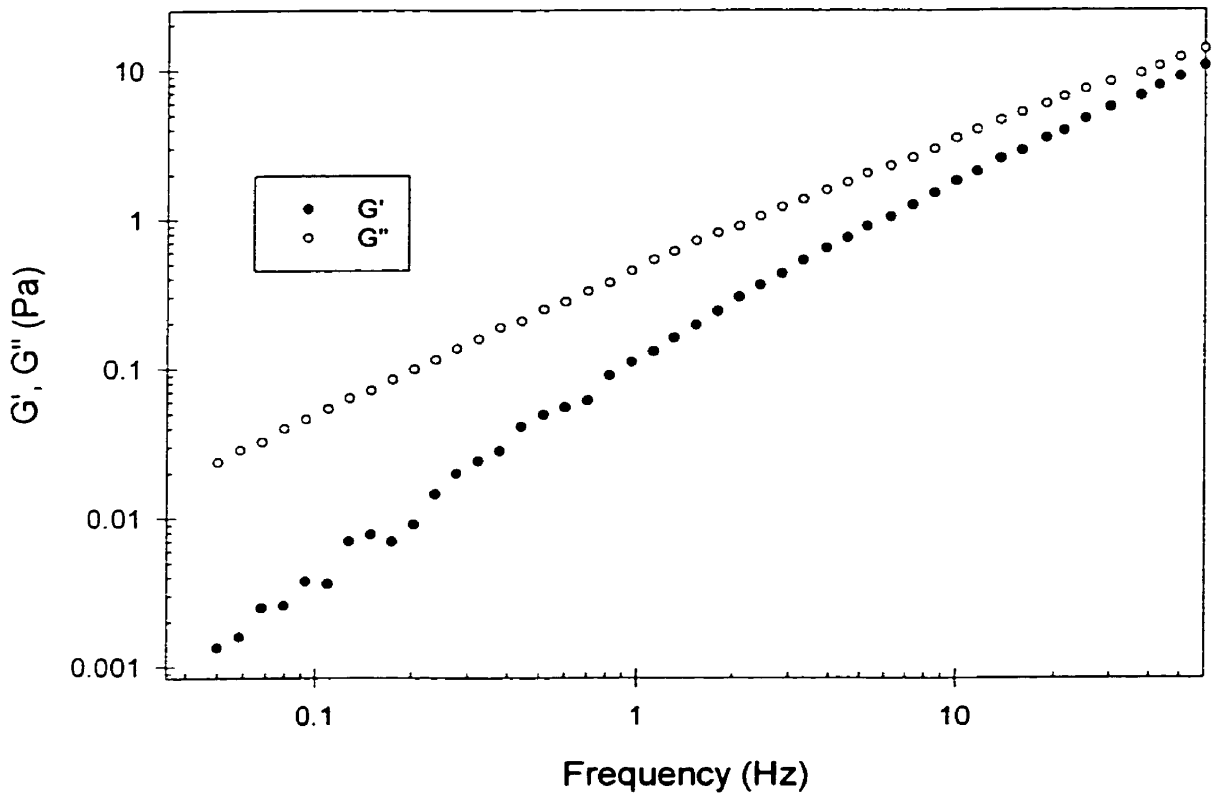


Figure 4.12: Frequency dependence of G' and G'' for 0.2% w/v of chitosan solution at 25°C

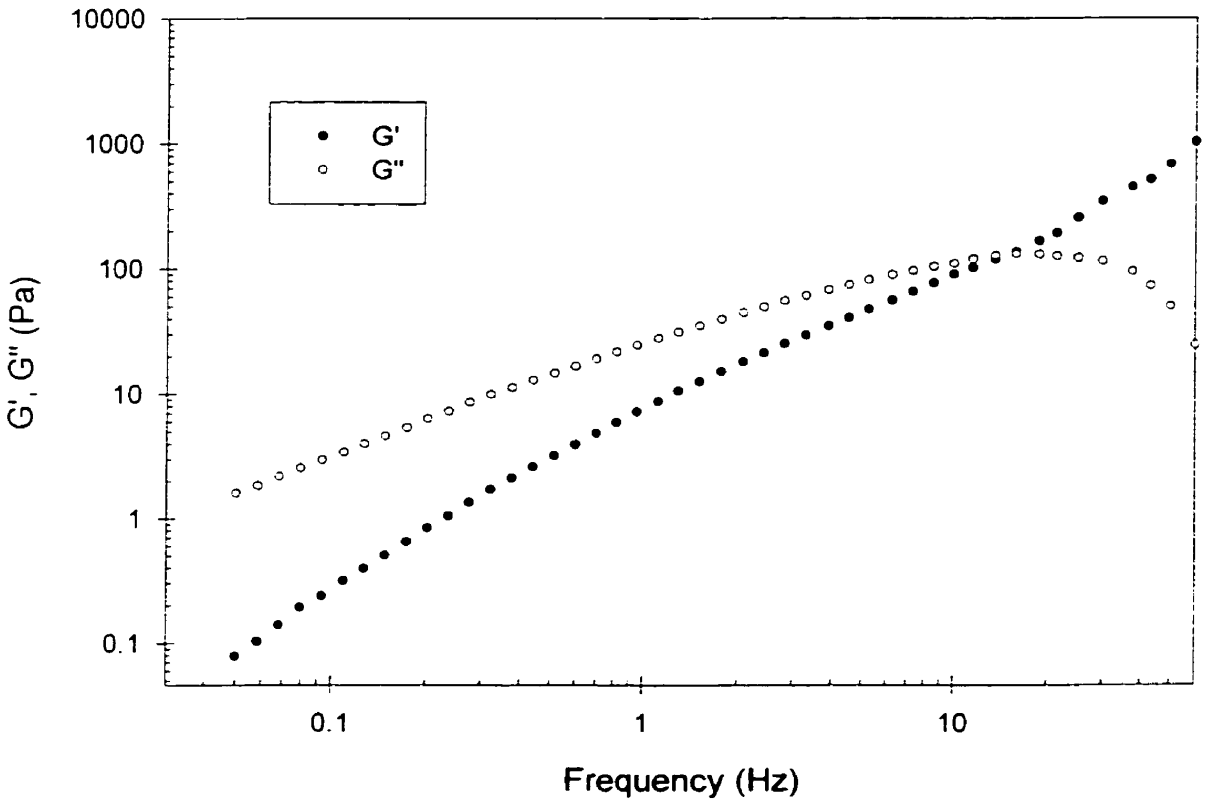


Figure 4.13: Frequency dependence of G' and G'' for 2% w/v of chitosan solution at 25°C

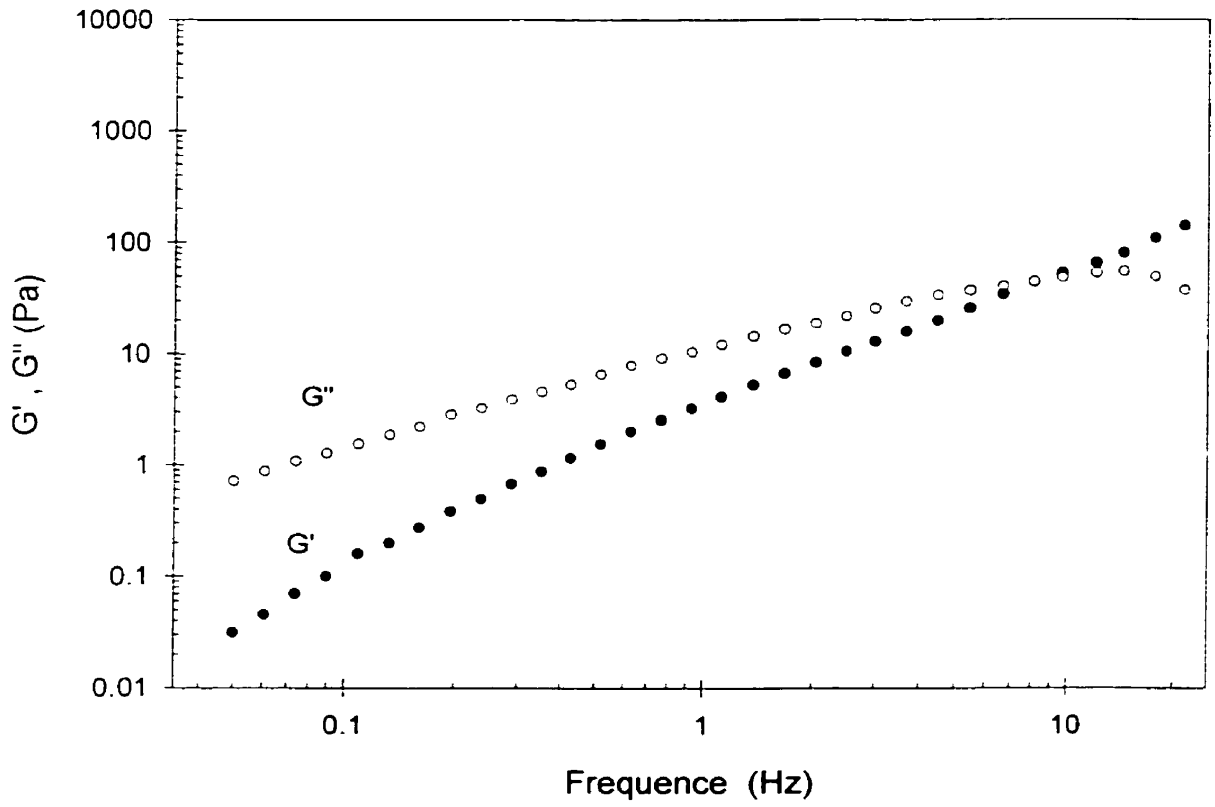


Figure 4.14: Frequency dependence of G' and G'' for chitosan (2%)/β-GP (8%) solution at 25°C

solution of chitosan shows a dilute solution behavior typical of flexible linear polymers, $G'' > G'$ for all accessible frequencies, and both moduli increase with increasing frequencies (Figure 4.12). A 2% solution of chitosan shows a crossover of G' and G'' at 25°C; $G'' > G'$ at lower frequencies but $G' > G''$ at higher frequencies (Figure 4.13). This is typical of so-called concentrated solution behavior. The molecular chains disentangle during a long period of oscillation at low frequencies, and the solution behaves as a viscous liquid, whilst the molecular chains do not disentangle during a short period of oscillation at high frequencies, and their entanglement points play a role of temporary knots of a three-dimensional network, and as a result the solution behavior tends to that of an elastic solid. Comparing a pure chitosan solution (Figure 4.13) with a C/β-GP solution (Figure 4.14), it is quit clear that the viscoelastic properties of the chitosan solution are not changed by adding β-glycerophosphate disodium salt.

The rheological properties of chitosan gels at 37°C at different concentrations are shown in Figure 4.15. The storage modulus G' shows a plateau, a behavior, which has been observed for many elastic gels. All preparations behaved as gels, increasing the concentration of chitosan increased the elastic modulus. In terms of the strength of a thermoreversible gel, it is quite clear that the storage modulus G' has temperature dependence as shown in Figure 4.16 that the storage modulus G' decreased with decreasing temperature.

It is well known that rheological properties of ionic polysaccharides are strongly influenced by the addition of salts. Figure 4.17 shows the mechanical spectra of gels (2% of chitosan solutions in the presence of β-Glycerophosphate disodium salt) at 37°C. Addition of β-Glycerophosphate disodium salt has two main effects as mentioned before: 1) increases the pH to the physiological level without gelation at low temperature, and 2) induces the solution to gel when heating. The anions shield the electrostatic repulsion between amino groups in chitosan molecules, and hence promote the chitosan multichain helix formation and with the association of helices, a three-dimensional gel formed. The

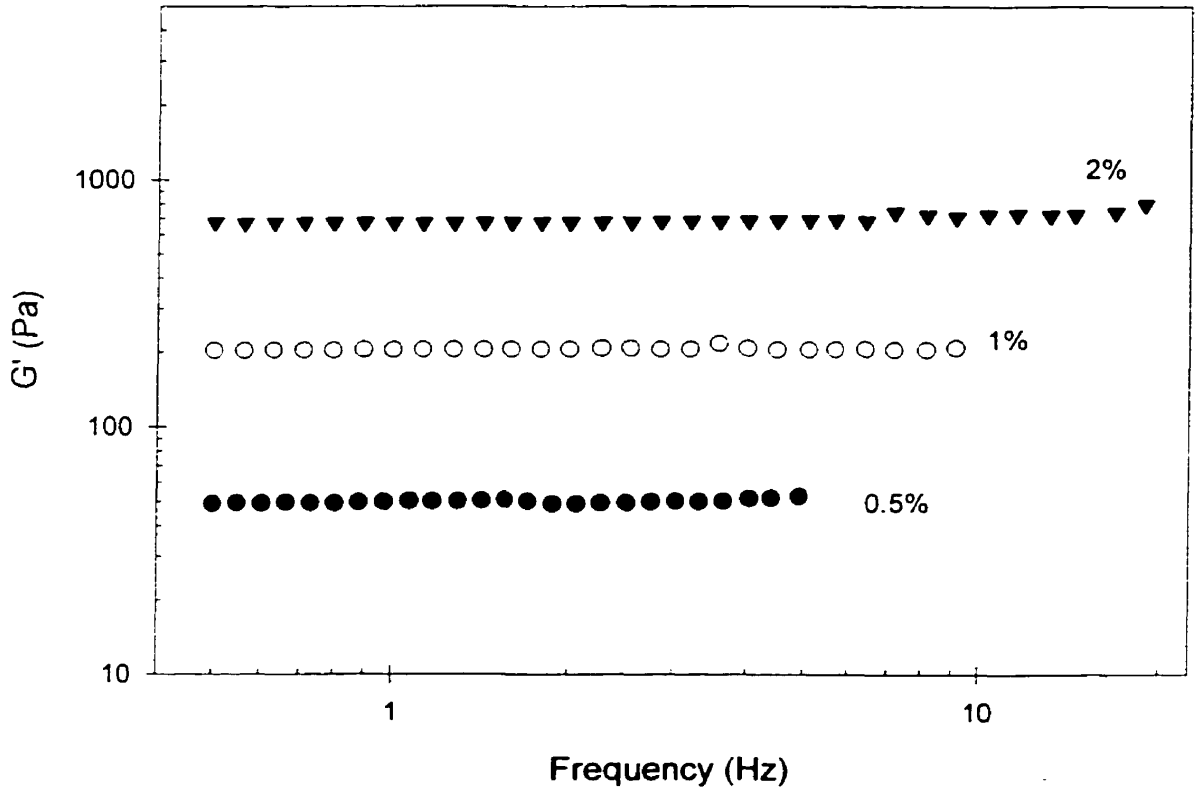


Figure 4.15: The elastic modulus of chitosan gels with different concentrations of chitosan (Aldrich, HMW) versus oscillation frequency at 37°C.

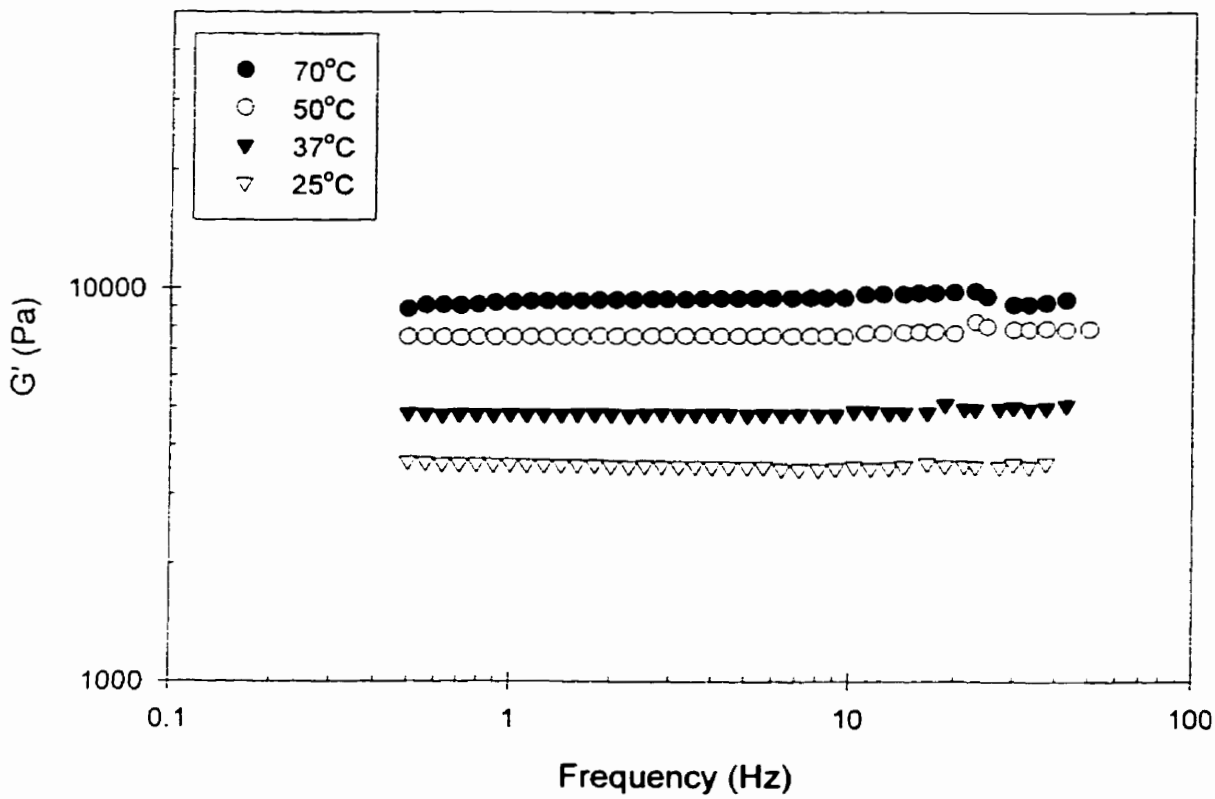


Figure 4.16: The elastic modulus of chitosan (Maypro Ind.) gel (chitosan 2%, β-GP 8%) versus oscillation frequency at different temperatures.

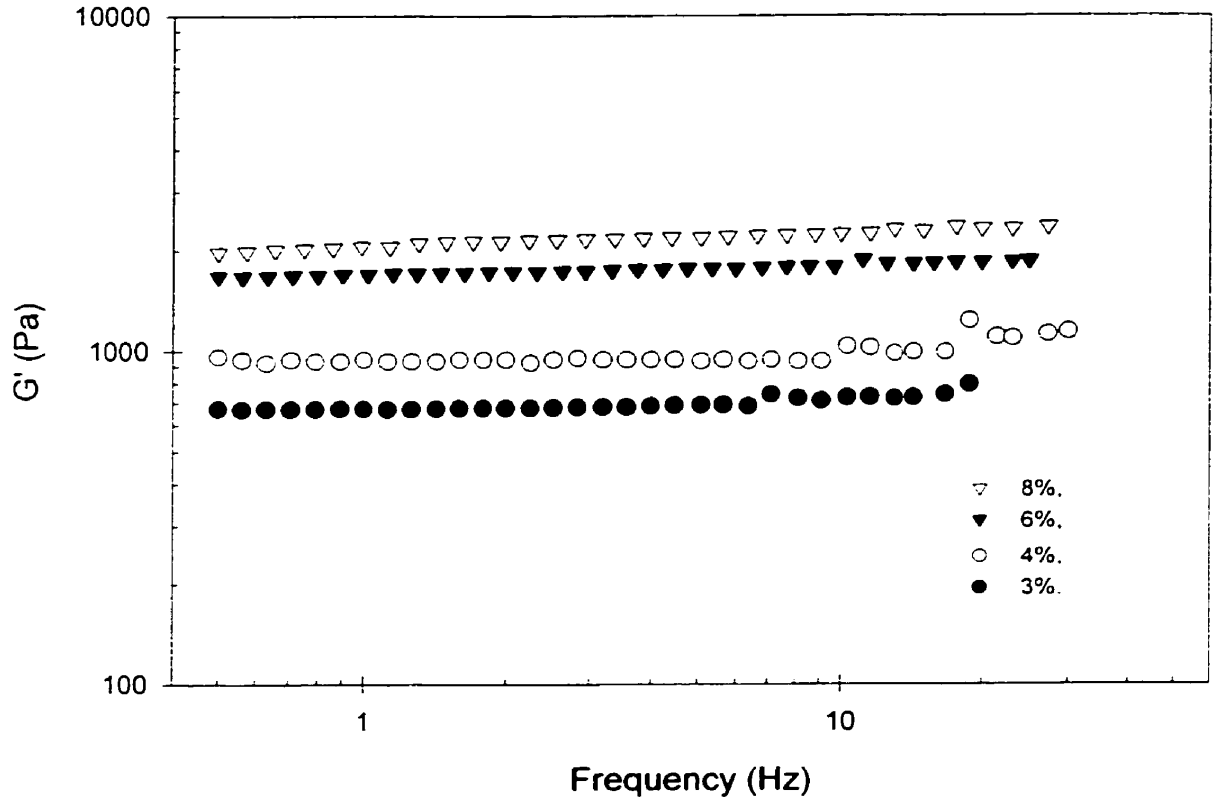


Figure 4.17: The elastic modulus of 2% chitosan (natural biopolymer, DDA≈85) gels with different concentrations of β-GP versus oscillation frequency at 37°C.

higher concentration of β-GP presented, the stronger gel was formed. Therefore, the action of β-Glycerophosphate ions to shield the electrostatic repulsion between cationic groups is more important than the breaking of hydrogen bonds.

4.4.4 Temperature dependence of spectra

For a thermo-setting gelation process such as chitosan/β-Glycerophosphate disodium system, the temperature plays a major role by favoring a less polar conformation and thus allows chitosan-chitosan associations *via* hydrophobic attractions and hydrogen bonding. The temperature scan rate can strongly influence the rheological results. Generally, it should be slow enough to be not too far from equilibrium at any given temperature.

1 Effect of concentration of chitosan and β-GP

Thermal scanning rheological measurements of G' and G'' for a solution of chitosan shows that the elastic modulus, G', is low for a solution and increases drastically with temperature as a result of the gel formation process. The phase transition temperature increases with decreasing concentration of chitosan. The relationship between the sol-gel transition temperature and concentration is shown in Figure 4.18. At high concentrations the preparations would be gels at low temperature. This is presumed that the hydrophobic effect becomes more accentuated with increasing polymer concentration.

The association behavior of chitosan/β-GP system can also be influenced by the concentration of β-Glycerophosphate disodium salt as mentioned above. In this study on semidilute solutions of chitosan, an interaction between chitosan and ionic β-Glycerophosphate was observed. In general, the gel formation is stronger in the presence

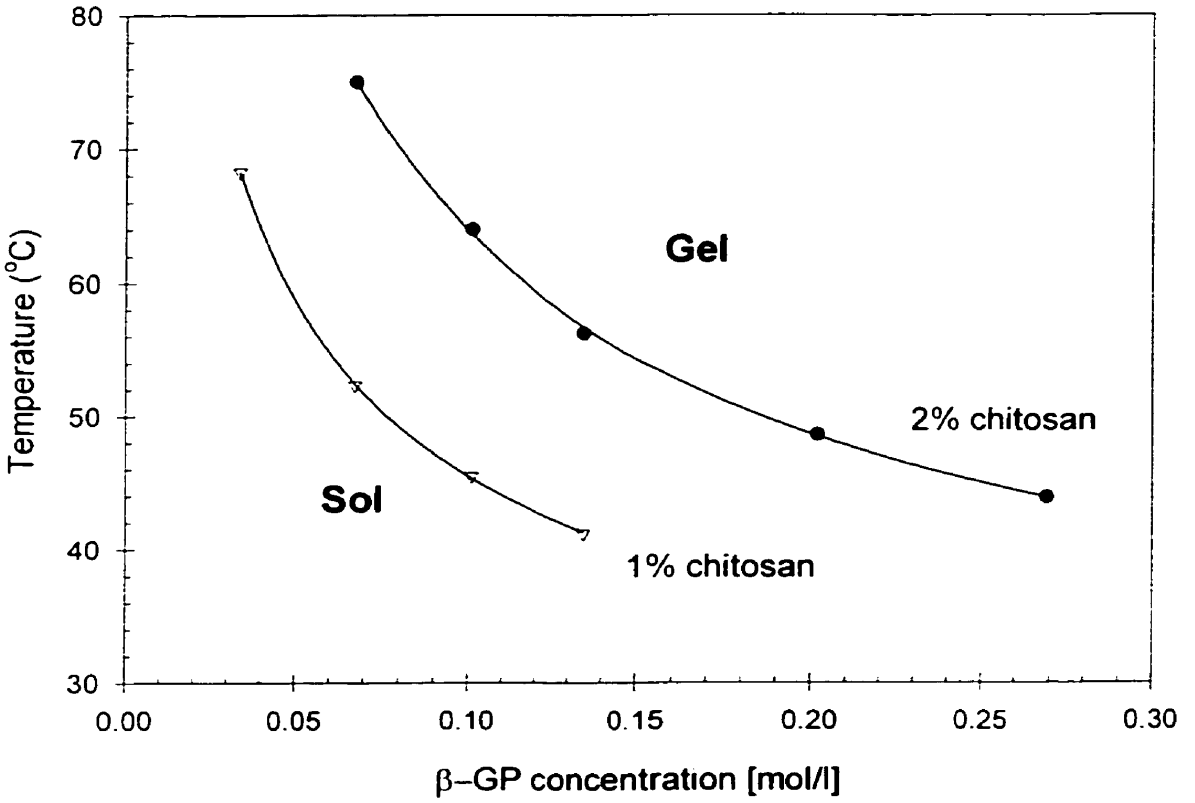


Figure 4.18: Sol-gel transition temperature as a function of the concentration of β-GP in chitosan solution.

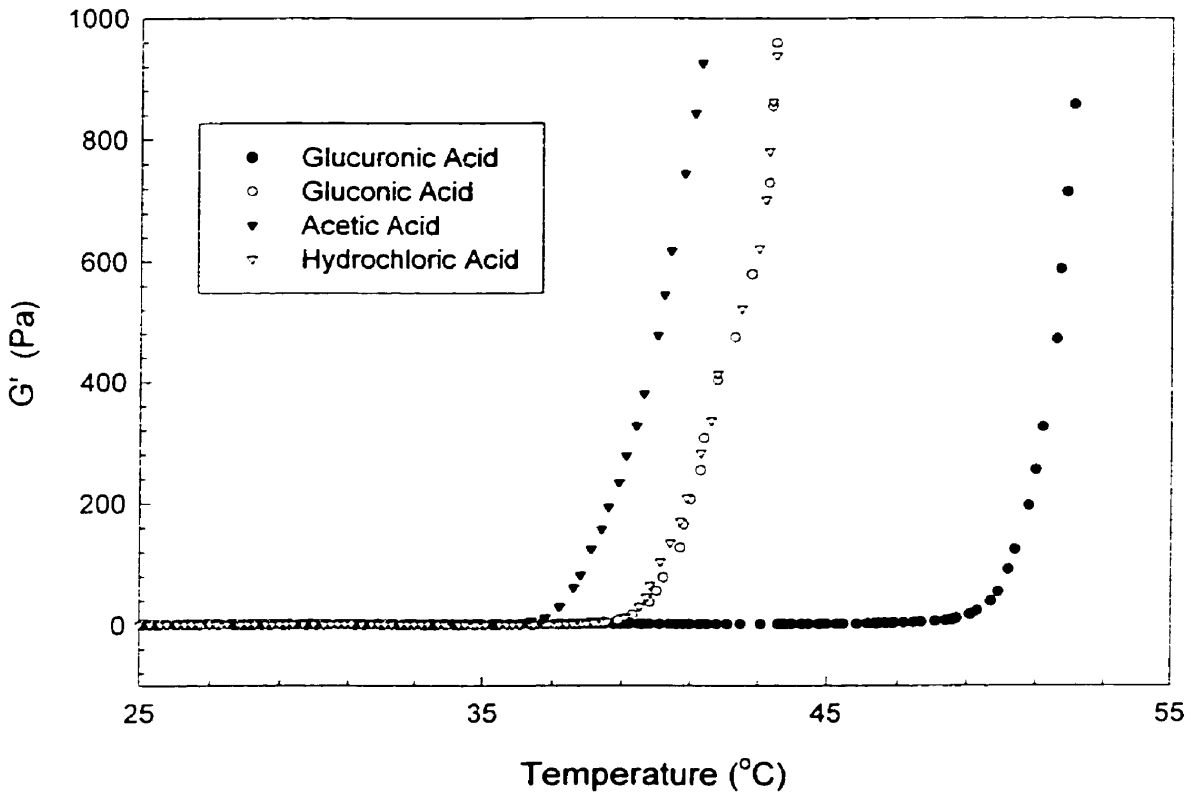


Figure 4.19: Thermal scanning rheological measurements of 2% w/v chitosan (from Ardrich Co., HMW, treated by NaOH 50% w/v) in different solvents results in different sol-gel transition temperatures.

of more β-Glycerophosphate salt, and sol-gel transition temperature is seen to decrease with increasing the concentration of β-GP as shown in Figure 4.18.

2 Effect of solvent

It is known that the sol-gel transition temperature could be changed with different acidic solvent as shown in Figure 4.19. Changing the solvent, the only change in rheology is the change in sol-gel transition temperature. It is assumed that the different steric effect of counter-ions of solvent (Cl^-, CH_3COO^- or $Gluconic^-$) attributed to the different shielding effect on the protonated amino groups. The shielding effect reduced the electrostatic repulsive force between adjacent charged segment.

3 Effect of degree of deacetylation

Because the properties of chitosan solutions are greatly influenced by the characteristics of the particular chitosan, such as the molecular weight and the degree of deacetylation, different samples with various molecular weight and degree of deacetylation have been used to form C/β-GP thermogelling systems at physiological pH. However, it is worth to note that the temperature of the incipient gelation increases as the degree of deacetylation decreases, while the molecular weight showed no significant effect on the temperature of gelation.

The observed effect is that gelation occurs through aggregation of the polysaccharide chains, presumably through hydrophobic interaction. This is aided by the reduction in electrostatic repulsion as decreasing DDA. Thus the gelation process depends upon the DDA of chitosan. Thermal scanning rheological measurements of chitosan solutions in different DDA show sol-gel transition temperature increases with decreasing the DDA of chitosan. The relationship between sol-gel transition temperature and DDA is shown in Figure 4.20.

4.5 DSC study

Differential scanning calorimetry (DSC) has been used to study the sol-gel transition. The heat absorbed per unit time dQ/dt is given by $C_p dT/dt$, where C_p is the heat capacity, T is the temperature, and t is the time. In the heating DSC measurement, dT/dt is positive, and the endothermic peak is equivalent to the maximum of the heat capacity. In cooling DSC measurements, dT/dt is negative, and the exothermic peak is again equivalent to the maximum of the heat capacity. It is generally recognized that an endothermic peak appears when the system changes from the ordered state to the disordered state such as the melting of crystals or the transition from gel to solution, whilst an exothermic peak appears when the system changes from the disordered state to ordered state such as crystallization or gel formation.

Figure 4.21 and Figure 4.22 show the heating DSC curves of 1% w/v and 1.5% w/v chitosan with 8% w/v β-glycerophosphate at different scan rates. Increasing scan rate shifted slightly the endothermic peak to higher temperatures. Comparing these two figures, a similar situation as that of the rheological studies is also recognized for higher concentration solutions have a greater tendency to form a gel than those of lower concentrations, therefore, a concentrated solution showed an endothermic peak accompanying gel formation at a lower temperature than dilute solutions. The endothermic peak temperatures of 1% w/v and 1.5% w/v chitosan with 8% w/v β-glycerophosphate solutions coincided with the solution-gel transition temperatures which were observed by thermal scanning rheology at the same scan rate (Figure 4.23).

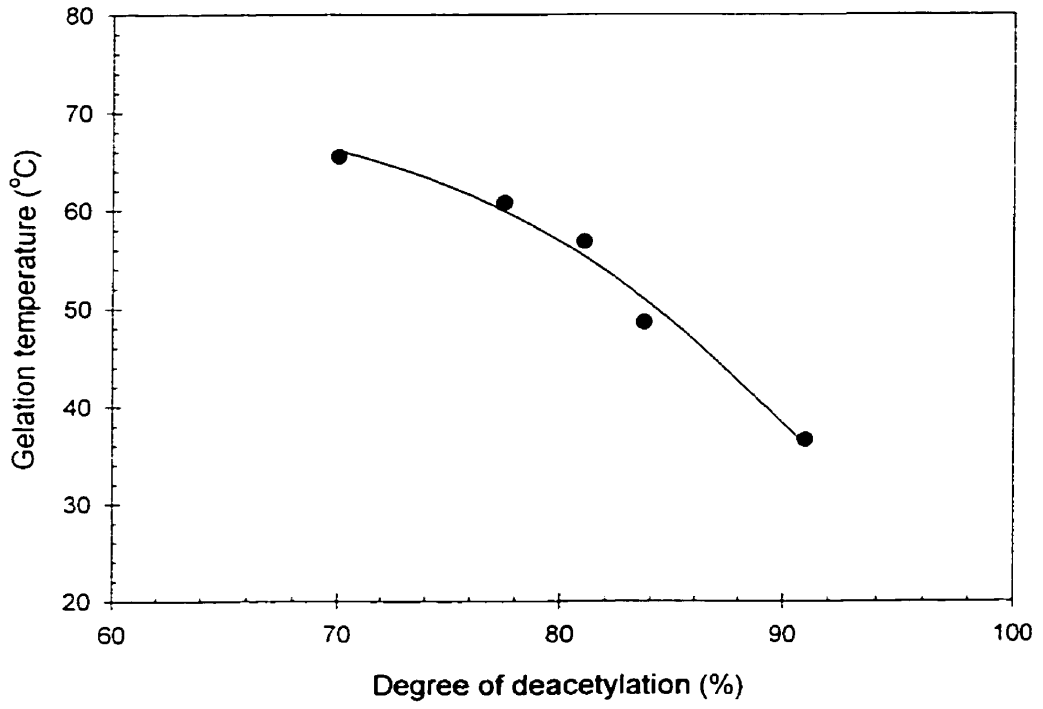


Figure 4.20: Relationship between DDA and sol-gel transition temperature for chitosan (2%)/β-GP (8%) system.

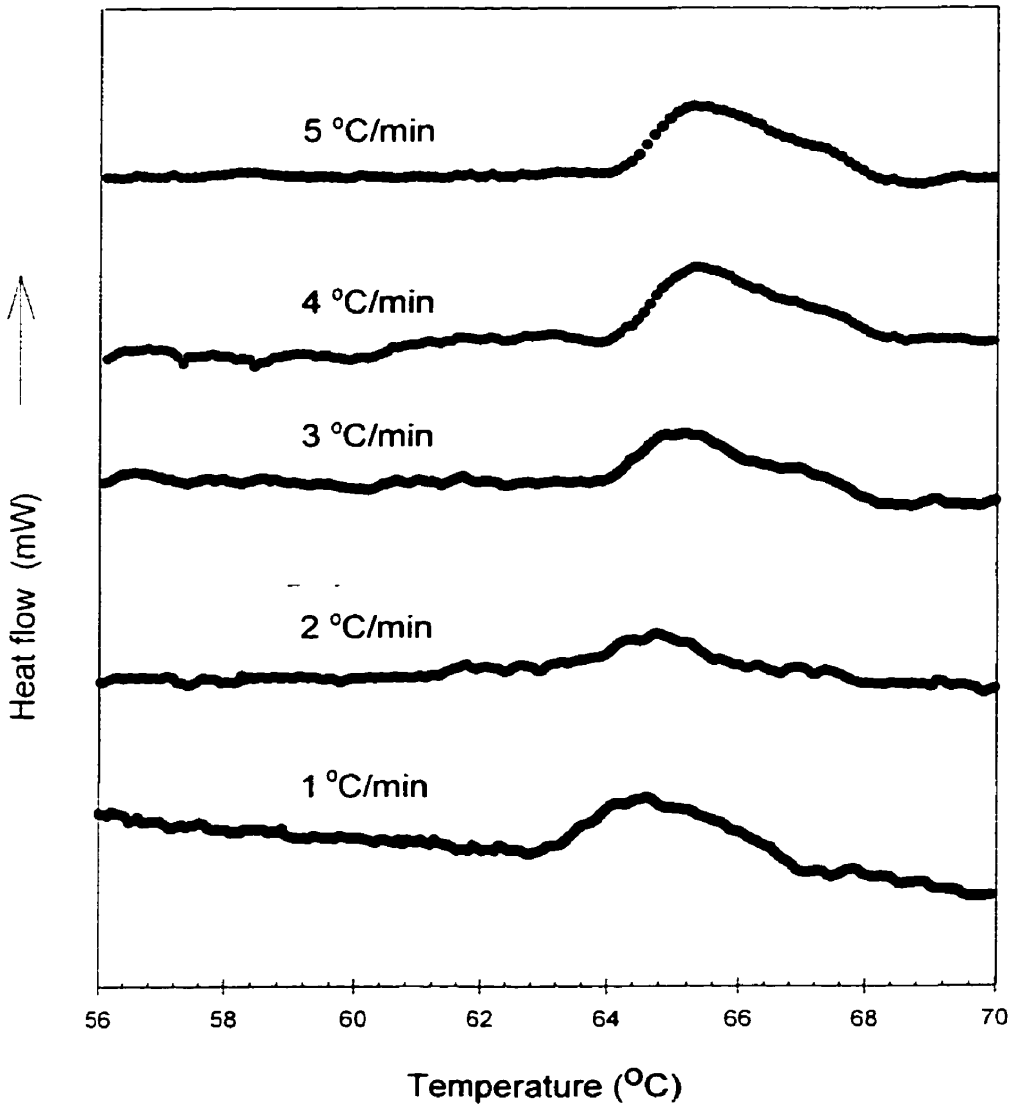


Figure4.21: Heating DSC curves of 1% w/v chitosan with 8% w/v β-Glycerophosphate at different scan rates.

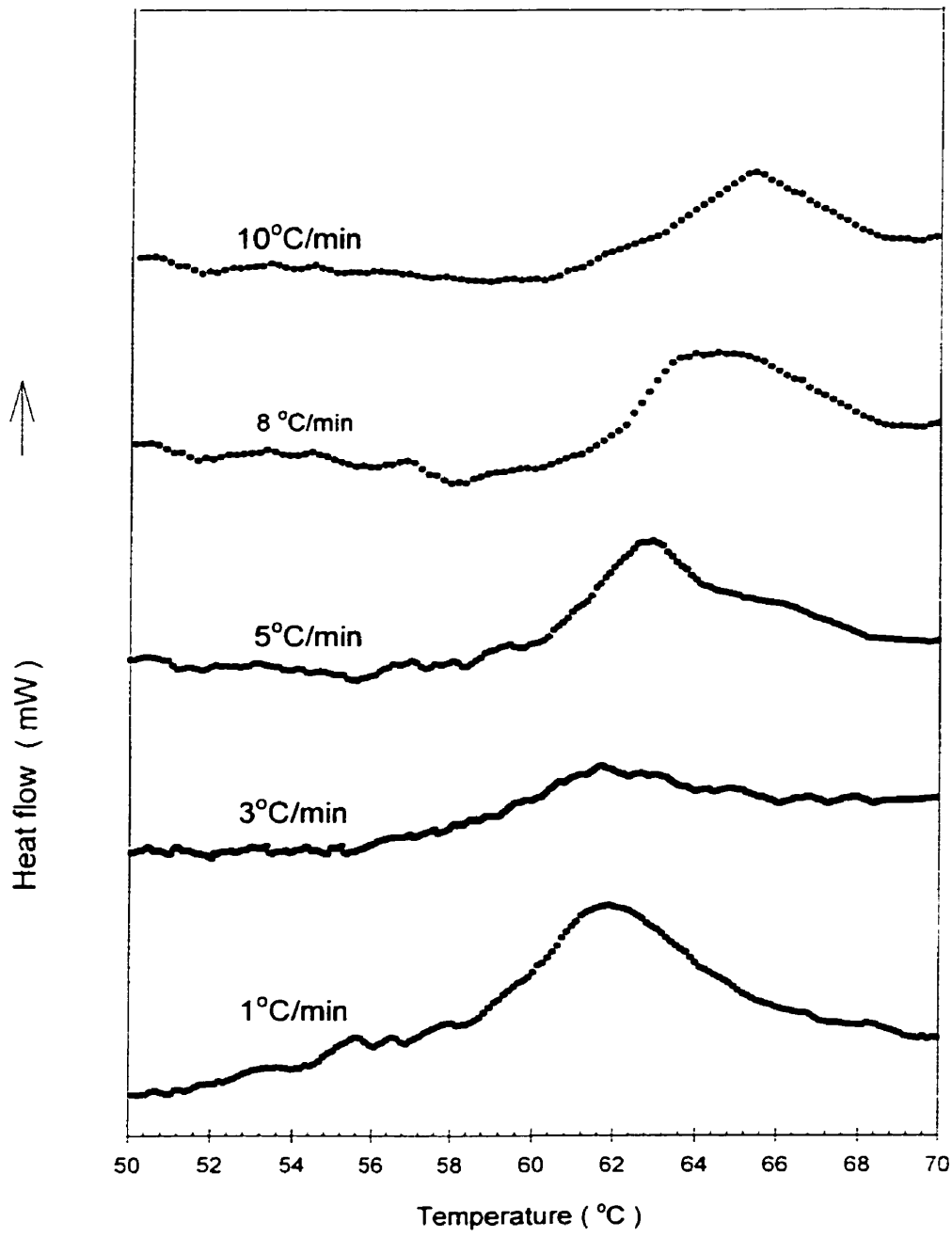
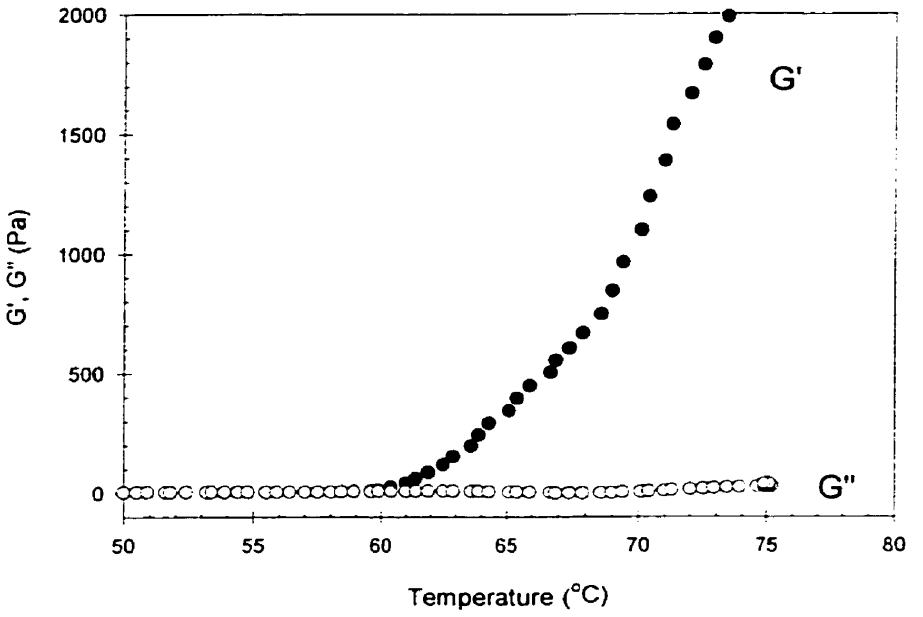


Figure 4.22 Heating DSC curves of 1.5% w/v chitosan with 8% w/v β-Glycerophosphate at different scan rates.

(a) Chitosan 1.5% / β-GP 8%



(b) Chitosan 1% / β–GP 8%

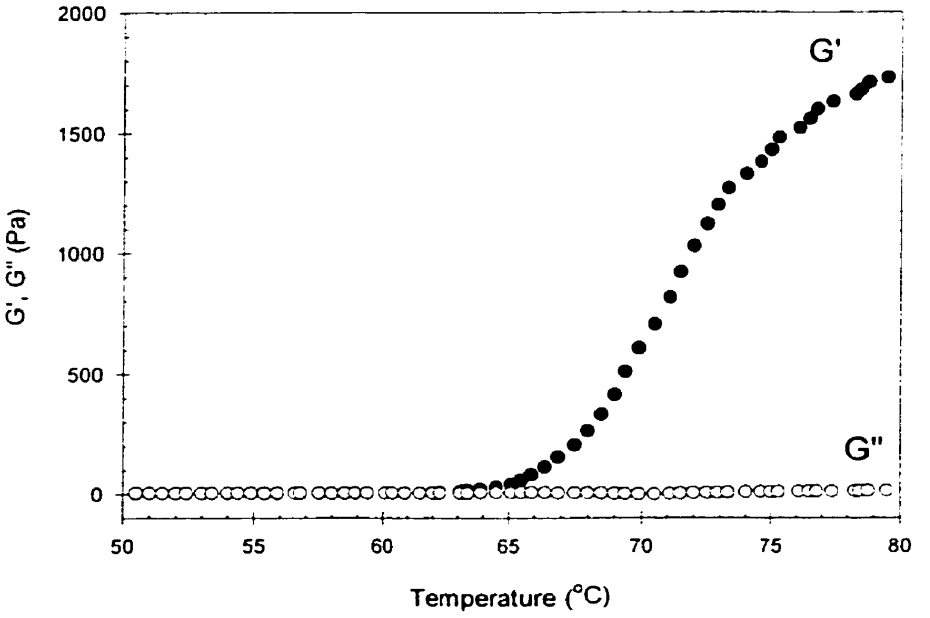


Figure 4.23: Thermal scanning rheological measurement at 1 °C/min.

CHAPTER 5 – CONCLUSIONS

The utility of polymer-based systems for biomedical applications is well known. As implants, they are used in various forms and particularly as films, particles or gels. Because of their mechanical compatibility with the soft living tissues, hydrogels are becoming more and more the appropriate polymeric systems to deliver therapeutics such as drugs, proteins and living cells. For the minimisation of the surgical intervention, a particularly useful system can be thought of as a liquid aqueous formulation, which turns into gel when injected into the body. The gelation should be induced by the environmental changes, for example the body temperature or electrolyte contained in physiological fluids.

The present study has been performed on a novel chitosan-based system recently discovered. This system consists of liquid formulations of chitosan capable of forming a gel upon heating. Such a formulation is a chitosan aqueous solution with β-glycerolphoshate, added a particular salt which increases the pH of the chitosan solution because of the salt basic nature, and which prevents the immediate aggregation of chitosan chains, even at neutral pH where normally chitosan can't exist in solution. Chitosan solutions neutralized with β-glycerophosphate disodium salt, to pH between 6.5 and 7.2, can be transformed to gel upon heating to 37°C and above. The resulting gels are completely thermo-reversible for C/β-GP systems with pH in the range of 6.5~6.9, while systems with pH above 6.9 show only a partial thermo-reversibility. This suggests the domination of hydrophobic forces, able reverse with the temperature, in the formation of C/β-GP gels. The resulting C/β-GP solutions have been proven thermosensitive and pH-dependent gel forming aqueous formulation. This characteristic is expected to receive great affection in the biomedical field. The liquid formulation can furthermore be loaded with a therapeutic material and injected into the body to form a gel *in situ*.

The C/β-GP systems have been studied by using rheological experiments. Thus,

the temperature of gelation or gel points were established by observing the frequency-independent value of loss tangent obtained from a multi-frequency plot of tan(δ) *vs* temperature. The power law exponent at the gel points was found between 0.428 and 0.445. The temperature-dependence and the time-dependence of elastic modulus (G') and viscous modulus (G''), allowed observation of the Sol/Gel transitions, and at the same time gave information on the gel strength as well as the kinetics of the gelation. The temperature-dependence of G' and G'' has also been investigated as the temperature decreases in order to investigate the thermoreversibility of C/β-GP gels. These types of measurements showed clearly that the C/GP gels become clear liquid when cooled down to ~ 4°C at a pH within 6.5~6.9. The gelation temperature and gel strength was determined as a function of DDA, the concentrations of chitosan and β-GP. The gelation temperature decreases with increasing the concentration of chitosan, the concentration of β-GP or the degree of deacetylation of chitosan. The strength of these gels is time dependent, increases with increasing temperature, the concentration of chitosan or the concentration of β-GP.

The likely mechanism behind the non-trivial behaviour of C/β-GP systems, and the role of β-GP in transforming the chitosan-based solution from pH-dependent to temperature sensitive, has been discussed in the light of the published literature for similar systems. The special role played by β-GP is attributable to its two moieties, the phosphate group and the glycerol part. The dibasic form of the glycerophosphate group serves to adjust the pH to neutral, while the glycerol part makes the chitosan more soluble at low temperature possibly, because of its structuring effect on water. At high temperature, this structure is broken and the chitosan chains tend to associate and form a gel. The Sol/Gel transition can be seen in this sense as hydration-dehydration equilibrium of chitosan chains.

Since chitosan is obtained by alkaline deacetylation of chitin, it is obvious that the extent of the deacetylation and the molecular weight are the two main physical

characteristics, which affect the behaviour of chitosan solutions. We investigated the influence of these two factors on the C/β-GP system and we found that DDA affects significantly the temperature of gelation, while the molecular weight has little or no effect. Thus, we showed that the temperature of gelation decreases as the DDA increases.

In this study, we have also been concerned with the alkaline treatment of chitosan in order to increase its DDA, because despite the abundance of chitin, only chitosans with DDA of ~80% are commercially available. Therefore, we established the conductimetric titration of chitosan as a fast, easy and efficient method to determine the degree of deacetylation of locally produced chitosan and we showed that the results obtained compared well with those determined by NMR spectroscopy.

In addition to that, we established a protocol for the purification of chitosan in order to provide a material free from insoluble matters and endotoxins, which is required for biological needs.

Future work could include further elucidation of the gelation mechanism, influence of solvent and other species. It would also be of interest to find substitutes for β-GP and chitosan, and thereby develop distinct but analogous thermogelling systems.

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