Titre: Title:	Optimization of LAL Assay in Plate Reader and Methods to Reduce Interference in Chitosan for Endotoxin Detection
Auteur: Author:	Almas Siddiqui
Date:	2016
Type:	Mémoire ou thèse / Dissertation or Thesis
Référence: Citation:	Siddiqui, A. (2016). Optimization of LAL Assay in Plate Reader and Methods to Reduce Interference in Chitosan for Endotoxin Detection [Mémoire de maîtrise, École Polytechnique de Montréal]. PolyPublie. https://publications.polymtl.ca/2366/

URL de PolyPublie: PolyPublie URL:	https://publications.polymtl.ca/2366/
Directeurs de recherche: Advisors:	Michael D. Buschmann
Programme: Program:	Génie biomédical

UNIVERSITÉ DE MONTRÉAL

OPTIMIZATION OF LAL ASSAY IN PLATE READER AND METHODS TO REDUCE INTERFERENCE IN CHITOSAN FOR ENDOTOXIN DETECTION

ALMAS SIDDIQUI

INSTITUT DE GÉNIE BIOMÉDICAL

ÉCOLE POLYTECHNIQUE DE MONTRÉAL

MÉMOIRE PRÉSENTÉ EN VUE DE L'OBTENTION

DU DIPLÔME DE MAÎTRISE ÈS SCIENCES APPLIQUÉES

(GÉNIE BIOMÉDICAL)

DÉCEMBRE 2016

UNIVERSITÉ DE MONTRÉAL

ÉCOLE POLYTECHNIQUE DE MONTRÉAL

Ce mémoire intitulé:

OPTIMIZATION OF LAL ASSAY IN PLATE READER AND METHODS TO REDUCE INTERFERENCE IN CHITOSAN FOR ENDOTOXIN DETECTION

présenté par : SIDDIQUI Almas

en vue de l'obtention du diplôme de : Maîtrise ès Sciences Appliquées

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

M. L'HOCINE Yahia, Ph. D, président

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

M. BARALLET Jake, Ph. D, membre

DEDICATION

For my father in heaven, my mother, uncle, brother and husband,

For my friends and family in both the continents.

ACKNOWLEDGEMENTS

I would like to thank Professor Michael Buschmann, my supervisor for his excellent guidance and support throughout the course of my M.Sc. A program. For granting me this opportunity to showcase my abilities and explore the inter-disciplinary fields of chemistry and biology.

I would also like to equally thank Dr. Vincent Darras and Dr. Marc Lavertu who have been integral part of my project and guided me along the way for successful completion of my thesis. The discussions I had with them were very helpful.

Next, I would like to thank my colleague and friend Mohamad Gabriel Alameh for his rigor and guidance through difficult parts of my project. I am very grateful for his continuous support and encouragement.

I am also grateful for the support of my friends at polytechnique Garima Dwivedi, Yuan Chang, Colleen Mathieu, Genevieve Picard, Leili Ghazizadeh, Ashkan Tavakoli and others.

RÉSUMÉ

Le lipopolysaccharide (LPS) ou endotoxine est un composant essentiel de la face externe de la membrane externe des bactéries à Gram négatif qui provoque, après administration, des symptômes dangereux chez les humains tel le choc septique et la mort.

La contamination des matériaux cliniques par les endotoxines pendant les étapes de fabrication est courant, ce qui rend les ingrédients pharmaceutiques actifs non utilisables. Avant de commercialiser le produit final sur le marché, les compagnies pharmaceutiques réalisent des tests LAL (lysat d'amibocytes de *Limulus polyphemus*) approuvés par la FDA afin de déterminer la concentration en endotoxines. L'évaluation de la contamination des matériaux de départ à partir de chitosane pour la fabrication de nanoparticules injectables CD-ARNsi et des échafaudages CS/HA par des endotoxines est compliqué du fait des interactions positives avec les endotoxines. Un résultat faussement négatif lors du test LAL peut entrainer l'approbation du matériel mais provoquer un choc cytokinique lorsqu'il est injecté dans le corps humain.

Nous avons d'abord déterminé la quantité d'endotoxines dans le chitosane de départ par des essais de gels coagulés sur LAL, une méthode semi-quantitative qui permet d'estimer la quantité d'endotoxines. Nous avons trouvé une valeur inférieure à 0.3 EU/mg pour des chitosanes de degré de désacétylation de 92% avec une grande ou petite masse molaire (10 kDa), ce qui est en dessous des limites. Si les valeurs obtenues par les essais de gels coagulés sont considérées comme absolue par la pharmacopée américaine, cette quantité limite représente un risque significatif lors de l'administration de doses plus fortes lors des tests cliniques. Cela conduit donc à utiliser des essais LAL plus sensibles, tel les tests LAL cinétiques par turbidité ou chromogène, afin d'obtenir des valeurs plus précises d'endotoxines.

Dans la seconde partie, nous avons développé les essais cinétiques où des étalons sont utilisés afin d'établir une courbe d'étalonnage. La détermination de l'équation de la régression linéaire de la courbe d'étalonnage log (concentration) en fonction log (time) est déterminée, le temps reporté étant le temps nécessaire pour que l'absorbance à 405 nm atteigne une valeur prédéterminée, typiquement 0.1 pour les tests par turbidité et 0.2 pour les tests chromogènes. A cause de la nature biologique de l'enzyme LAL, la variabilité autorisée par la pharmacopée américaine est de ± 2 fois la valeur théorique, ce qui veut dire que la valeur quantifiée peut avoir une variation de 50 à 200%. Du fait de l'utilisation d'un lecteur de plaques pour effectuer les essais, différents

paramètres techniques, tel la longueur du chemin optique, l'identification du taux d'évaporation dans les puits de la plaque 96 puits, le prétraitement des échantillons et la gamme de concentration de la courbe d'étalonnage ont été optimisés afin d'obtenir des équations de courbes d'étalonnage répétables.

Dans la troisième partie des essais cinétiques, la quantification des endotoxines par des tests de turbidité dans un chitosane de degré de désacétylation de 92% et de grande et petite masse molaire (10 kDa) ont été développés. A cause de l'interaction entre le chitosane et les endotoxines, il a été observé une inhibition de la détection des endotoxines, appelé interférence d'inhibition. Dans le but de réduire cette inhibition, plusieurs prétraitements du chitosane ont été testés, tel le traitement par NaOH, digestion par des chitosanases aussi bien que la dépolymérisation par acide nitreux ou encore le traitement par de l'acide chlorhydrique concentré, traitement recommandé par la pharmacopée américaine. Nous avons trouvé que tous ces traitements sont délétères pour l'endotoxine libre et entrainent une réduction de la détection d'endotoxines mais nous avons découvert que le chitosane jouait un rôle de protecteur, ce qui permet d'obtenir un taux acceptable mais non total de recouvrement en endotoxines ajoutées. A partir de ces résultats, l'utilisation d'un agent neutre est préconisé afin de dissoudre le chitosane sans détruire les endotoxines libres ainsi que les libérer du chitosane pour les doser. L'utilisation d'agents dispersants, détergents ainsi que des cations afin d'affaiblir les liaisons entre le chitosanes et les endotoxines sont aussi recommandés pour les prochaines études.

ABSTRACT

Endotoxin or Lipopolysaccharide (LPS) is a component of gram-negative bacteria cell wall, which causes hazardous symptoms in humans such as septic shock and death after administration. Endotoxin contamination in clinically administered materials/ nanoparticles is prevalent, typically introduced during production and processing steps, which renders the active pharmaceutical Ingredient (API) unsuitable for use. Before releasing the end product in the market, the companies perform endotoxin detection by FDA approved LAL (Limulus Amoebocyte Lysate) tests. Evaluation of endotoxin contamination in chitosan raw materials used for the production of injectable Chitosan-siRNA nanoparticles and Chitosan/Hyaluronic Acid scaffolds in vivo is complicated due to its positive interaction with endotoxin. A false negative result by the LAL test may lead to approval, but may cause the release of life-threatening cytokines such as interferons when injected in human body.

We first determined the amount of endotoxin in the chitosan raw material(s) by gel-clot LAL assay, a semi-quantitative assay, which provided an estimated value of endotoxin contamination. We found values $\leq$0.3 EU/mg for chitosan with 92% DDA at high and low (10kDa) molar mass, which is under the acceptable limit. If the gel-clot derived endotoxin value are considered as absolute by USP, then this limit amount represents significant risk going into the clinical phase with the higher administration doses. This leads to the use of more sensitive LAL assays for the accurate and absolute quantification of endotoxin, such as kinetic turbidimetric and kinetic chromogenic LAL assays.

In the second part, we developed the kinetics assays, where standards are used to establish a standard curve. A linear regression equation of a Log conc. vs. log onset time standard curve is then determined, the onset time being obtained when the measured absorbance at 405nm of each calibrator reaches a threshold O.D., typically 0.1 in turbidimetric and 0.2 for chromogenic LAL assay. Because of the biological origin of LAL enzyme, the inherent variability in the assay is set as $\pm$ 2-fold defined by the USP, i.e. a quantified value may have a variation of 50-200%. Because of the use of a plate reader to perform the assay, different technical parameters, such as pathlength correction, identification of wells with high evaporation in 96 well plates, pre-treatment of samples and calibration range were then optimized in order to obtained repeatable standard curves equation.

In the third part of the kinetic assay, the endotoxin titration in chitosan with 92% DDA at high and low molecular weight by kinetic turbidimetric LAL assay were developed. Because of interaction between chitosan and endotoxin, inhibition of detection of endotoxin called as inhibition interference was observed. In order to reduce this interaction, various pre-treatments were tested, such as NaOH treatment, chitosanase enzyme digestion as well as chemical depolymerisation of chitosan by nitrous acid and chemical treatment by concentrated hydrochloric acid as recommended by USP. We found that all these treatments were deleterious for the free endotoxin and lead to reduced detection, but a protective effect of chitosan on endotoxin was discovered which allowed good but not total recovery of spiked endotoxin. Based on this, the use of a neutral reagent is recommended for dissolving chitosan that does not disintegrate free endotoxin and also releases the bound endotoxin. The use of dispersing agents, detergents and cations to weaken the bonds between chitosan and endotoxin are also recommended for future use.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF SYMBOLS AND ABBREVIATIONS

ACCI	Associates of Cape Cod
API	Active Pharmaceutical Ingredient
BET	Bacterial Endotoxin Test
CARD	Caspase Activation and Recruitment Domain
CD14	Cluster of Differentiation 14
CMC	Critical Micelle Concentration
CRO	Contract Research Organization
CSE	Control Standard Endotoxin
DDA	Degree of Deacetylation
E.coli	Escherichia coli
EP	European Pharmacoepia
FDA	US Food and Drug Administration
HA	Hyaluronic Acid
HCl	Hydrochloric Acid
HED	Human Equivalent Dose
IFNα	Interferon alpha
IFNβ	Interferon beta
IL-6	Interleukin-6
JP	Japanese Pharmacoepia
Kdo2	3-deoxy-D-manno-octulosonic acid
LAL	Limulus Amoebocyte Lysate
Lambda (λ)	Sensitivity of LAL assay
LBP	Lipid A Binding Proteins

LD50	Median Lethal Dose
LPS	Lipopolysaccharide
LRW	LAL Reagent Water
MD-2	Myeloid Differentiation Factor 2
MSC	Mesenchymal Stem Cells
MTD	Maximum Tolerated Dose
MW	Molecular Weight
MyD88	Myeloid differentiation primary response gene 88
NOAEL	No Observed Adverse Effect Level
NLRP3	Nod-like receptor protein 3
PAMPs	Pathogen Associated Molecular Patterns
p-NA	p-NitroAnilide
PRR	Pathogen Recognition Receptors
Ra-LPS	Rough Lipopolysaccharide
RSD	Relative Standard Deviation
RSE	Reference Standard Endotoxin by USP
RT	Room temperature
S-LPS	Smooth Lipopolysaccharide
Sec	Seconds
siRNA	Small Interfering Ribonucleic Acid
TIR	Toll/Interleukin-1R Resistance domain
TLR4	Toll-Like Receptor 4
TNFα	Tumor Necrosis Factor alpha
USP	US Pharmacoepia

CHAPTER 1 INTRODUCTION

Chitosan is a chemically modified biodegradable polymer with application in multiple fields as food, cosmetics, biomedical and pharmaceutical industries (Rinaudo, 2006). Chitosan is derived from chitin, a natural polymer extracted from natural sources like crab and shrimp shells, mushrooms and yeasts. Because of its origin from natural sources, several side products are removed from chitin during processing, such as lipids, proteins and calcium carbonate (Wooten, 2003). The use of chitosan for its application in pharmaceuticals requires clean products without contaminants. One of the unwanted contaminants that can hinder chitosan's application in clinical and biological processes is endotoxin. Endotoxins or Lipo-polysaccharides are naturally occurring outer cell wall component of disease causing gram-negative bacteria, and are toxic pyrogens capable of inducing septic shock when they enter in the human immune system. Due to the ubiquitous presence of bacteria in the environment; it is very challenging to eliminate endotoxin from the working stations during chitosan production and the LPS contamination may be introduced at any of the processing stages.

US, European and Japanese Pharmacoepias (USP, EP and JP) (EP, 2005; JP, 2001; USP, 2005) have issued guidelines for the bacterial endotoxin test (BET) for endotoxin quantification and have set out the maximum allowable limit for endotoxin contamination in drugs and medical devices. The Bacterial Endotoxin tests identified by the pharmacoepias are the three versions of Limulus Amoebocyte Lysate (LAL test): gel-clot, turbidimetric and chromogenic LAL tests; that are based on the principle of coagulation of the horseshoe crab blood following its encounter with bacterial endotoxin.

The high affinity of positively charged chitosan to the negatively charged phosphate and carboxylic groups respectively in the Lipid A and core region of the LPS along with other weak interactions such as hydrogen bonding and Vander Waals interaction (I. M. Y. V. N. Davydova*, V. I. Gorbach, I. N. Krasikova, and T. F. Solov'eva, 2000), makes the endotoxin removal from chitosan even more challenging.

In order to accurately quantify the endotoxin content, certain pre-treatments of the chitosan have to be performed as preventative measures for interference and for absolute quantification of

endotoxin. Such preventative measures are preeminent in the pursuit of development of a reliable method for quantification of endotoxin in chitosan by LAL assay.

The nature and structure of endotoxin and the importance of endotoxin detection due to immune system activation will be discussed in detail in the next chapter. It also entails different types of assays used for endotoxin determination and mechanism and principle of the LAL assay functioning.

The characteristics of chitosan and mechanism of chitosan interaction with endotoxin, and various methods adapted to remove it from the processing steps to the end-product preparation are also discussed in detail in the critical literature review.

CHAPTER 2 CRITICAL LITERATURE REVIEW

2.1 Endotoxin

Endotoxin is a lipopolysaccharide complex component of outer cell walls of gram negative bacteria that can induces inflammatory responses such as cytokine activation leading to fever, septic shock and death after introduction to host immune system (Akira, Uematsu, & Takeuchi, 2006). Endotoxins are also called as pyrogens due to their fever inducing property. The term endotoxin for the unknown pyrogen of bacterial origin was first used by Richard Pfeiffer, one of Robert Koch's students in 1892, after he successfully extracted "heat resistant substance" upon cellular disintegration of *Vibrio cholerae* bacterium (Williams, 2001).

2.1.1 Structure and function of LPS

Endotoxins or Lipopolysaccharides (LPS) are composed of hydrophilic polysaccharide part and a hydrophobic Lipid A part. *E. coli* LPS is widely characterized and used as a model to identify the structure of LPS. The structure of *E. coli* LPS shown in **Figure 2-1** below shows the structural arrangement of Lipid A and hydrophilic core sugars and O-antigen The LPS is located on the outer membrane of the cell wall of the gram negative bacteria, but synthesized at the cytoplasmic region of the inner membrane. The structure of LPS is divided in to three parts: 1) Lipid A 2) core oligosaccharide and 3) O-antigen. The Lipid A is the hydrophobic component, which resides on the outer membrane, while the core sugars and O-antigen is displayed on the surface of the bacteria. Lipid A is the widely conserved region in different bacterial species compared to the core oligosaccharides and O-antigen and is responsible for the toxicity and virulence of the gram-negative bacteria. Some bacteria contain only Lipid A and core oligosaccharides known as rough-LPS (Ra-LPS) while others flag O-antigen on their surface, giving them smoother structural morphology (s-LPS). Lipid A-Kdo_2 is the basic structure required for survival by many bacteria (X. Wang & P. J. Quinn, 2010; Xiaoyuan Wang & Peter J. Quinn, 2010).

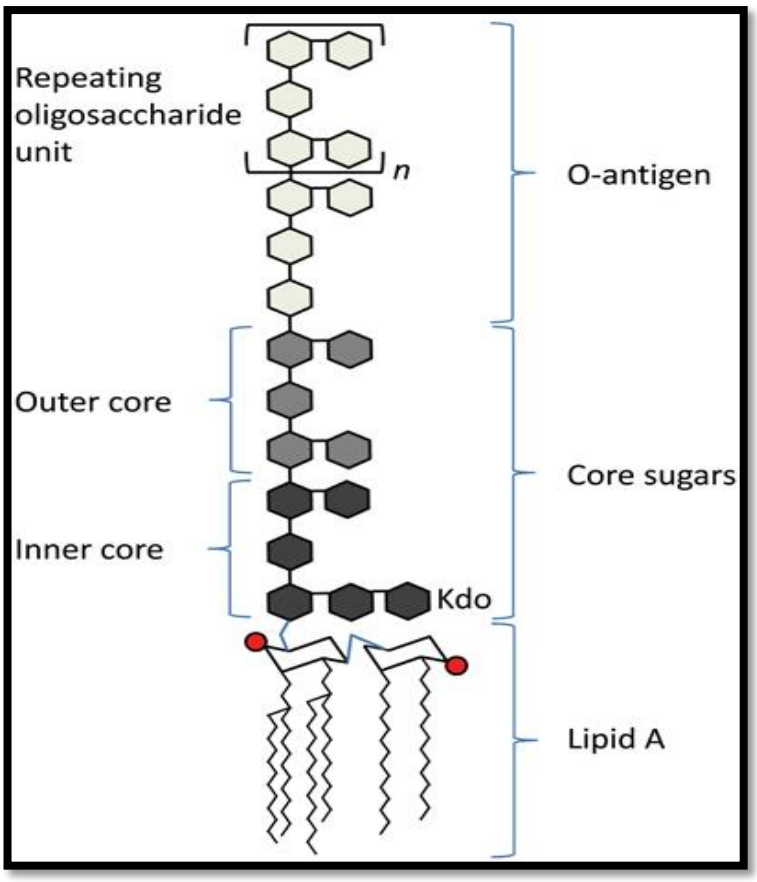


Figure 2-1: Structure of *E. coli* LPS (Maeshima & Fernandez, 2013)

2.1.1.1 Factors affecting toxicity of LPS

The detailed structure of LPS specifically O-antigen varies in different serotypes of bacteria (Wang, Wang, & Reeves, 2010), but changes in Lipid A chemistry in different bacterial species leads to differences in their virulence properties. The fine structure of LPS monomers shows difference in response of TLR4 cytokine induction. Depending on the induced pyrogenecity, the LPS are classified in to agonists and antagonists. The hexacylated Lipid A is considered most toxic compared to pentacyl and tetracyl substituted Lipid A; the chemical structure, length of the acyl chains and the presence or absence of phosphate group also classifies the LPS as agonist and antagonist respectively (Steimle, Autenrieth, & Frick, 2016).

The LPS can form aggregates/micelles in aqueous solutions when they reach their critical micelle concentration (CMC) considered to be at 11µg/mL (Wistrom, 1998), and the geometry depends upon the pH, temperature, presence of detergents or divalent cations of the

medium and the charge present on the LPS itself. The divalent cations like Ca^{2+} and Mg^{2+} stabilizes the aggregate structure and its conformation (Garidel et al., 2005). Depending upon the condition, the LPS mainly Lipid A can form three distinct structures at monomeric as well as supramolecular aggregate level, which determines the pyrogenecity of the LPS: a) Lamellar structure (L) b) Cubical structure (Q) c) Hexagonal structure (HII) (Klaus Brandenburg, 1996). The three structures are shown in **Figure 2-2** below.

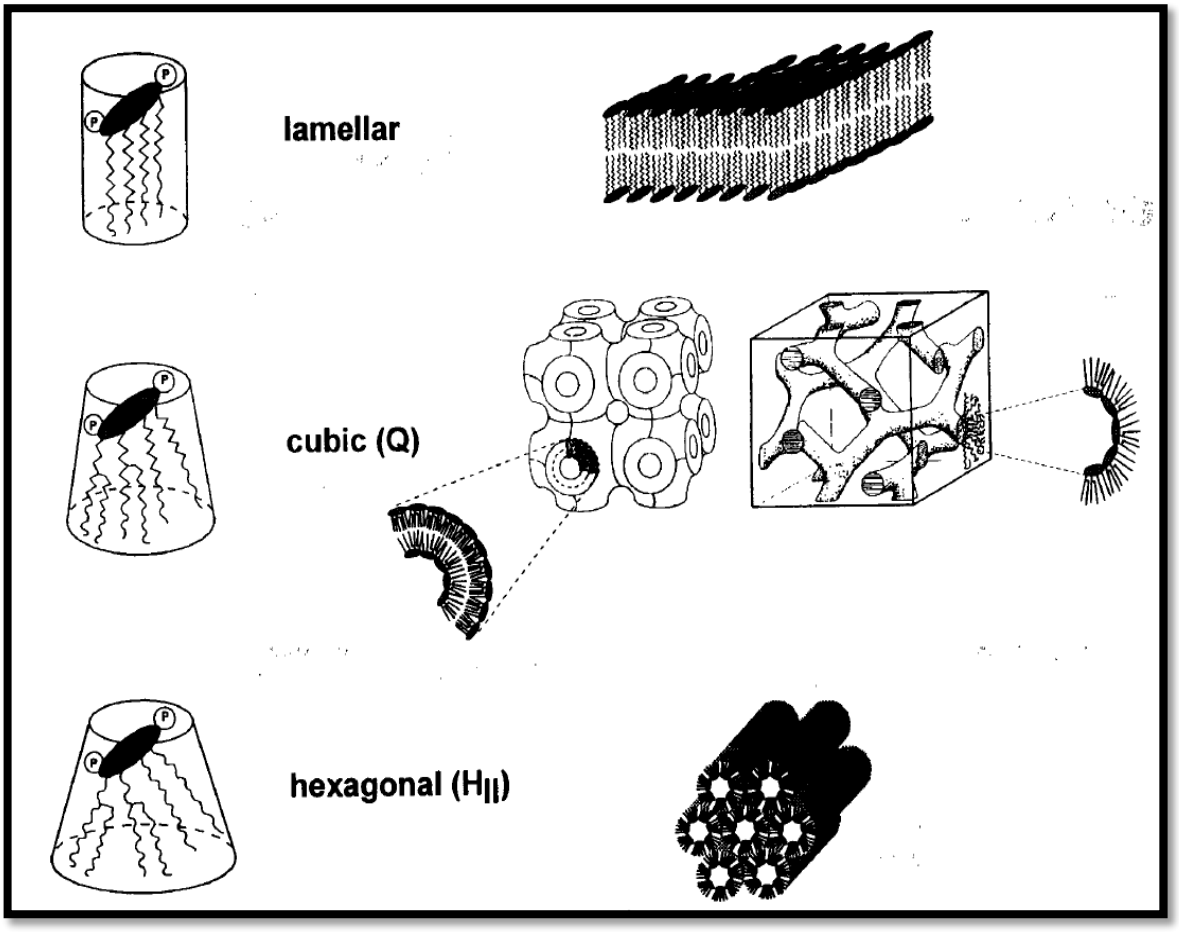


Figure 2-2 : Conformation of LPS monomer and the resulting supramolecular 3-D aggregate structure, which determines the pyrogenecity of the LPS (Klaus Brandenburg, 1996).

The lamellar structure due to planar arrangement is the least toxic and does not induce cytokine response; it also has the tendency to form micelles(Andra B. Schromm, 1998). The cubically aggregated form of LPS is moderately toxic and activates the immune system to a medium level; but the hexagonal structure which has the non-lamellar inverted cubic form is the

most toxic activating the TLR4-MD2 complex and amplifying the cytokine response to a dangerous extent, with even pico gram levels flaring up the response (Williams, 2001).

2.1.2 Immune induction by LPS

Lipopolysaccharide (LPS) or endotoxins originating from the prokaryotic bacteria are identified by mammalian immune system as one of the biggest threats. The endotoxin and the bacteria itself are recognized by the innate immune system of the mammals. The cells of the innate immune system such as macrophages and dendritic cells recognize the endotoxins by their pathogen recognition receptors (PRRs). The invading bacteria or endotoxin express specific pathogen associated molecular patterns (PAMPs) which are recognized by different PRRs that activate specific signalling pathway. The PRRs in the mammalian immune system that recognizes pyrogenic Lipid A portion of LPS are the Toll Like Receptor 4 (TLR4) which activates the signalling pathway and result in the release of pro-inflammatory cytokines (Akira et al., 2006). The summarized TLR4 signalling pathway is shown in **Figure 2-3** below:

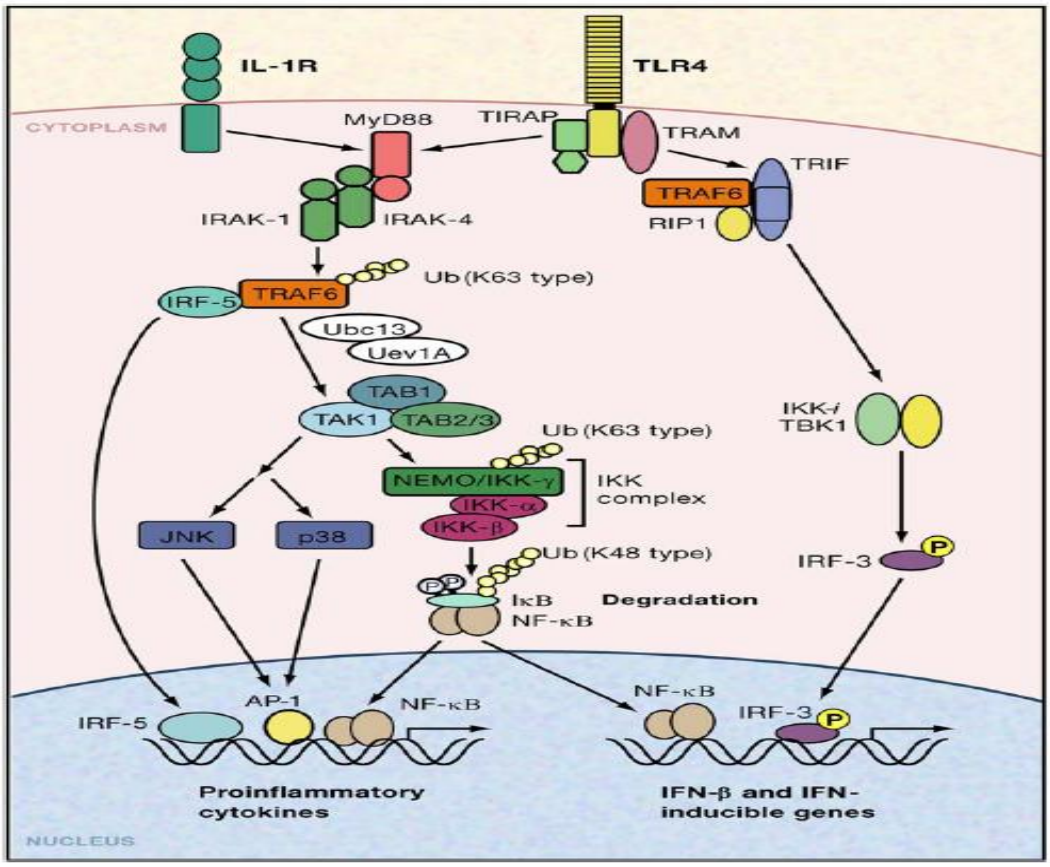


Figure 2-3 : TLR4 signalling pathway induced by recognition of LPS leading to pro-inflammatory cytokine release (Akira et al., 2006)

The TLR 4 is an extracellular recognition receptor lying on the transmembrane of macrophage or phagocytic cells, the recognition of LPS is aided by the Lipid A binding proteins (LBP) present in the serum to sense the bacterial presence in the blood (Hansen, Rasmussen, Niels-Christiansen, & Danielsen, 2009; Vreugdenhil, Snoek, Greve, & Buurman, 2000). LBP transports the LPS in its monomeric form to CD14- a soluble protein and a membrane anchored protein of TLR 4 expressing cells, which increases the susceptibility of TLR4 towards LPS by a factor of 10^2 to 10^3 (Wright, 1990). The CD14 then shuttles the LPS to MD-2, which is a TLR4 co-receptor and could be either bound to TLR4 monomer via hydrogen bonds or present as soluble protein into the cell exterior analogous to CD14. MD-2 dimerizes the TLR4 monomers and forms a complex with two MD-2 and 2 TLR4 domains. The mechanism of TLR4-MD2 dimerization is that five of the six acyl chains of LPS buries inside a binding grove of MD-2 (Park et al., 2009), the sixth acyl chain exposed on the MD-2 surface in combination with hydrophobic residues of MD-2 promotes intermolecular interaction with TLR4 (Jin & Lee, 2008; Ohto, Fukase, Miyake, & Shimizu, 2012). The binding of MD-2 with TLR4 and the resulting conformational changes leads to the dimerization of c-termini of TLR 4 ecto-domains (Jin & Lee, 2008; Kim et al., 2007; Ohto et al., 2012). This dimerization leads to the dimerization of cytosolic TIR domains which can induce downstream signalling of cytokine by either Myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter -inducing interferon-β (TRIF)-dependent downstream signalling (Molinaro et al., 2015). CD14 are considered to be important for LPS sensing at low LPS concentration, but are indispensable at high conc. The TLR 4 signalling apart from cytokine activation also result in enhanced production of chemokines, free radicals and nitric oxide (Ohto et al., 2012; Poltorak, Ricciardi-Castagnoli, Citterio, & Beutler, 2000) which increases toxicity symptoms like pain and low blood pressure leading to septic shock and death.

Different signalling pathways for cytokine activation have also been elucidated in recent research apart from TLR4 pathway. An intracellular signalling pathway including “inflammatory caspases” also exists for release of cytokines after LPS recognition. The inflammatory caspases are proteolytically inactive zymogens, activated after proteolytic cleavage mediated by caspase activation and recruitment domain (CARD) present on the enzyme N-terminal responsible for

protein-protein interactions. Intracellular signalling pathway takes place in the cytosol, and results in the activation of inflammatory caspases 1 termed “inflammosome” leading to the production of cytokines as well as pyroptosis- a special kind of cell death. Two pathways exist to activate inflammosomes: 1) Classical or canonical pathway 2) Non-canonical pathway. The classical pathway activates inflammosomes in a caspase-dependent manner by an assembly of Nod-like receptor protein 3 (NLRP3) or NLR family CARD domain-containing protein 4 (NLRC4) with intracellular adaptor protein NLR family CARD domain-containing protein 4 (ASC) which leads to activation of caspase 1 resulting in the cleavage of IL-1β and IL-18 and finally secretion of cytokines (Mariathasan et al., 2006; Martinon, Petrilli, Mayor, Tardivel, & Tschopp, 2006; Schroder & Tschopp, 2010). The non-canonical pathway includes activation of caspase 1 by the activation and aggregation of caspase 11, which can directly sense cytosolic Lipid A independently from TLR4; the activation of Caspase 11 leads to cytokine production as well as pyroptosis.

The interaction of Lipid A portion of LPS with the extracellular TLR4 as well as intracellular cytosolic caspase 11 leading to the production of cytokines is rigorously demonstrated in **Figure 2-4** below:

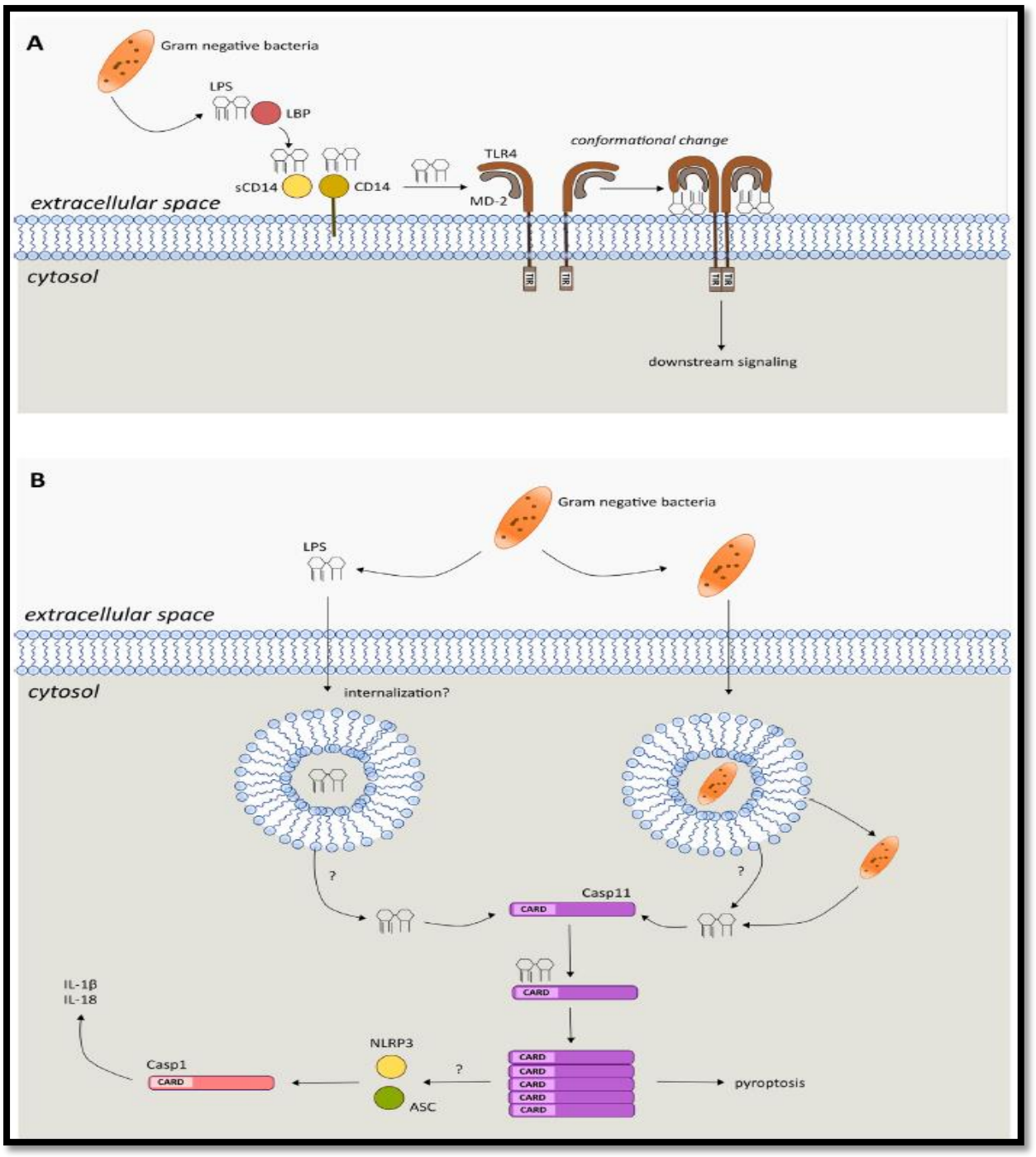


Figure 2-4 : The extracellular and intracellular mechanism of molecular sensing of bacterial LPS. The MD2-TLR4 complex formation after activation by LPS results in downstream signalling and cytokine production (A) The LPS present in the cytosol is internalized by caspase 11 expressing vacuole and binds to the CARD domain of caspase 11 resulting in oligomerization and activation of caspase 11. Caspase 11 activation results in assembly of inflammosome leading

to secretion of pro-inflammatory cytokines by activated caspase-1; the oligomerized caspase 11 also leads to pyroptosis, a special kind of cell death. (B) (Steimle et al., 2016)

The cytokines like IL-6, IFNα, β and TNFα released by innate immune system leads to precarious physiological response such as fever, chills, hypotension and most threatening septic shock. The downstream effect of the innate immune response stimulated by LPS leading to display the dangerous physiological response is shown in the **Figure 2-5** below:

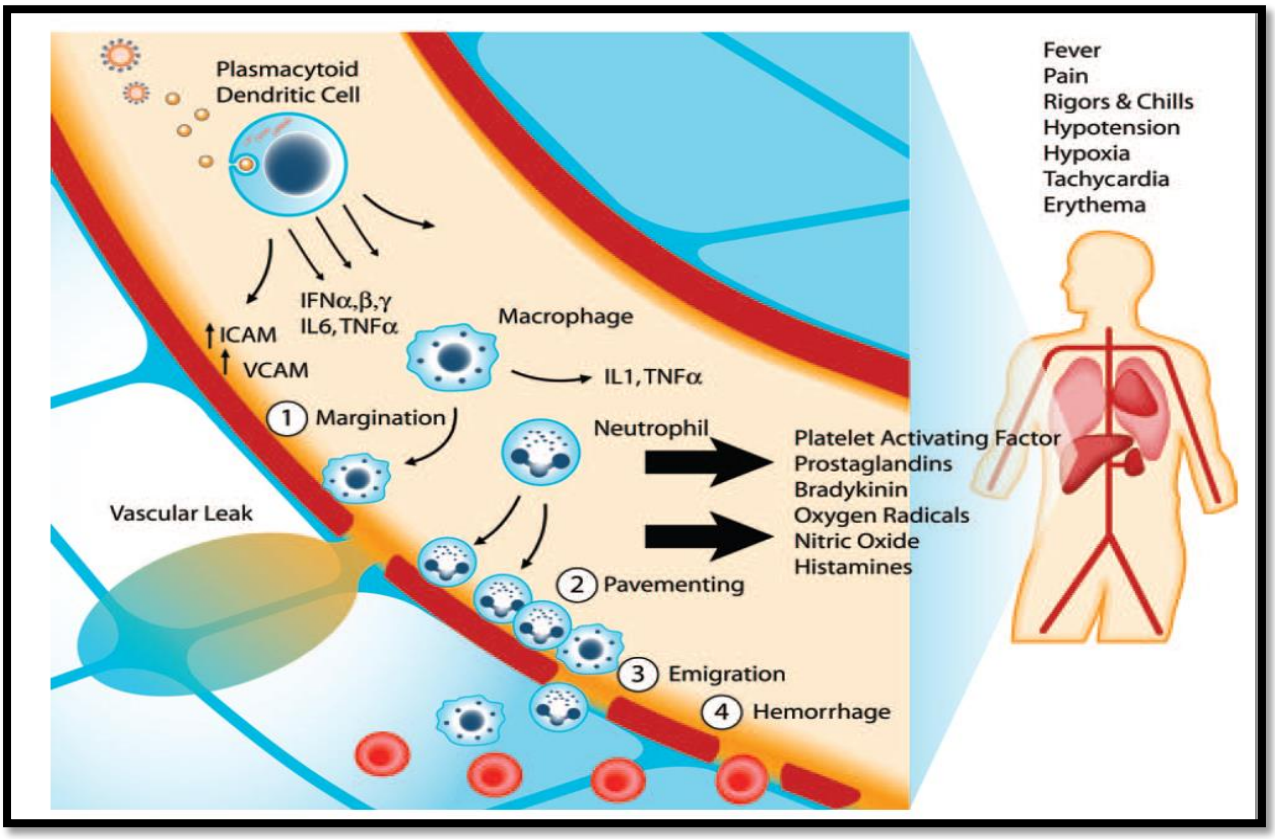


Figure 2-5 : LPS shows similar downstream effect of immune stimulation (Judge & MacLachlan, 2008)

Cytokines released in the blood stream activates the endothelial cells and result in a cascade of effects on blood cells like margination, pavementing, emigration and hemorrhage leading to thrombocytopenia. The effect of IL-1β and TNFα on vascular endothelial cells results in hypotension. The myriad of reaction is observed in septic shock leading to death in late stages (Judge & MacLachlan, 2008).

Due to such exaggerated response of human immune system after coming in contact with LPS, it is absolutely essential to eliminate endotoxin from the active pharmaceutical ingredient (API) and the nano-vectors (chitosan nanoparticles) to ensure the compliance with the accepted LPS limit of 5 EU/kg/hour issued by the USP before application in humans (USP, 2005).

2.1.3 Importance of endotoxin detection

In the manufacture of parenteral drugs, the control of endotoxin contamination is very important because of the following aspects: 1) The endotoxin is ubiquitous in nature due to omnipresence of bacteria, hence it is capable of contaminating any given formulation. 2) Endotoxins are potent inducers of inflammation compared to any other microbial and non-microbial pyrogen 3) The stability of the endotoxin after being subjected to extreme conditions like high heat and changing pH is very high 4) The relative likelihood of the endotoxin contamination in the parenteral formulations is very high, as they go through multiple production and manufacturing process (Williams, 2001). Hence, a regular testing of pharmaceutical waters and in process material for ensuring low endotoxin contamination in the end-product before releasing is a pre-requisite for any pharmaceutical product. The detection and removal of endotoxin contamination is one of the predominating concerns in the pharmaceutical manufacturing industry.

2.1.3.1 Harmonization of endotoxin units [EU/mL] of different species

Typically, the FDA defines 100 picogram LPS as equal to one endotoxin unit (EU) (GmBH, 2013), but the endotoxin units in different species varies depending upon the pyrogenecity of the LPS which is determined after calibration with the USP Reference Standard Endotoxin (RSE). The potency of LPS of even the same species from different lots/ batches also varies when standardized against the RSE (times_CRL, 2006; USP, 2005). The potency of CSE against RSE is expressed in EU/ng, also known as RSE / CSE ratio. It is not a good practice to convert the EU values to weight (picogram) as the pyrogenecity also depends upon the molecular weights and size of LPS; i.e. different molecular weight and sizes of LPS may produce same pyrogenecity (MJ, 1997).

2.1.4 Methods of endotoxin detection

Traditionally the measurement of biological activity of the LPS has been qualitatively determined by the rabbit pyrogen test (RPT), in which the test article was injected intravenously into the rabbits and the body temperature was monitored, an increase in body temperature by ≥0.5°C, proved the presence of LPS in the test article. The test was performed on 3 animals in the first phase, if any 1 animal showed an increase in basal body temperature then the test article was injected in 5 animals in phase 2 (Dobrovolskaia, Germolec, & Weaver, 2009). However, the risk of false positive always loomed because of the intrinsic pyrogenecity of bio formulations independent of the LPS. In order to reduce the amount of testing in animals, an in vitro testing method was recommended by the FDA, which was accurate, sensitive and precise. The in vitro testing method, which has replaced the RPT in detection of endotoxin approved by FDA, is the Limulus Amoebocyte Lysate assay. Levin and Bang discovered the limulus amoebocyte lysate abbreviated as LAL in 1964 during investigation of the effects of gram-negative bacteria on the blood of horse shoe crab (Limulus Polyphemus) (Levin J, 1964). The blood from the horseshoe crab was found to be clotting in the presence of gram-negative bacteria due to activation of a serine-protease zymogen named factor C triggering a clotting cascade.

The LAL clotting was found to be in direct correlation to the amount of endotoxin (FDA, 1987); this property was utilized to quantify the endotoxin contamination of bio-pharmaceuticals soon after LAL discovery. LAL testing is an effective method to detect endotoxin contamination in vitro and it greatly reduces the burden of cost and labour used for the RPT testing (Dobrovolskaia et al., 2009). The LAL assay was approved for pyrogen testing by the FDA in 1987, owing to its efficacy and sensitivity in the detection of endotoxin in the pharmaceutical products (FDA, 1987). There are three types of LAL assay used in modern times for the endotoxin detection: Gel-clot LAL, Turbidimetric LAL and Chromogenic LAL assay. There are other in vitro tests available that are not approved by the FDA, but are used for detection of endotoxin which utilizes macrophages, mononuclear cells, whole blood or TLR 4 reporter cells (Haile, Puig, Kelley-Baker, & Verthelyi, 2015; Smulders et al., 2012; Warren et al., 2010) sensitive to small amounts of endotoxin, which could be used to qualitatively assess the presence of endotoxins in the test samples. There are two tests recently approved by the FDA as confirmatory tests to LAL: Macrophage Activation test (MAT) and Recombinant Factor-C LAL, which qualitatively confirms the pyrogenecity of the test samples.

2.1.4.1 Discovery and origin of LAL enzyme

As mentioned previously, the Limulus Amoebocyte Lysate was discovered by Bang during the study of coagulation disease in the horseshoe crab Limulus Polyphemus. Initially, it was thought to be initiated by an invertebrate pathogen, but it was discovered that the Limulus crabs were showing intravascular coagulation even after injection of endotoxin from gram-negative bacteria (Williams, 2001). The serum from the haemocytes/ amoebocytes of the crabs when added to the bacteria produced a stable clot. The formation of the clot was compared to the human fibrinogen clotting response. The enzyme is extracted commercially by collecting the haemolymph from horseshoe crab, then the haemolymph is centrifuged and the haemocytes/ amoebocytes are collected (Annamaria, 2012). The amoeobocytes are 15μm in width and contain small and large granules; the large granules contain the gel-clotting serine protease and clotting factors along with a bactericidal anti-LPS factor, which bursts after coming in contact with the bacteria and forms a clot (B. Akbar John, 2010). The small granules contain antibacterials such as tachyplesin, which kills the bacteria after gelation. The mechanism of activation of gel clotting after coming in contact with the bacteria by an amoebocyte is shown in **Figure 2-6** below:

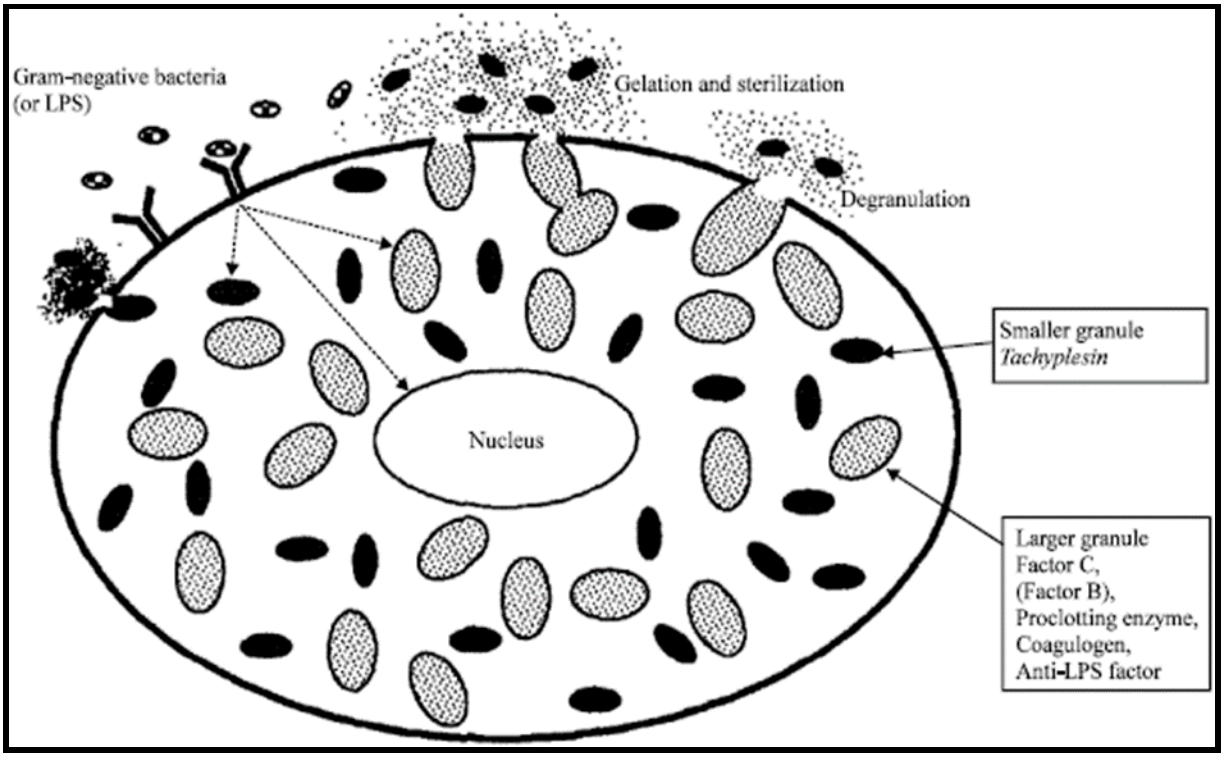


Figure 2-6 : Mechanism of activation of a horseshoe amoebocyte after bacterial invasion. The LPS binding proteins detects the invading LPS, which activates the fusion of large granules to the plasma membrane. The large granules disperse their components in the plasma resulting in the formation of clot when LPS comes in contact with the intracellular components; this gelation leads to immobilization of invading bacteria and then the Anti-LPS factor from large granules and tachyplesin from small granule destroy the bacteria by bactericidal action (B. Akbar John, 2010).

Due to the clotting mechanism by bursting of amoebocyte, the amoebocytes from the haemolymph of horse-shoe crab are washed and lysed in a solution by osmotic pressure during the commercial preparation, and the coagulant, coagulation factors etc. released in the solution are lyophilized at -80˚C to form a powder, which is sold as commercial LAL enzyme (Annamaria, 2012).

2.1.4.2 LAL assay: Biochemical mechanism, different formats and their principles and applications

The LAL enzyme is in fact a complex of factors that activates in a cascade to show clotting or turbidity, there are various components present in this complex as: Factor C, factor B and proclotting enzyme that are serine protease and gel-forming protein coagulogen (Iwanaga, 2007). The extent of clotting depends on the conc. of endotoxin activating the enzyme. The LPS molecule triggers the coagulation cascade by activating factor C a serine protease zymogen that is converted in to activated factor C, which then binds to Factor B and activates it, the activated Factor B converts the proclotting enzyme into clotting enzyme, which in turn converts soluble coagulogen to the insoluble coagulin gel producing turbidity (Sandle, 2013). This mechanism is endotoxin specific, and the degree of clot formation depends on the amount of LPS activating the cascade.

There is an alternative mechanism, which could also activate the LAL complex to produce similar clotting or gel-formation. This mechanism involves Factor G, which is activated by β-(1-3)-D-Glucan- a component commonly present in cellulose and glucosamine based substances, the activated factor G triggers the proclotting enzyme that activates the clotting enzyme, which then subsequently convert insoluble coagulogen into coagulin gel (Sandle, 2013). Since, this pathway is unspecific to endotoxin; the presence of glucans containing substances could lead to false-

positive results when performing Bacterial Endotoxin Tests (BET). There are commercially available buffers which inhibit the β-(1-3)-D-Glucans activation of LAL enzyme and makes the complex specific for endotoxin activation, examples of such buffers include the glucashield buffer supplied by Associates of Cape Cod and β-G glucan blocker supplied by Lonza, the ingredient inhibiting the glucans in these buffers are propriety and undisclosed.

The mechanism of activation of LAL complex by endotoxin and alternative pathway are shown in **Figure 2-7** below:

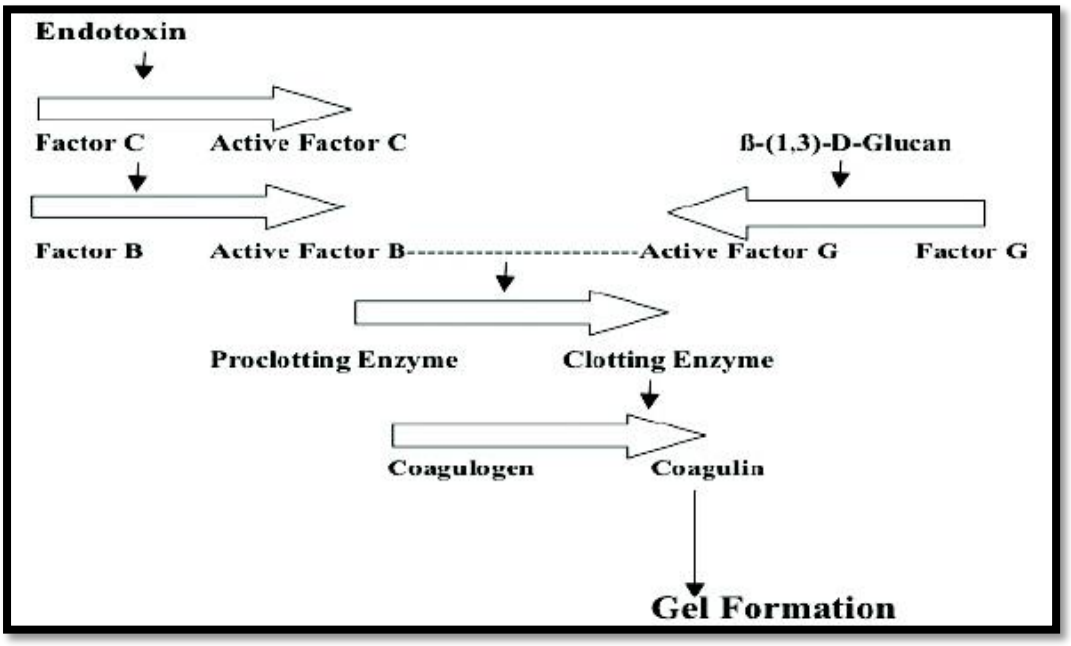


Figure 2-7 : Biochemical mechanism of LAL cascade activation after introduction to endotoxin (Sandle, 2013), exploited for semi-quantitative and quantitative determination of endotoxin by the extremely sensitive LAL assays.

The cysteine rich coagulin formed at the end of the cascade are released as monomers with NH2-terminal A chain which is connected by disulfide bonds with the COOH terminal B chain and an extended hydrophobic core covered by peptide C helix which is uncovered after cleavage and release of peptide C. (Iwanaga, 2007). This newly uncovered hydrophobic cove of one molecule is hypothesized to interact with the hydrophobic edge of the second molecule in a head to tail fashion and forms dimers, trimers and multimers (Iwanaga, 2007) as shown in the **Figure 2-8** below, these chains are the basis of formation of turbidity in a LAL reactive solution:

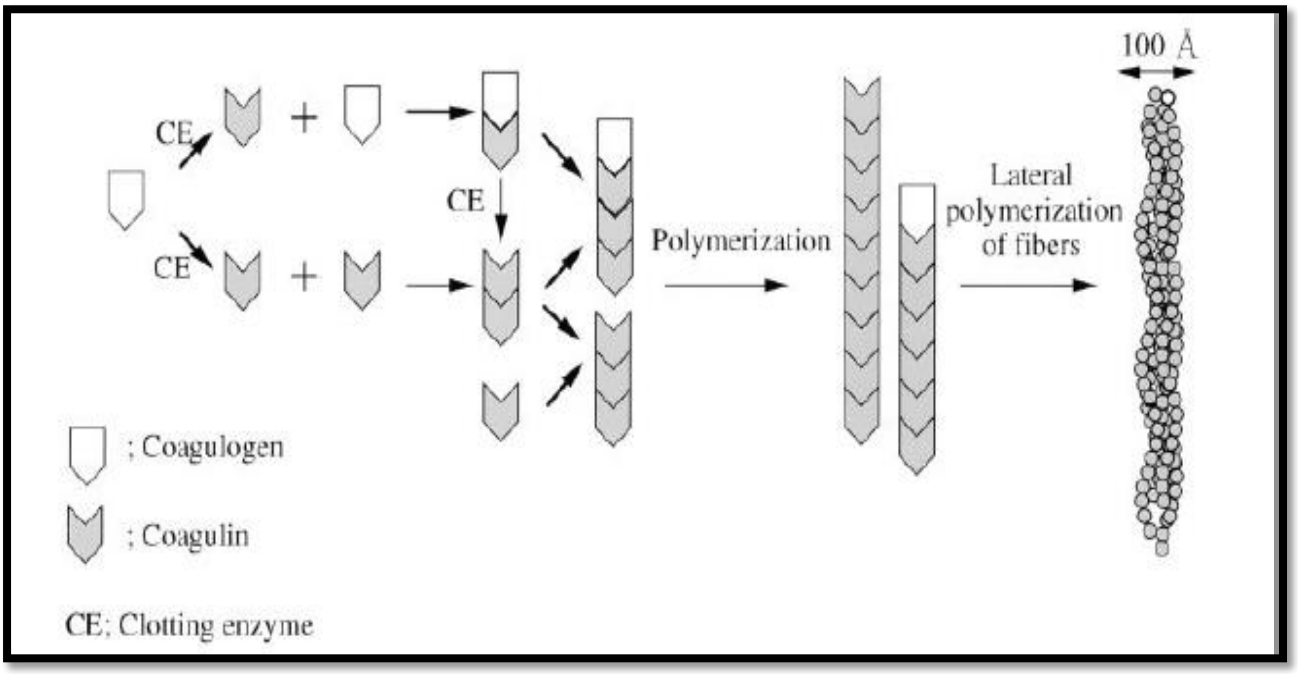


Figure 2-8 : Hypothetical polymerization of coagulin monomers for the formation of a coagulin chain- the basis of turbidity formation in a LAL reactive sample (Hiroko Kawasaki, 2000).

The property of LAL complex to form gel-clot in the presence of LPS, is exploited for quantification of endotoxin, and as mentioned in previous section, it is the FDA approved method for Bacterial Endotoxin Testing (BET) and is most effective and sensitive in vitro method, the results of which are corroborated by other assays (RPT). The sensitivity of the assay towards endotoxin is manipulated commercially depending upon the format of LAL. There are different formats of LAL used currently, pertaining to the development of synthetic chromogenic substrate; different LAL formats are described below (Joiner, Kraus, & Thomas C. Kupiec, 2002):

1) Gel-clot assay: The gel-clot was the first format of LAL testing used by the pharmaceutical companies, and until recently has been widely used method for endotoxin testing (Dobrovolskaia et al., 2009). The LAL enzyme after coming in contact with endotoxin of the test samples undergoes coagulation cascade and forms a firm gel-clot, which could be classified as negative or positive depending on its integrity upon inversion. The commercial gel-clot kits are supplied with different sensitivity labelled Lambda (λ) and ranges from 0.03-0.25 EU/mL (ACCI, Product Sheet) . The test is semi-

quantitative and helps in estimation of the endotoxin content of the test samples above or below the labelled sensitivity. If the gel-clot test is positive for a sample, then the endotoxin conc. can be affirmed more than or equal to the labelled sensitivity or $\geq$ Lambda (λ), and if the gel-clot is negative then the endotoxin conc. in a test sample is designated less than or equal to the labelled sensitivity or $\leq$ Lambda (λ) (ACCI, Instruction guide).

2) Kinetic Turbidimetric LAL assay: The kinetic turbidimetric LAL assay is a quantitative method of endotoxin detection and depends on the formation of turbidity of LAL enzyme upon coming in contact with endotoxin. The development of turbidity can be observed kinetically at given time intervals for a specified period of time in a spectrophotometer at a wavelength of 405nm, this feature prevents false results and aids in interpretation. In order to quantify the sample endotoxin, a standard of known amount of endotoxin is constructed from the commercially supplied Control Standard Endotoxin (CSE). The sample endotoxin conc. is interpolated from the linear equation obtained from the regression analysis of log conc. vs. log onset time (defined as time to reach a pre-determined threshold O.D). The sensitivity of kinetic turbidimetric LAL assay is 0.005 EU/mL. The sensitivity of the assay is enhanced by inhibiting the alternative Factor-G pathway activated by β-D glucan causing false positive results due to activation of coagulation cascade, this pathway is inhibited by specialized buffers supplied by vendors Eg; Glucashield Buffer supplied by ACCI. A flowchart of endotoxin quantification by kinetic turbidimetric LAL assay detailed by Lonza aids in the understanding of the assay at the bench level:

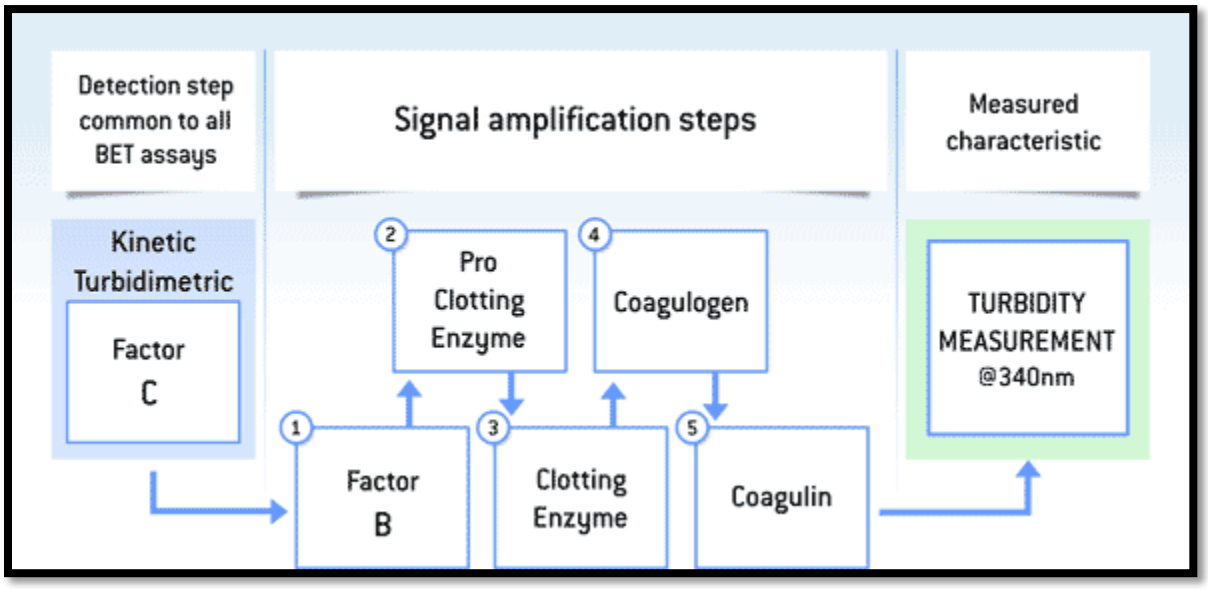


Figure 2-9 : Flowchart of the kinetic turbidimetric LAL assay, the development of turbidity and measurement of turbidity, which is accomplished at 405nm in this thesis (recommended by ACCI) without any drawbacks, cited from www.lonza.com.

3) Chromogenic LAL assay: Like turbidimetric, chromogenic LAL assay has the sensitivity of 0.005 EU/mL in a plate reader. The chromogenic assay is a modified version of turbidimetric LAL assay, in that the clotting enzyme is linked to a synthetic chromogenic substrate Gly-Arg-p-Nitroanilide (pNA), which is cleaved in to Gly-Arg and p-Nitroanilide forming yellow colour after coming in contact with endotoxin. The formation of yellow chrome is directly correlated with the concentration of endotoxin and measured by the spectrophotometer at 405nm. The chromogenic LAL assay has two formats: Kinetic and Endpoint. In a kinetic reaction, the development of chromogenic substrate is recorded over time, and the endotoxin conc. of a test sample is determined by interpolation from a linear equation after regression analysis of a Log conc. vs. Log onset time standard curve. There are two formats of Endpoint chromogenic LAL available commercially: p-Nitrolaniline and the Diazo coupling option; the p-Nitroaniline works on the same principle as explained above and the color development is measured at the end of the reaction time; but in the second option, the p-Nitroaniline is coupled with a Diazide, which gets converted in to a Magenta colored Azo dye after cleavage by the cascade in presence of endotoxin. The absorbance of the p-NA is measured at 405nm and the Diazo-coupled LAL is measured at 545nm at the end of the reaction time, which could

be used in the measurement of colored samples with absorbance maxima near 405nm. Endpoint chromogenic is a faster method and allows absorbance measurement after a fixed amount of time, but the disadvantage is the high probability of a false positive/ false negative result at a particular measurement time, these results could occur in a sample with fluctuating absorbance at different times. Depending upon the type of samples, the option of kinetic or endpoint must be chosen to detect the endotoxin contamination. The method of standard construction and result interpolation in kinetic chromogenic assay is similar to the kinetic turbidimetric assay (Joiner et al., 2002).

The flowchart of the chromogenic assay, demonstrated by Lonza on their website helps in understanding the mechanics of the assay:

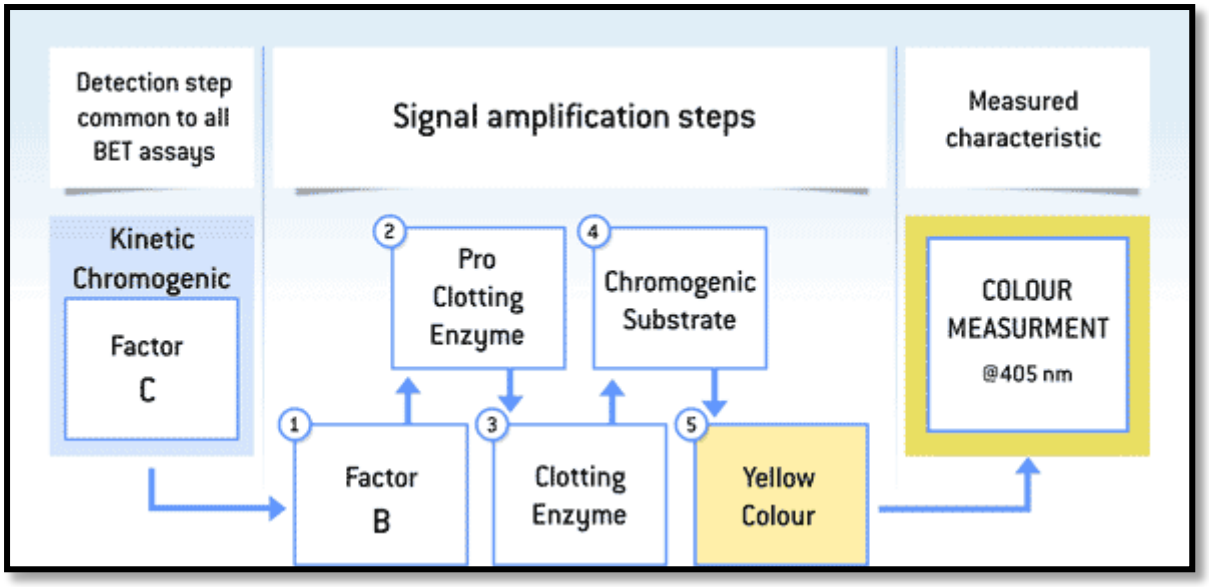


Figure 2-10 : Flowchart of the kinetic chromogenic assay and development of color after activation and its measurement at 405nm, cited from www.lonza.com

2.1.4.3 Selection of LAL assay for Endotoxin detection

Traditionally, the LAL assay was used to detect the endotoxin contamination in medical devices and active pharmaceuticals. With the advent of nanotechnology and application of nanoparticles for several purposes such as nucleotide cargo delivery (Moghimi & Szebeni, 2003), drug delivery (Jong & Borm, 2008), use as sensors and probes (Shi, Li, & Ma, 2014) etc. the nanoparticles can well be classified as new class of active pharmaceuticals. There have been

many reports of interference by nanoparticles in the detection of endotoxin by LAL assay (Smulders et al., 2012) such as quantum dots and metal nanoparticles that are colored and have an absorbance wavelength at 405nm which interferes with chromogenic LAL assay and liposomes, emulsions and PLGA nanoparticles that have turbid formulations interfering with turbidimetric LAL assay (Dobrovolskaia et al., 2009). There are other new materials, previously uncharacterized that also interact and interfere with the endotoxin detection by LAL assay. The interfering substances and nanoparticles interfere with endotoxin reactivity, LAL enzyme cascade/ LAL proteins or the reaction products (turbidity/ chromogenecity) (Neun & Dobrovolskaia, 2011). These interferences may present as enhancement or inhibition of the respective endotoxin concentration of a sample; this manifests as false positive and false negative and may lead to erroneous rejection or acceptance of the sample batch (Neun & Dobrovolskaia, 2011). A false positive result may lead to rejection of nanoparticle or material with good potential in therapeutics, whereas if the sample with false negative is advanced to clinical application, it may lead to preposterous immune reaction in higher mammals, leading to septic shock and even death.

Hence, in order to overcome the interferences and choose the best method for the accurate and absolute detection of endotoxin contamination of the nanoparticles and/or interference producing samples, a group from National Institute of Health (NIH) have laid out the guidelines to follow:

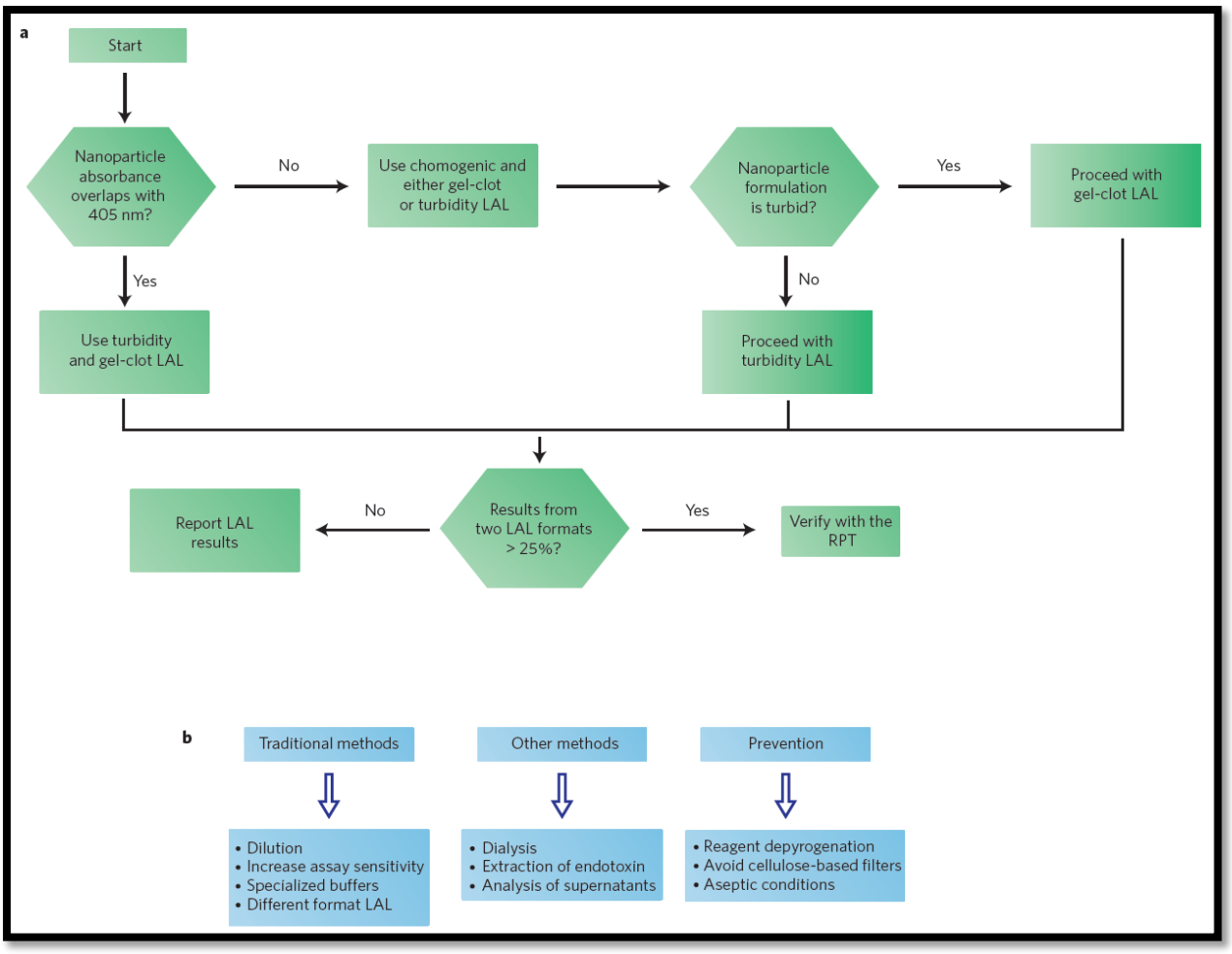


Figure 2-11 : Decision tree for selecting appropriate LAL method for testing endotoxin contamination in nanoparticles (a). Different approaches to remove interference are also shown in part b (Dobrovolskaia et al., 2009).

The selection of an appropriate method depends on the physicochemical properties of the test sample, and the possible interaction mechanism it might have with the LAL chemistries. In case of chitosan, the only interaction predicted are with the endotoxin, the characteristics of which are explained in the next section. Hence, the chitosan endotoxin was tested by all the three formats of LAL assay.

2.2 Chitosan as a gene delivery vector and tissue regeneration scaffold

Chitosan is a positively charged biodegradable polymer, which has many applications in biomedical and tissue engineering. Chitosan is most commonly used as natural or artificial scaffold for cartilage tissue regeneration, due to its property to act as glycosamino glycans (GAGs) one of the important cartilage components (Fan, 2014). They also find their application in osteo-arthritic models. The scaffolds are used in combination with growth factors such as Platelet-Rich Plasma (PRP) (Oktay et al., 2010) and autologous cells such as Mesenchymal Stem cells (MSCs) (Thomas G. Koch, 2009) or blood to effectively act as a tissue regeneration scaffold. The scaffolds have been shown to successfully induce the tissue repair in various animal models such as rabbits (Oktay et al., 2010), sheep and horses (Thomas G. Koch, 2009).

Chitosan also finds application in nanotechnology, where chitosan at high DDA and low molecular weight (Alameh et al., 2012) are used as vectors for the delivery of nucleic acids such as siRNA, mRNA and plasmid DNA. Chitosan nanoparticles have established a base in applied research, and extensive use of Chitosan nanoparticles has been observed from past ten years (Pubmed citations). The positively charged chitosan forms a complex with negatively charged nucleic acids, the nanoparticles after intravenous, intraperitoneal or subcutaneous administration in vivo enter the target cells, where the nucleic acid cargo is released by the action of endosomes (Wittrup et al., 2015) In our group, the efficiency of Chitosan-siRNA nanoparticles and chitosan-pDNA nanoparticles have been demonstrated in small animals such as mice (Jean et al., 2012).

Chitosan is derived from chitin, which comes from crustaceans and fungi. Chitin is a naturally occurring polysaccharide extracted from exoskeleton of marine crustaceans such as shrimps and crabs or cell walls of fungi. Chitin is deacetylated and depolymerised to produce chitosan with different degrees of deacetylation (DDA) and molecular weight (MW) having different potentials for delivery of nucleic acid and performance of scaffolds in vivo (Buschmann et al., 2013). In order to understand the characteristics of chitosan important for its application as a scaffold and a delivery vehicle, various characteristics must be understood as discussed below.

2.2.1 Degree of Deacetylation (DDA)

DDA is a very important parameter allowing the knowledge of average amount of amine and so positive charge available to interact with the negative charge of the nucleic acids. DDA

influences the chitosan nanoparticle stability, inflammation and immune modulation in biological systems (Buschmann et al., 2013) and the degree of biodegradation, healing capacity and performance of chitosan scaffolds for tissue engineering application (Hoemann et al., 2007). The importance of DDA in chitosan nanoparticle preparation is emphasized, as the endotoxin interaction with chitosan mimics that of nucleic acid. The DDA is an important parameter that can be manipulated to improve the nucleic acid delivery. A wide range of DDA can be produced by adjusting the time and temperature of alkaline deacetylation reaction of chitin. The release of acetyl group from chitin exposes the NH_2 group, on the glucosamine D-unit, which, after protonation, binds to the phosphate group of DNA/RNA by ionic linkage. Higher deacetylation of chitosan leads to elevated toxicity of the chitosan nanoparticle in biological systems, but it is also dependent on molecular weight and concentration (Buschmann et al., 2013).

Molecular weight (MW) of the chitosan can also be manipulated to improve biocompatibility and bio distribution of chitosan nanoparticles in in vivo systems.

2.2.2 Molecular Weight (MW)

Molar mass determination of chitosan is also an important characteristic to understand the average number of monomers per chain and the dispersity related to actual distribution of chain lengths in the chitosan (Buschmann et al., 2013).This important parameter will influences the structural properties like size and morphology; and the physicochemical properties of chitosan polyplexes. Molecular weight also influences chitosan polyplex aggregation, a property that plays a major role in entrapping endotoxin between the chitosan polyplexes. Aggregate formation is influenced by acetyl group and degree of ionization (Buschmann et al., 2013; Lavertu, Methot, Tran-Khanh, & Buschmann, 2006).

2.2.3 Binding affinity of chitosan

Chitosan binds to lipopolysaccharide (LPS) by both electrostatic interaction and hydrogen bonds. The lipid A portion of LPS is hydrophobic which does not interact with chitosan, but the core-oligosaccharide and the O-antigen binds to the chitosan due to the negative charge on the sugars; the chitosan may also interact with the negatively charged phosphate group present on the Lipid A. The degree of interaction of negatively charged oligosaccharides with the glucosamine chains of chitosan depends upon the concentration of positively charged amine residues on each

monomer exposed by the degree of deacetylation of chitosan. The structure of LPS from E. coli, bacteria from which the reference standard endotoxin (RSE) is prepared by USP is shown in **Figure 2-12**.

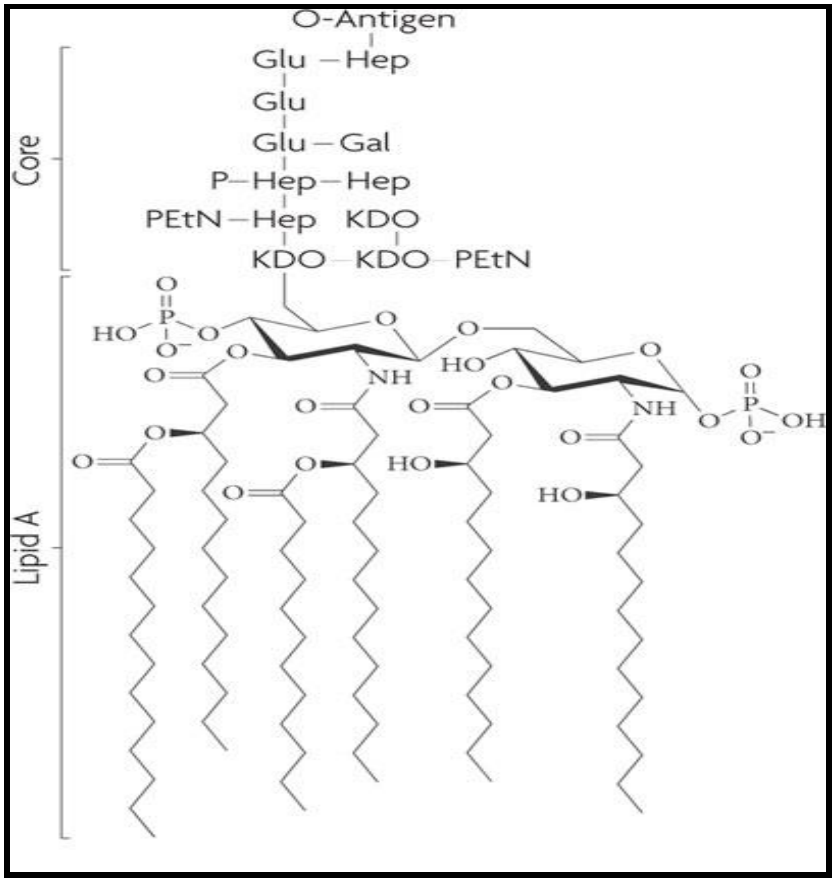


Figure 2-12 : Structure of E. coli amphiphilic LPS (Ruiz, Kahne, & Silhavy, 2009) present on outer membrane with hydrophobic Lipid A portion linked to the core oligosaccharides by the 8-carbon sugar 3-Deoxy-D-manno-octulosonic acid (Kdo). The core oligosaccharides are the repeat of 6 basic sugars as: Hep (L -glycerol- D -manno-heptose), Glu (Glucose), Gal (Galactose), Gal-N-Ac (N-acetyl-Galactosamine), GlcN (glucosamine) and GlcNAc (N-acetylglucosamine). The characteristic of a smooth LPS is the presence of O-antigen, which contains 5-40 repeats of specific sugars, that varies with different bacterial species (Bagheri, Keller, & Dathe, 2011).

The LPS can bind to chitosan with Low molecular weight (LMW) and High Molecular weight (HMW) chitosans at variable rates. The chitosan-LPS complex leads to reduction in the toxicity of LPS due to the screening of the toxiferous group (Lipid A) and changes in the macromolecular organization of endotoxin by disaggregation or monomer formation before

binding (I. M. Y. V. N. Davydova*, V. I. Gorbach, I. N. Krasikova, and T. F. Solov'eva, 2000). The hydrophobicity of LPS also plays a complicated role in the interaction with chitosan; decrease in the length of carbohydrate chain as in rough LPS leads to formation of aggregates with different LPS molecular mass. The binding of LPS with chitosan depends on the different macromolecular organizations of the LPS and the ability of chitosan to destroy the carbohydrate-carbohydrate interactions between long O-polysaccharide chains of LPS by its hydrophilicity (I. M. Y. V. N. Davydova*, V. I. Gorbach, I. N. Krasikova, and T. F. Solov'eva, 2000).

2.2.4 Preparation and treatment of chitin for production of chitosan and resulting endotoxin removal

The raw material obtained from the shells of crustaceans and insects is processed for the production of chitin using the process illustrated in the flowchart in **Figure 2-13**. The grinding and sieving of the raw material is necessary to improve the yield of chitosan and removal of unwanted product. Depending on the targeted molecular mass and DDA of chitin, the subsequent steps may be adjusted; the demineralization and deproteinization steps are performed next to remove the minerals and proteins respectively. The deproteinization step is performed in mild alkaline conditions, where typically, NaOH is used that may influence the DDA of resulting chitosan; the demineralization step removes salts such as CaCO3 and Ca_2PO_4 by acidic treatment typically by HCl, which may result in hydrolysis of polymers if the treatment is too strong. Depending upon the source, the decoloration step is carried out before drying or further treatment (Buschmann et al., 2013).

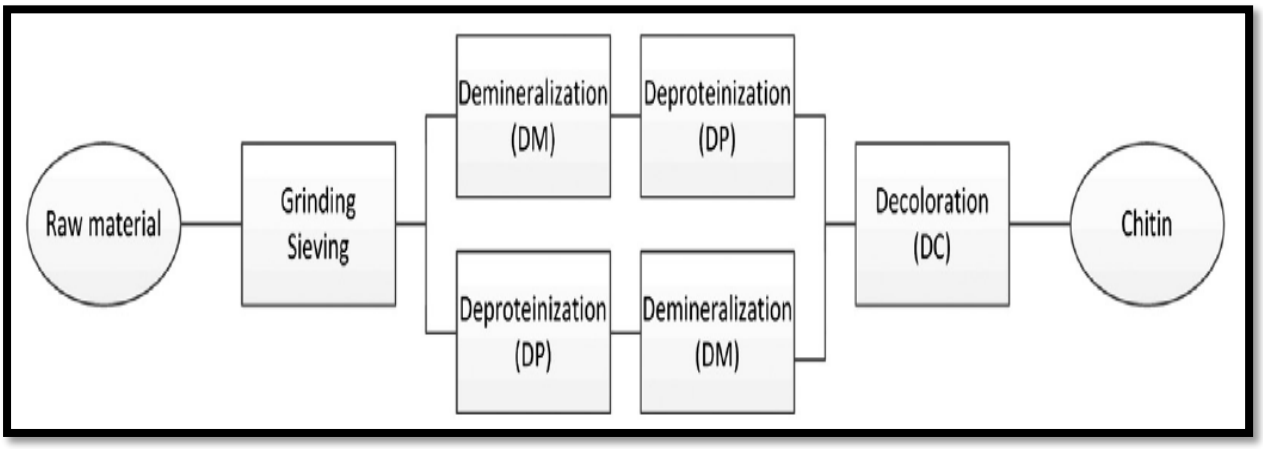


Figure 2-13 : Production of chitin from the raw materials obtained from marine source. The steps of are illustrated as a flowchart (Buschmann et al., 2013).

After production, the chitin is deacetylated and depolymerized for the production of chitosan. The chitosan is produced by alkaline deacetylation of chitin in a hot alkaline solution of 40-50% w/w NaOH, the time, temperature of the treatment will affect the distribution of N-acetyl, and amine chains on the chitosan chain i.e. different degree of deacetylation (Buschmann et al., 2013). The use of high conc. of NaOH leads to the removal of endotoxin present indigenously on the chitin (note, 2001). The depolymerisation of chitin is performed to obtain short monomeric chains with small molar masses. The depolymerisation cleaves the glycosidic linkage between the disaccharide units of the chitosan glucosamine chain by the catalytic action of nitrous acid in the presence of HCl at moderate temperature(Buschmann et al., 2013). The application of acid and heat on the chitosan may lead to acidic hydrolysis of endotoxin present on the chitin or chitosan, resulting in the production of low endotoxin chitosan (Sandle, 2011). Such harsh chemical treatment during the production of chitin as well as chitosan ensures the fulfillment of quality control mandate for low endotoxin contamination in the final product. The chitosan produced is used in various formulations such as Chitosan-siRNA nanoparticles and Chitosan-Hyaluronic Acid (HA/CS) or Chitosan-glycerol-phosphate (GP/CS) scaffolds for application in gene delivery and tissue regeneration respectively. The process of production of chitosan from chitin and subsequent formulations for different application is shown in **Figure 2-14**.

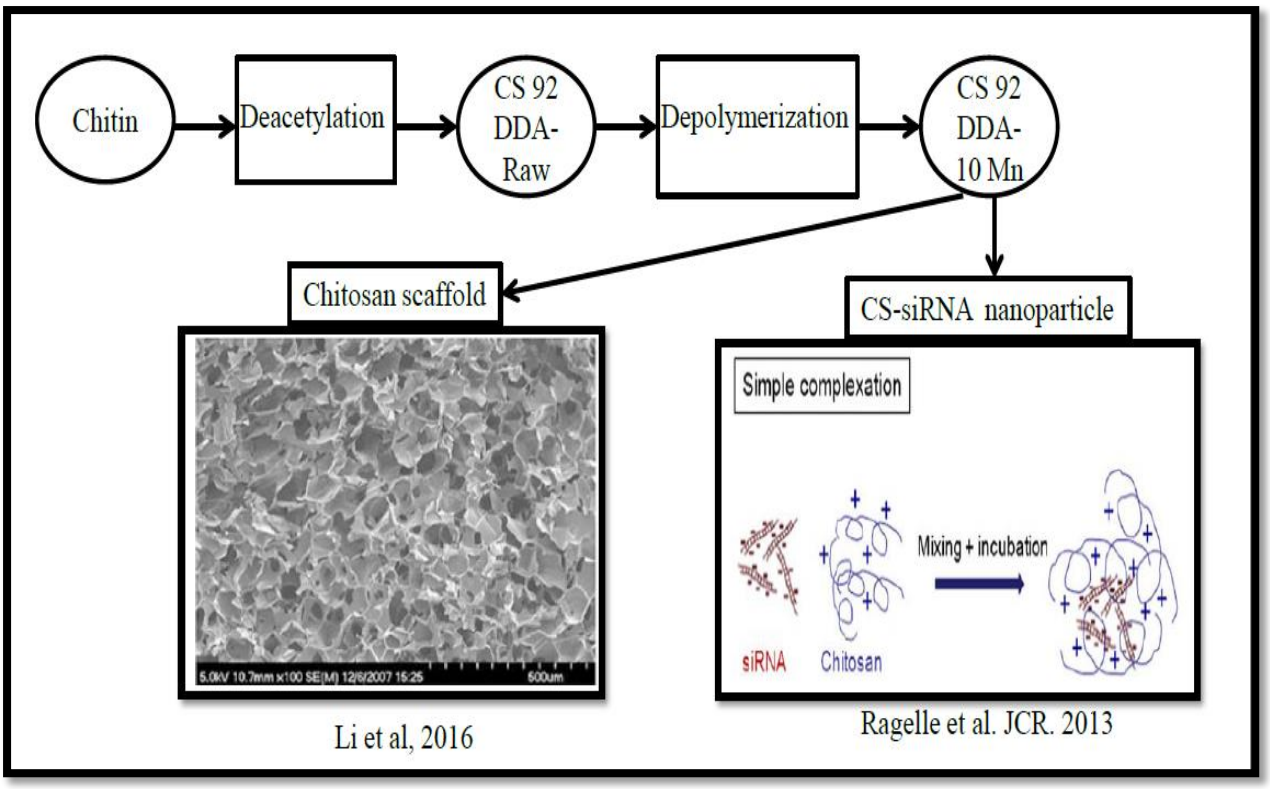


Figure 2-14 : Steps of chitosan production from chitin and its formulation in CS-siRNA nanoparticles and chitosan scaffolds for the gene delivery and tissue regeneration applications respectively. Figures adapted from (Liu et al., 2015; Ragelle, Vandermeulen, & Preat, 2013).

After the quantification of the chitosan raw materials, if the EU conc. is detected at elevated level then other methods targeting the electrostatic binding and hydrogen bonds between chitosan and endotoxin must be adapted and may be used just before endotoxin quantification by LAL assays. Such treatments are called as pre-treatments, denoted so because of their use before the endotoxin quantification by LAL assay.

Based on the problems discussed in this section, the hypotheses and objectives addressed in this thesis are outlined in the next chapter.

CHAPTER 3 THESIS OBJECTIVES AND HYPOTHESIS

Due to the ubiquitous nature of endotoxin, it can be naturally found on any surface, solution, substance and even on human body; however, the contamination of endotoxin during the production process of an active pharmaceutical ingredient (API) is a nightmare for the quality control units and without detection may cause life-threatening immune reaction if the API is administered in humans. With the in vivo use of chitosan and intended application in human subjects, the chitosan is regarded as an API in this thesis, and the absolute quantification of endotoxin contamination is essential for controlling the dose of administration of scaffolds and CS nanoparticles as well as for the verification of the manufacturing process of the chitosan. Hence, the overall objectives of this thesis are described below:

3.1 Overall Objectives

1) To quantify EU concentration of powdered chitosan raw materials, especially CS with 92% degree of deacetylation with high and low molecular weight (10kDa) used for the preparation of chitosan scaffolds and CS-siRNA nanoparticles administered in horses and mice respectively.
2) Obtain a repeatable standard curve range in kinetic LAL assays for absolute quantification of chitosan endotoxin.
3) Reduce the interference of chitosan in endotoxin detection by performing chitosan pre-treatments for absolute quantification of chitosan endotoxin.
4) Provide recommendations for future endotoxin testing in chitosan.

3.1.1 Specific objectives for obtaining repeatable ranges of standard curves for chitosan endotoxin quantification

Objective 1: To investigate the endotoxin conc. of chitosan raw materials by gel-clot LAL assay to obtain an estimation of contamination and test different ranges to establish the most repeatable and less variable standard curve for absolute quantification of chitosan endotoxin.

Objective 2: To investigate the technical parameters such as reagent evaporation and pathlength contributing to standard curve variability and mitigation of variation by optimization of these parameters.

3.1.2 Specific Hypotheses of this study

Hypothesis 1: A repeatable range of standard curve with minimum allowable variation will be determined after examination and optimization of all parameters prone to variation.

Hypothesis 2: Adopting a small range of standard curve after optimization matching the estimated value of chitosan endotoxin will be highly repeatable.

After the reduction of the variation accounted by different parameters and their optimization, and establishing a standard curve range with lowest variation and high repeatability, the absolute quantification of chitosan will be performed.

However, chitosan is known to interact efficiently with the LPS due to the presence of negative charge and hydrogen bonds, similar to the mechanism observed with nucleic acids. This interaction signifies the interference of chitosan in the detection of endotoxin, which may lead to false negatives having the potential to elicit alarming level of cytokines once administered in clinical subjects. However, there are certain pre-treatments adopted for reduction of interference between chitosan and endotoxin for absolute quantification. Therefore, the objective for Chapter 4 is as follows:

3.1.3 Specific objectives for identification and reduction of interference in endotoxin detection by chitosan Pre-treatments

Objective 1: To identify different types of interference caused by chitosan to endotoxin and/or LAL by incorporating controls such as challenge samples and spiked water samples.

Objective 2: To reduce the inhibition interference in endotoxin determination by performing chitosan pre-treatments targeting electrostatic binding and monomer size for absolute quantification of endotoxin content in chitosan raw materials.

3.1.4 Specific hypotheses of this study

Hypothesis 1: The pre-treatment of chitosan will release endotoxin bound to the positively charged and large glucosamine chains and facilitate absolute quantification of endotoxin.

Hypothesis 2: The harsh chemical treatment used in depolymerisation reaction will be deleterious to the endotoxin integrity and reduce detection.

This approach will allow the quantification of otherwise difficult chitosan raw materials' EU conc. This approach will broaden the understanding of complexities of interaction between chitosan and endotoxin and carries the potential to provide recommendations for establishing the quality control process for quantification of endotoxin in chitosan.

CHAPTER 4 OPTIMIZATION OF PARAMETERS TO OBTAIN REPEATABLE LAL STANDARD CURVE

This chapter presents the estimation of chitosan endotoxin values by gel-clot LAL assay, determination of endotoxin conc. of chitosan nanoparticles and scaffolds dose after simulation to Human equivalent dose (HED). Testing of various standard curve ranges of the more sensitive turbidimetric LAL assay for absolute quantification of chitosan endotoxin and optimization of parameters to obtain a repeatable standard curve in a plate reader for turbidimetric LAL assay.

4.1 Introduction

Due to application of chitosan in various biomedical applications in the form of nanoparticles and scaffolds, the quantification of endotoxin in raw material is necessary. The Chitosan (CS) with 92% degree of deacetylation (DDA) with High (Raw) and Low molecular weight (10kDa) are tested for endotoxin with the gel-clot LAL assay. The gel-clot LAL assay provides an estimation of endotoxin value above or below the range of its sensitivity. This estimated value is used for calculation of the endotoxin conc. of clinical/ in vivo doses in chitosan nanoparticles and scaffolds. Further efforts have been taken to quantify the absolute conc. of endotoxin present in the chitosan raw materials by the use of highly sensitive turbidimetric LAL assay. However, the variation of standard curves at different ranges interfered with absolute endotoxin quantification, but allowed the investigation in to contributing parameters to variation. A significant problem in quantifying the endotoxin level of any test article by LAL assay is the non-repeatability of standard curve due to inherent variations imperative in the biological assays, compared to the chemical assays. The error of a LAL test is two-fold, which represents a range of 50-200% variation (McCullough, 2010). Various troubleshooting methods are utilized in this section to produce a repeatable and reliable standard curve.

4.2 Materials and Methods

4.2.1 Estimation of Chitosan Endotoxin value by Gel-Clot LAL assay

The gel-clot assay was performed to estimate the endotoxin contamination of chitosan 92DDA- Raw and chitosan 92 DDA-10kDa MW, the latter was processed from the former and used in the animal studies. The chitosan samples were dissolved according to the method recommended by the supplier i.e. Associates of Cape Cod (ACCI) for endotoxin detection in chitosan in 0.2% acetic acid at 37°C for 1 hour at a conc. of 10mg/mL.

The gel-clot estimation of EU conc. was performed using the Pyrosate kit (Kit# PS14005-10) obtained from Associates of Cape cod (ACCI); the sensitivity of the kit was labelled at 0.03 EU/mL, denoted as lambda (λ) which is the EU conc. resulting in the formation of a firm clot unbreakable after inversion, called as positive clot. The kit contained 10 blue capped vials with lyophilized LAL enzyme for direct dissolution in sample, instead of reconstitution in pH adjusting glucashield buffer, and 10 red capped Positive Product Control (PPC) vials with the CSE at EU conc. of 2λ, the PPCs act as the detectors of inhibition or enhancement interference by the samples to endotoxin during the LAL assay. The samples were diluted 1:100 times to a final conc. of 0.1mg/mL in the glucashield buffer to adjust the sample pH for optimal LAL reactivity, then 0.5mL pH adjusted diluted sample was added in duplicate in blue capped vials with LAL enzyme. The LAL enzyme was mixed in the sample solution by shaking the tube rack for 20-30 seconds and from these vials, half the reconstituted volume of sample+ LAL at 0.25mL was transferred to the red capped PPC vials and mixed thoroughly with the CSE at a conc. of 2λ [0.06 EU/mL] to prepare the PPCs. Similar procedure was followed for LRW without buffer dilution to obtain a negative and positive control with blue capped LRW+LAL vials acting as negative control and red capped LRW+LAL+ 2λ CSE forming the PPCs, the inclusion of positive and negative control is the pre-requisite for validation of any format of LAL assay. The vials were then incubated in a non-circulating water bath at 37˚C for the recommended time of 48 minutes to observe the clot formation, disturbance from the surroundings could lead to misrepresented results and all the measures were taken to reduce it. The formation of gel clot in the vial was inspected and the vials with no visible clot were scored as negatives, while the ones with a firm gel-clot unbreakable by 180˚ inversion of the vial were scored positive. When a vial shows a gel-

clot that looked firm, but is disrupted after 180˚ inversion then the sample is deemed to have endotoxin below sensitivity level, and scored negative.

Validation criteria:

a) The positive control i.e. 2λ CSE+ LRW+ LAL must always have positive clotting for the assay to be valid.

b) The negative control (LRW +LAL) must always show negative clot in a valid assay.

Interpretation of results:

i) The PPCs of a non-interfering sample will show positive clotting; a negative PPC result indicates interference by the sample towards endotoxin or LAL enzyme.

ii) In case of negative clotting, the sample EU conc. was reported as less than or equal to labelled sensitivity $\leq$0.03 EU/mL

iii) In case of positive clotting, the sample conc. was reported as greater than or equal to labelled sensitivity i.e. $\geq$ 0.03 EU/mL

Based on the estimated value of EU conc. of CS 92-Raw and CS 92-10 samples, the concentration of endotoxin in the minimum and maximum dose of injected CS-siRNA nanoparticles was simulated after conversion from mouse to Human Equivalent Dose (HED).

4.2.2 Simulation of chitosan injected dose in animals to Human Equivalent Dose (HED) and estimation of endotoxin conc.

The most important parameter to regulate in the active pharmaceutical Ingredient (API) injected in humans is the amount of endotoxin entering the body. As emphasized in the literature review, even the smallest amount of endotoxin entering the human circulatory system can cause lethal immune response leading to septic shock and death. Therefore, there are strict limitations imposed by the USP for the endotoxin content of medical devices and formulations used in humans. The EU limit for formulations used in intravenous application is 5 EU/kg/hour and 0.2 EU/kg/hour for intrathecal application.

The minimum dose of chitosan-siRNA nanoparticles formulated for estimation of cargo delivery efficiency and chitosan toxicity in mouse models was 1mg/kg of siRNA; this dose

translated to 2.8mg/kg of injected chitosan at an N: P [Amine (N) from CS: Phosphate (P) from siRNA] ratio of 5. The chitosan was used at five-fold higher conc. than siRNA to increase polyplex uptake by the cells (Thibault et al, 2011). Both the chitosan and siRNA doses were converted to the human equivalent dose (HED) using the conversion factor of 12.3 recommended by the FDA. The HED of chitosan was then multiplied by the estimated EU conc. obtained from gel-clot assay and that of siRNA was multiplied with the EU conc. obtained from the certificate of analysis. The additive EU conc. of the HEDs was then compared with the acceptable limit of 5 EU/kg/hour approved by the USP for its suitability as an API formulation for intravenous injections.

Similarly, the maximum tolerated dose (MTD) of chitosan-siRNA nanoparticles in mice coated with Hyaluronic acid (HA) to facilitate the uptake, reduce chitosan-induced toxicity and increase the cargo load was 8 mg/kg of siRNA. The N: P: C ratio [Amine (N) from CS: Phosphate (P) from siRNA: Carboxylic acid (C) from HA] used for this formulation was 2:1:1.5, this translated the dose of chitosan to 8.85 mg/kg and that of HA to 15.27 mg/kg. All the siRNA, chitosan and hyaluronic acid doses were converted to HEDs and the EU conc. in EU/kg of each component was calculated, for chitosan EU content the estimated EU conc. obtained from gel-clot assay was used. The suitability of this dose for human application was then confirmed by comparing the additive HED EU conc. with the acceptable EU limit issued by the USP for intravenous injection (5 EU/kg/hour).

The endotoxin concentration of the 0.1mg/kg dose of chitosan used as scaffolds in the intra-articular injection of horses was also calculated by multiplying with the estimated 0.3 EU/mg value of chitosan obtained from gel-clot assay; this dose was not converted into HED, because the acceptable EU limit for human intra-articular injections is unavailable. The calculated EU value was then compared to the harmonized limit (0.1 EU/kg) accepted for intra-articular injection in LPS sensitive horses by different authors (Carregaro et al. 2014 and Lucia et al. 2013).

4.2.3 Use of more sensitive kinetic LAL assays for chitosan endotoxin determination

As the EU conc. of chitosan samples obtained from the gel-clot is an estimated value, it was necessary to quantify the absolute concentration of endotoxin in chitosan, because of its application in LPS sensitive animals like horses and final clinical application in rather sensitive humans. The harmonized acceptable EU limit for intra-articular injection in horses is very low (0.1 EU/kg) and in order to have a conservative EU value to avoid an immune response in future clinical use in humans this value will be further reduced by a safety factor of 10 (Lazar et al, 2009). For a 70 kg human, the upper acceptable limit will be just 0.7 EU, instead of 30 EU for an average 300kg horse which will lead to rejection of chitosan for use as a pharmaceutical ingredient based on the calculated EU conc. of chitosan dose (9 EU in 30mg dose) from the estimated EU value by gel-clot. In order to reach such level of precision with endotoxin quantification of chitosan used as scaffolds, using an estimated EU value from gel-clot assay seem very risky.

On the other hand, from the perspective of quality control, the value of 300 EU/g is very high for a clinically applicable product; the cleanest chitosan available commercially, has an EU value of 100 EU/g i.e. 3 times lower than our product. In order to compete commercially, it is a pre-requisite to have the information about absolute EU conc. of the chitosan produced in-house. If the EU conc. after precise quantification is at a lower limit, then the product specification would claim lower endotoxin content; but if the absolute EU conc. of in-house chitosan is higher than the commercial one, then optimization of manufacturing, deacetylation and other downstream processing may be initiated to produce a chitosan batch with ultra-low endotoxin. Indeed, it is essential to obtain an accurate and absolute conc. of endotoxin to establish the product specification of the in-house chitosan batches.

The disadvantage of using an estimated EU value is that it may lead to overestimation of the chitosan EU conc., which may lead to its rejection before the market release. Hence, in order to avoid false-positive results, it is extremely important to quantify the absolute EU conc. of chitosan to help reduce the likelihood of rejection due to the estimated values.

4.2.3.1 Principle of LAL assay

In order to accurately quantify the absolute EU conc. of chitosan, the kinetic turbidimetric and chromogenic assays were used. The kinetic formats of the LAL assays are most sensitive for quantification of chitosan. The sensitivities of both the assays are 0.005 EU/mL, which could easily validate or reject the EU value estimated from gel-clot assay. As shown in Chapter 1 section 2.1.4.2, the principle of quantification by turbidimetric LAL assay relies upon the development of turbidity following activation of enzyme cascade by endotoxin. The formation of insoluble coagulin protein from the soluble coagulogen after activation of Factor C cascade results in direct correlation with the amount of endotoxin present in the test solution. Similarly, in the chromogenic LAL assay, the clotting enzyme is conjugated with a protein-dye substrate i.e. Gly-Arg-p-Nitroaniline, which when cleaved releases the yellow colored p-Nitroaniline substrate. The quantity of this chromogenic substrate is directly proportional to the actively pyrogenic endotoxin in the sample.

4.2.3.2 Generating standard curves from raw data

The development of turbidity or development of yellow color is quantified over a 2-hour time period at a wavelength of 405nm at a specified time interval (30s- 100s) by a spectrophotometer or plate reader. An absorbance (O.D) vs. time graph is obtained after completion of a run.

The vital information extracted from the originated data is the onset time of the standard calibrators and test samples, the onset time refers to the number of seconds it took a sample to reach a pre-set threshold O.D. Depending on the raw data of each assay, the threshold O.D is set either at 0.1 or 0.2 from the starting O.D of each calibrator, to remove excess background for the analysis. The samples with high endotoxin conc. reach the threshold O.D of 0.1 in shorter time and the ones with lower conc. reach the mark in longer time resulting in small and high onset times respectively. For plotting the standard curve, a linear regression of the log of onset times and log of corresponding endotoxin concentrations is plotted, the linear equation in the form of $y= mx + b$ is used for calculating the sample endotoxin concentration. The acceptance criteria set out by USP for the standard curve is that it must have minimum of three standard calibrators and a correlation coefficient of ≥ 0.98. The validity of the assay is also measured by observing the recovery of a CSE control sample, which is LRW+ spike of known amount of endotoxin between

75-125% (STE-1.4, 2015; USP, 2005). If these acceptance criteria were unmet, the assay was considered invalid and was repeated.

4.2.4 Testing of various standard curve ranges for establishing a repeatable range

For valid quantification of chitosan EU conc. various standard curve ranges were tested to match the expected and estimated EU conc. of chitosan and also to establish the most repeatable range for future use in endotoxin detection. The variation arises due to the inherent 2-fold error in the assay, technical and manual errors. A 1000 EU/mL CSE stock was used to prepare the standard curve ranges. The standard curve ranges tested were: -

a) **50-0.005 EU/mL**- This range was tested based on the certificate of compliance issued by the supplier (Associates of Cape Cod) suggested for use of this range in a plate reader and because of its widespread use by another eminent supplier Lonza (Lonza, 2014). No. of repetitions **(N)** = six.

b) **10-0.01 EU/mL**- Due to the use of chitosan raw material with 92% DDA, which might have high EU contamination. Because of the recommendation from another major LAL supplier Charles River Laboratories (CRL), that states that a test of four-log of assay sensitivity (log 0.005 *4) standard curve from 10-0.005 EU/mL that may be suitable for the in process and raw materials testing (CRL, 2006); for the initial quantification of absolute chitosan EU conc. this range was applied. No. of repetitions **(N)** = three.

c) **5-0.005 EU/mL**- Higher calibrator was reduced 2-fold for testing. No. of repetitions **(N)** = six.

The variation in the repeatability of each range was calculated by comparing the relative standard deviation %RSD (%CV) = $\frac{|Mean|}{SD} * 100$ for slope and intercept of the linear equations of each range with the acceptable percentage coefficient of variation (%CV) value of slope and intercept issued at 10% and 3% respectively for all the ranges by the lysate supplier i.e. ACCI.

4.2.5 Optimization of parameters to obtain repeatable LAL standard curve and equivalent reactivity in plate reader as tube reader

4.2.5.1 Calculation of Pathlength in a Plate reader

Pathlength determination of the reagents, samples, solutions etc. tested in the plates instead of a cuvette is very crucial, as the plate reader measures the absorbance by transmitting the beam from the bottom of the well, which is captured by the detector at the top. While passing through the sample, the absorbance is determined by the Beer Lambert's law with following factors:

Equation 4-1 : $Absorbance\ (Abs) or\ Optical\ Density\ (O.D) = \varepsilon * d * c$

Where ε= Molar absorptivity coefficient of solution ($M^{-1}\ cm^{-1}$) that changes with different wavelengths

d= pathlength (cm) determined by the distance travelled by a beam in a solution

c= concentration of the analyte (M)

In the **Equation 4-1, ε** is a constant, and **c** is determined by the measured absorbance, but **d** i.e. pathlength is a variable that changes with instrument. As shown in the **Figure 4-1** when a cuvette is used for absorbance measurement, the transmitted light beam passes through the solution in the cuvette horizontally i.e. through the width of the cuvette, which remains consistent with every measurement. However, in a plate reader, the light is transmitted by the plate reader in a vertical direction from bottom to the top of the solution in a well i.e. through the height of the sample:

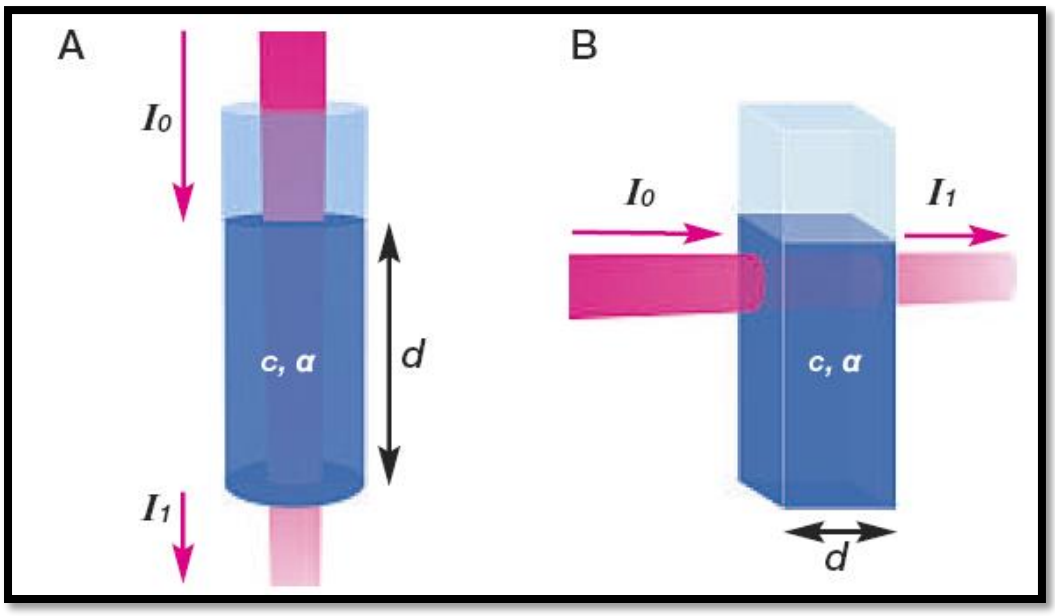


Figure 4-1 : The pathlength in a microplate well varies **(A)** compared to the fix pathlength in a cuvette/tube **(B)** (Bio-One, 2008)

When the pathlength is dependent on sample height, the pathlength could vary with small changes in sample volume. To harmonize the absorbance measurement, the raw absorbance values are corrected for the pathlength using the following formula:

Equation 4-2 : $$d\ (Pathlength) = \frac{Abs_{975} - Abs_{900}}{K-factor}$$

Where Abs975= Absorbance of water/ diluent measured at 975nm- wavelength producing highest absorbance peak in water (**Figure 4-3**)

Abs 900= Absorbance of water/diluent measured at 900nm- To reduce the basal level of absorbance in water (**Figure 4-3**), and

K-factor= Constant value of absorbance measured in 1cm cuvette, obtained by subtracting the Abs975 of water with Abs900. The value is expressed in an equation as:

Equation 4-3 : $$K - factor = Abs_{cuvette\ (975)} - Abs_{cuvette\ (900)}$$

By correcting for the pathlength, reliable absorbance values for the samples could be obtained.

4.2.5.2 Determination of evaporation during assay conditions and its effect on pathlength in 96 well plates

LAL assays are performed under the following conditions: 37˚C over a 2-hour period. Thus, the investigation of the effect of temperature on the reagent evaporation is crucial as the variance in sample height can directly affect the pathlength travelled by the vertical incident light and lead to variation in the absorbance measurement. The path travelled by the incident light through a well in a plate is vertical, contrary to the horizontal path in a cuvette. Due to the perpendicular direction, the path travelled by incident light depends on the height of the reagent in plate wells, which is easily affected by pipetting errors or evaporation. Indeed, the reagent evaporation could have a detrimental effect on the pathlength of reactants.

200µL LAL Reagent water (LRW- ACCI; Cat # WP050C) was pipetted in outer (A1, A12, H1, H12), side (D1, A6, D12, H6), inner (B2-G11) and middle wells (D4-F9) as shown in **Figure 4-2** below.

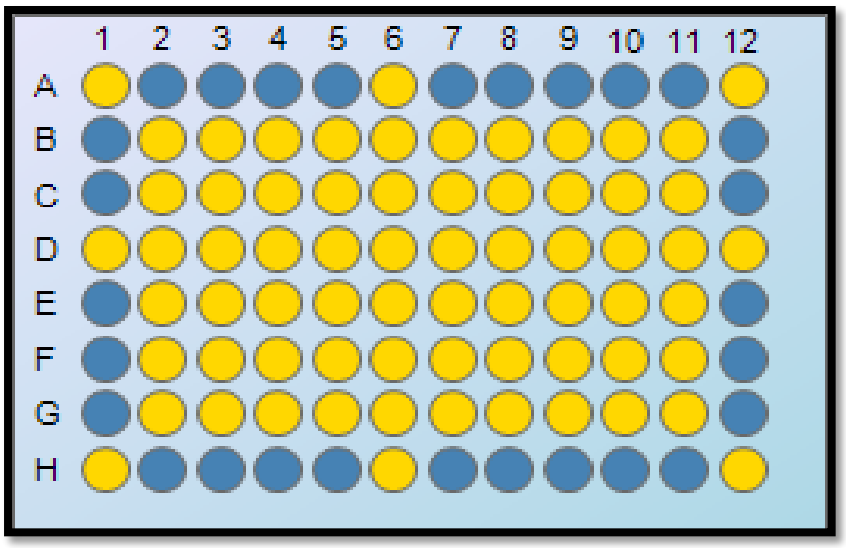


Figure 4-2: Area of plate used for filling LRW to evaluate the evaporation patterns in different areas of the plate.

The plate with lid was weighed for determination of lost water after the run. The optical density measurements were recorded at 975nm and 900nm wavelengths at high temperature- 37˚C for 2 hours, which are the conditions used in LAL assay. The Abs975nm- Abs900nm values were analyzed to determine the evaporation level of each well type as water molecules show

significant peak at 975nm (**Figure 4-3**) and reduction in the peak is representative of increasing evaporation due to loss of water molecules. The wells experiencing lowest evaporation rate was also determined by Abs975-Abs900 value. The percentage variability of evaporation between each well type was calculated over time by determining percentage coefficient of variation.

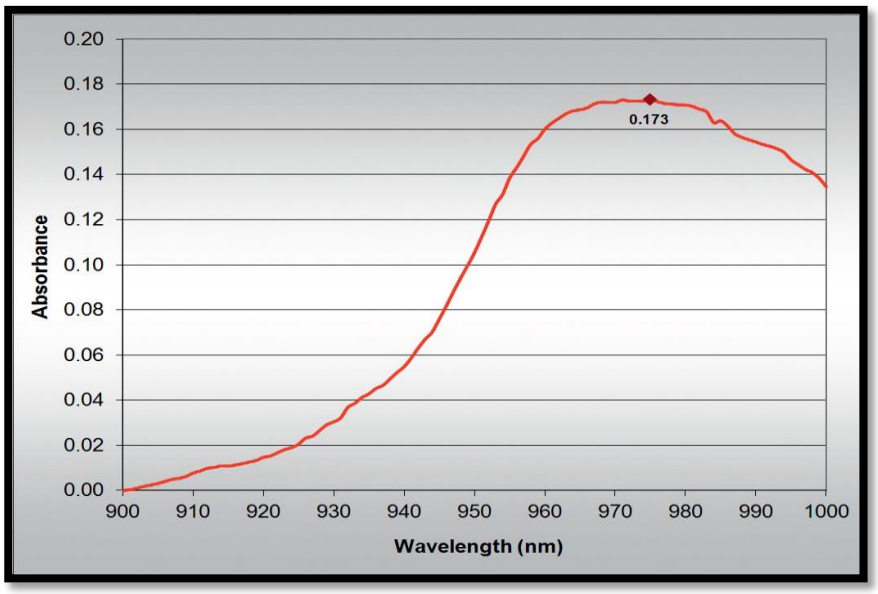


Figure 4-3 : Absorption spectrum of water in the near IR area (ThermoSci, 2012).

4.2.5.3 Standard curve Repeatability at high range

The repeatability of the standard curve is a major requirement for obtaining precise repeatable results from any assay. Biological assays are known for low repeatability, and hence have acceptable limits outlined for those assays. Many factors are responsible for inter-experimental variation of standard curve- such as difference in reaction by enzyme lot, low/high rate of reaction of CSE lot to enzyme, difference in onset time of same range due to fluctuations in reaction conditions. The variation generated due to inconsistent sensitivity of LAL enzyme and CSE lot is inevitable, but the reaction temperature conditions can be controlled to observe their effect on standard curve variation. An effort to reduce the variation in standard curve was undertaken by separately treating the samples and enzyme at 37°C and RT to establish the optimal reaction temperature required for producing lowest variation in standard curve.

As the LAL enzyme-endotoxin interaction is temperature dependent, the endotoxin standard dilutions were treated at room temperature (RT) and at 37˚C to determine the optimal

conditions for standard curve repeatability. The endotoxin standard dilutions were prepared from 1000EU/mL Control Standard Endotoxin (CSE) vial (ACCI Cat # E0005-1; Lot # 147) at a range of 50-0.005 EU/mL with 10-fold dilution. For RT treatment (N=2), the endotoxin dilutions and LAL enzyme reagent (Pyrotell T ACCI; Cat # T0051-5; Lot # 515-10-757-T) reconstituted in β-D glucan blocker -Glucashield buffer supplied by ACCI (Cat # GB051-5), were prepared at room temperature, pipetted in a 96 well plate (125µL sample +125µL LAL) and placed in the spectrophotometer for measurements at 37˚C for kinetic turbidimetric LAL reaction. For 37˚C treatment (N=3), the endotoxin dilutions and LAL enzyme reagent were prepared and incubated at 37˚C and then placed in the Tecan spectrophotometer (Tecan infinite- M200) for kinetic measurement of absorbance at 405nm of LAL-Endotoxin interaction at 37˚C for 2 hours every 60sec or higher. For the correction of Absorbance at 405nm of each sample by pathlength of LRW (sample diluent) + LAL, the absorbance at 975nm and 900nm was also measured. The pathlength of LRW+ LAL was calculated following **Equation 4-2**, and the Abs405 of each sample was corrected as follows:

Equation 4-4 : Correction of Raw O.D value: $Abs_{405}^{corrected} = \frac{Abs_{405}}{PL\ (cm)}$

At the end of measurements, the standard curve for each temperature condition was plotted following linear regression curve of Log endotoxin concentration [EU/mL] vs. Log Onset time (sec); the onset time for each standard dilution was calculated by taking the average of $Abs_{405}^{corrected}$ recorded at first 3 time points (t=0 to t=120s-200s) and adding the threshold O.D§ of 0.1 to the average $Abs_{405}^{corrected}$ value. The repeatability of the standard curves was determined by calculating the percentage coefficient of variation (%CV) of the intercept, slope and R^2 of each linear equation.

The difference in pathlength of LRW and LRW+LAL enzyme was observed. The Pathlength vs. temperature correlation for each reactant was also observed with time.

§ Threshold O.D value was set at a level, which reduced the background from each standard dilution.

4.2.6 Post-optimization of parameters: Repeatable standard curve ranges for Turbidimetric LAL assay

4.2.6.1 Turbidimetric: Use of smaller curve range of 0.16-0.005 EU/mL to obtain repeatable standard curve and match expected values in chitosan

After encountering multitude of challenges with the repeatability of the standard curve with a degree of ambiguity in the quantified EU values of chitosan simultaneously tested with variable standard curves, a small range of standard curve was used with highest calibrator at 0.16 EU/mL after applying all the optimized parameters. This tested range provided good repeatability and also enclosed the values of chitosan EU conc. obtained from gel-clot LAL assay i.e. 0.03 EU/mL perfectly within its detection range. Additionally, a smaller range provided flexibility in assay manipulation due to extended reaction time between sample and LAL enzyme Eg; the average onset time for 0.16 EU/mL is 1280 sec as compared to 300 secs for 50 EU/mL. An intermediate solution of 20 EU/mL was prepared from 1000 EU/mL by 50-fold dilution in LRW, subsequently followed by 6.25 times dilution for another intermediate solution of 3.2 EU/mL, this solution was then serially diluted 10-folds to prepare third intermediate solution at 0.32 EU/mL. The calibrators were then prepared by 2-fold dilution from 0.16 EU/mL, 0.08 EU/mL, 0.04 EU/mL, 0.02 EU/mL, 0.01 EU/mL and finally 0.005 EU/mL. The calibrators were pipetted in triplicate in a 96 well plate, and placed in a plate reader for incubation at 37°C for 20 mins to equilibrate the sample for rapid endotoxin detection after addition of pyotell lysate (LAL enzyme). The LAL enzyme made sensitive for endotoxin detection by reconstituting in glucan inhibiting glucashield buffer, and after the incubation time was added on top of each calibrator. Within 5 minutes of LAL addition, the absorbance measurement was started at 405nm for 2 hours at an interval of 1min 25 sec by the Magellan software; the absorbance at 975nm and 900 nm was also recorded for pathlength correction simultaneously. After the completion of run, the standard curve and onset times of calibrators were produced by software; the standard curve was plotted as log EU conc. vs. Log onset times by a linear regression function. The linear equation obtained as y= mx+ b was consequently used for the calculation of chitosan EU conc., which were prepared along with standard curves, but shown in chapter 4, for the purpose of clarity. This range of standard curve was repeated N= 3 times, and the linearity of each standard curve was observed to be $\geq$ 0.98 as per USP requirement, more importantly the repeatability of this range was

determined by calculating the percentage variability in the slope and intercept values between the three linear equations and compared with the acceptable limit of slope %CV= 10% and intercept %CV= 3% issued by ACCI.

4.3 Results

4.3.1 Estimation of chitosan endotoxin value by Gel clot assay

Gel clot is a semi-quantitative assay used for the estimation of endotoxin value in a sample. It is considered an official referee test by the Pharmacopeias if turbidimetric and chromogenic LAL gives indeterminate results. The results are classified negative or positive based on the formation of gel-clot, the negative control must show no clot and the positive control must show a firm clot for the test to be valid. If any test sample have a tendency of interference with endotoxin, then the Inhibition/Enhancement controls i.e. Positive product controls (PPCs) show negative clotting. A test sample with enhancement interference will show a positive clot in the test tube. The kits come with specified sensitive levels labelled "λ (lambda)" which is the lowest concentration of endotoxin required to produce a positive test result, the lowest sensitivity of 0.03 EU/mL was tested. A positive test result is when the enzyme is activated by the sample to form a clot, which does not break after inverting 180˚. The chitosans with 92% degree of deacetylation at low molecular weight and the raw material at high molecular weight were tested to estimate the endotoxin conc. by gel-clot LAL assay. Samples were prepared at a conc. of 10 mg/mL in 0.2% acetic acid after dissolution for 1 hour at 37°C according to ACCI method. For each sample, an interference control called positive product control (PPC) was tested which is included in the kit at twice the Control Standard Endotoxin (CSE) concentration required for producing a positive test result i.e. 0.06 EU/mL or 2λ.

According to the USP 85, the validity of gel-clot assay is demonstrated when duplicate vials of negative control i.e. LRW+LAL show negative clot, and the positive control (LRW+CSE 2λ+LAL) demonstrate a positive clot after a recommended incubation time (48 mins) at 37˚C. According to **Figure 4-4** and **Figure 4-5**, this criterion was met by the test.

Negative control

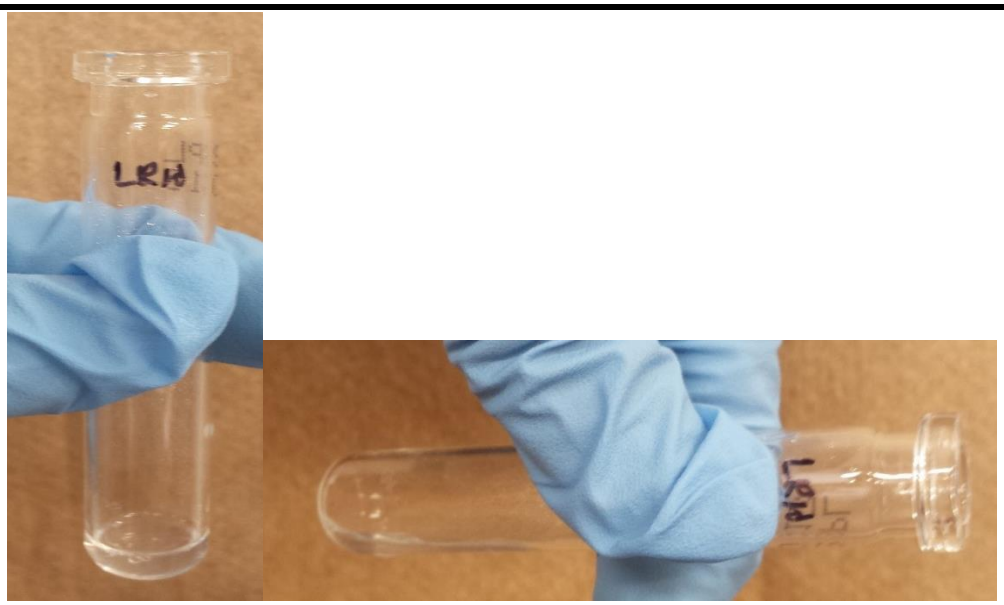


Figure 4-4 : The negative control (LRW+LAL) without CSE in the gel-clot LAL assay demonstrates the lack of gel formation confirmed by inversion, thus fulfilling the acceptance criteria required for assay validity as outlined by the USP.

Positive control

Figure 4-5 : The positive product control with CSE at 2 times the conc. Of sensitivity 2λ +LAL shows positive gel clotting confirmed after 180° inversions as required by the USP for validity of gel-clot assay.

Table 4-1 : The result of negative gel clot formation by chitosan test samples and positive clotting by their PPCs after incubation at 37˚C for 48 minutes by gel-clot assay.

Gel clot results (λ=0.03 EU/mL)		
Sample	Test	PPC
CS 92-10	-	+
	-	+
CS 92-Raw	-	+
	-	+

Additionally, the USP specifies that without any interference, the PPC of the samples must exhibit positive clotting, as illustrated in **Figure 4-6** and **Table 4-1** this USP criteria is entirely satisfied attesting that chitosan 92-10 and chitosan 92-Raw samples does not exhibit inhibition interference at a concentration of 0.1mg/mL after 1:100 dilutions.

Chitosan Positive product controls (PPCs) at 2λ

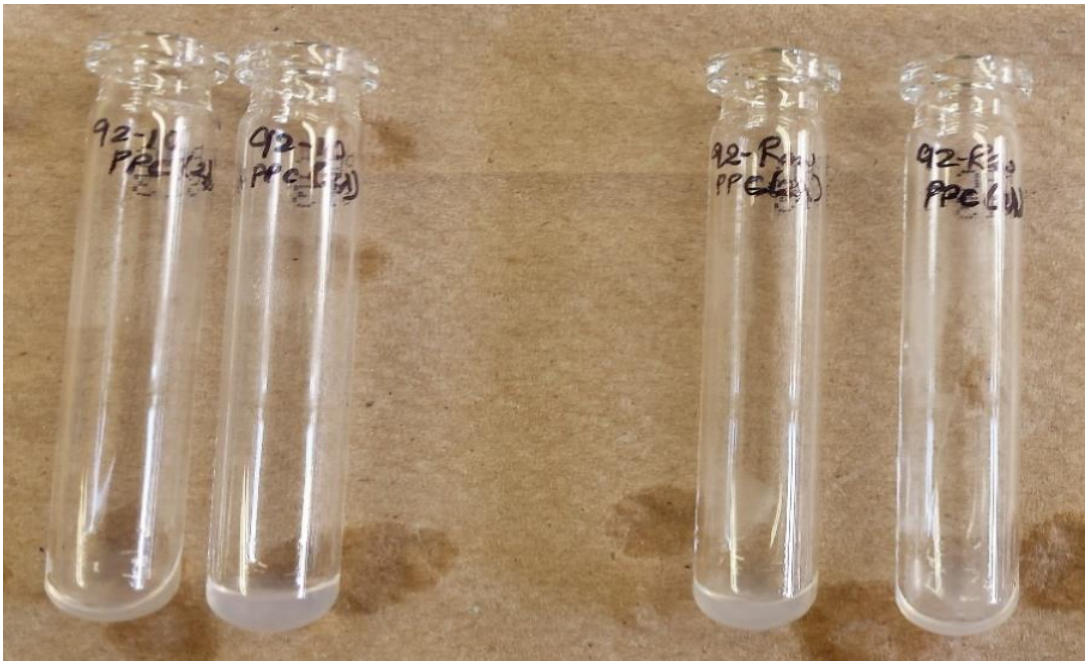


Figure 4-6 : The positive product controls of chitosan 92DDA-10Mn and CS 92-Raw at 2λ show positive clotting after incubation at 37˚C for 48 mins, exhibiting the lack of interference by the samples at 0.1mg/mL conc. after 1:100 dilutions.

The duplicates of chitosan 92-10 and 92-Raw test samples exhibit negative clotting (**Table 4-1**, **Figure 4-7** and **Figure 4-8**), indicating that the endotoxin content of both the chitosans at 0.1mg/mL is less than or equal to 0.03 EU/mL i.e. the labelled sensitivity of the gel-clot kit. Assuming that chitosan contains at least 0.03EU/mL, then endotoxin level in the raw material (powder) can be computed as follow: 0.03 EU/mL/0.1 mg/mL= 0.3 EU/mg* 1000 mg/g = 300 EU/g. Even though this limit is an indicative value, it ascertains the maximum level of endotoxin per gram of chitosan. This limit allows us to determine the amount of endotoxin present in the injectable chitosan nanoparticles and the cartilage repairing chitosan scaffolds whether it complies with the endotoxin limit allowed by the USP for parenteral drugs.

Chitosan 92DDA-10Mn

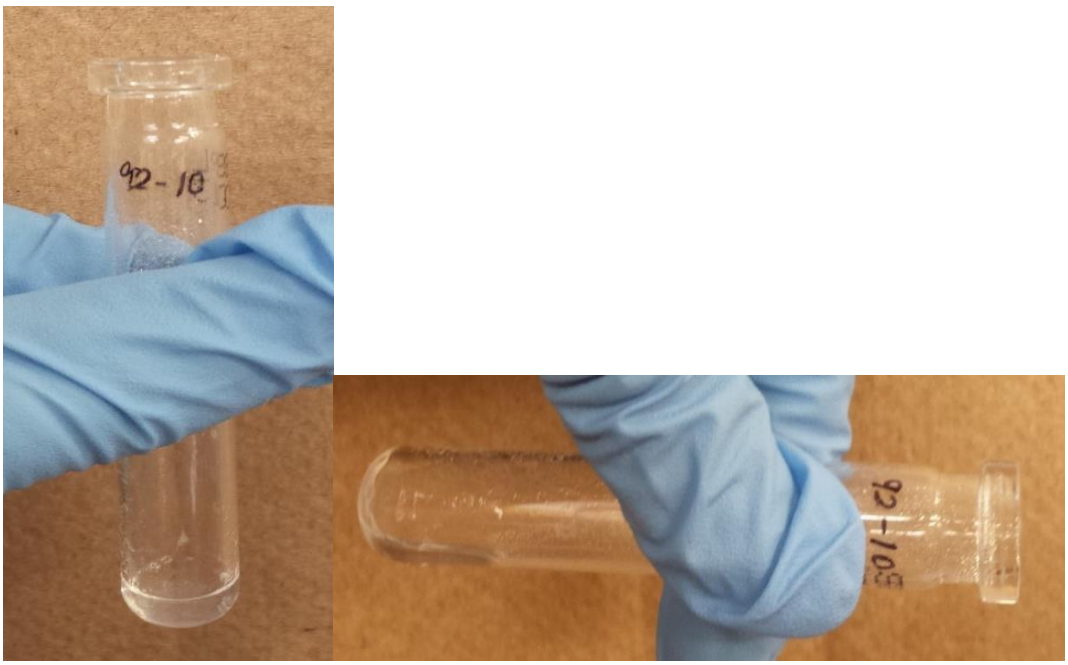


Figure 4-7 : Chitosan 92DDA-10Mn show negative gel clotting evident from unsettled clot after inversion indicative of endotoxin contamination below 0.03 EU/mL.

Chitosan 92 DDA-Raw

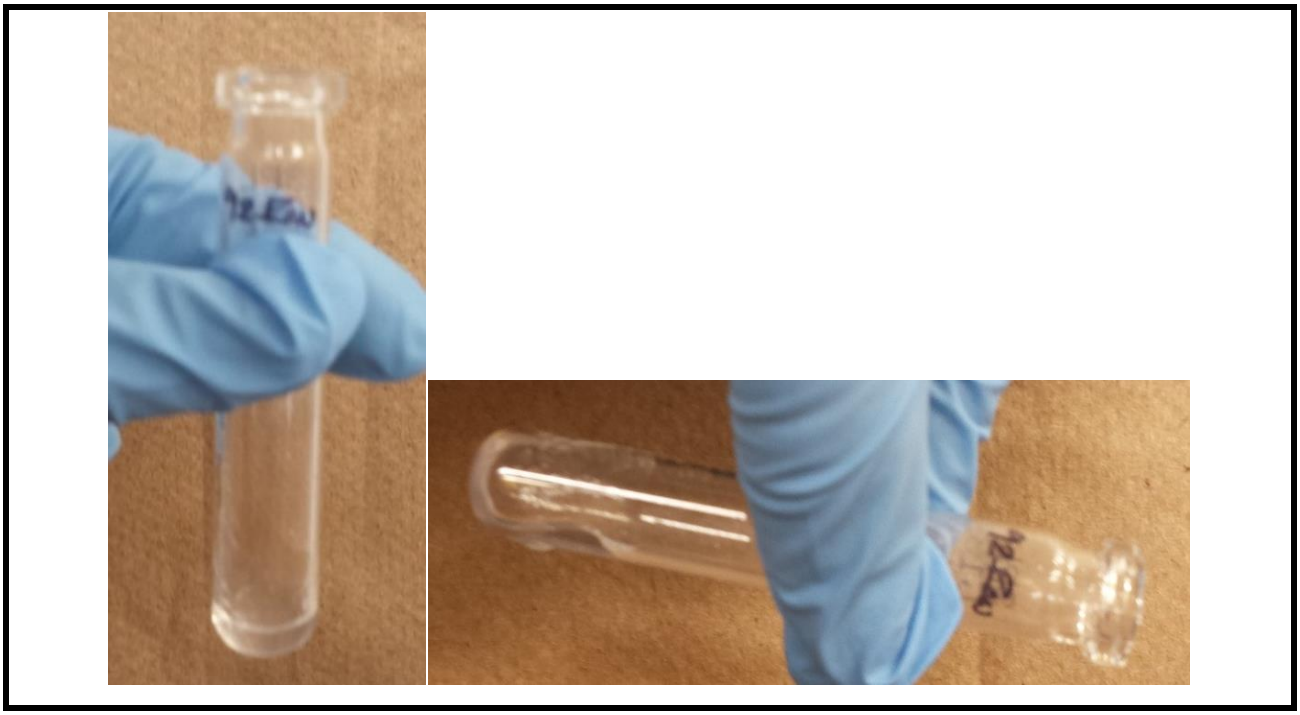


Figure 4-8 : The endotoxin level of chitosan 92DDA-Raw is found below 0.03 EU/mL as the sample endotoxin was insufficient to activate LAL complex demonstrated from the negative gel-clot.

4.3.2 Calculation of EU conc. of injected chitosan dose simulated to Human Equivalent Dose (HED)

With the advent of nanotherapeutics, an extensive niche of chitosan nanoparticles in the delivery of drugs and active compounds has been established. In our group, chitosan polyplexes are extensively used for the delivery of siRNA in mice models (Alameh et al., 2012; Jean et al., 2012) and as scaffolds to facilitate cartilage repair in various animal models (Hoemann CD, 2015; Méthot S, 2016) The aim of in vivo injections of chitosan polyplexes is to observe the efficiency of cargo delivery, as well as to assess toxicity of chitosan in animal systems; the assessment of toxicity may be biased if the chitosan is contaminated with LPS.

In animal models, many doses of chitosan nanoparticles complexed with siRNA have been used by our group, the endotoxin concentration of the minimum and maximum dose of chitosan-siRNA nanoparticles with an N: P ratio of 5 will be calculated based on the limit obtained from the gel-clot assay; where N in the N: P ratio denotes the amine groups from chitosan and P denotes phosphate groups from siRNA; this ratio determines the excess of positively charged

amine groups for high charge density that dictates the transfection efficiency of the nanoparticles in the tissues (Thibault et al., 2011).

4.3.2.1 EU limit of minimum intravenous (IV) dose of chitosan nanoparticles

The minimum dose of chitosan-siRNA nanoparticles administered in mouse model at an N: P ratio of 5 was 1mg/kg body weight; this dose translates to 2.8mg/kg of injected chitosan in mouse. According to the US FDA, the dose of the therapeutics used in the animals must be converted to the human equivalent dose (HED) to estimate the maximum safe starting dose for use in healthy human volunteers. Although currently the use of chitosan nanoparticles in human volunteers is a distant goal, the simulation of the animal dose of CS-siRNA nanoparticles to HED is performed for estimating the endotoxin value of chitosan used in intravenous injection, this is to ensure the compliance of chitosan EU value with the acceptable endotoxin limit of parenteral drugs used in humans set to 5 EU/kg/hour by the USP. The compliance of 1mg/kg CS-siRNA nanoparticles to this limit is shown by the calculation below:

Dose of chitosan administration derived from 1mg/kg nanoparticle dose in mouse= 2.8 mg/kg

Conversion of the chitosan dose of 2.8mg/kg in mouse to human equivalent dose (HED) by dividing with a factor of 12.3 recommended by the FDA (FDA, 2005):

$$\frac{2.8\ mg/kg}{12.3} = 0.227\ mg/kg$$

Ideally, for a 70kg human subject, total injected amount of chitosan= $70kg * \frac{0.227mg}{kg} = 15.93\ mg$

The endotoxin level of chitosan determined by the gel-clot assay= ≤300 EU/g or ≤0.3 EU/mg

To calculate the amount of endotoxin in EU of 15.93mg chitosan, the chitosan endotoxin value obtained by gel clot was multiplied= $15.93mg * 0.3\frac{EU}{mg} = 4.78\ EU$ for entire human body weight

Calculating the concentration of endotoxin per kg body weight of human for comparison with the acceptable endotoxin limit: $\frac{4.78\ EU}{70\ kg} = 0.068\ EU/kg$

The chitosan endotoxin value of ≤0.068 EU/kg is under the endotoxin limit of 5 EU/kg issued for parenteral drugs by the USP; the EU value for the lot of siRNA used in animal study is declared at ≤0.05 EU/mg by the supplier that translates to ≤0.004 EU/kg for 1mg/kg injected siRNA dose. The additive EU conc. of 1mg/kg CS-siRNA nanoparticles is ≤0.072 EU/kg signifying that the lowest dose of chitosan nanoparticle injection is safe for future use in humans.

4.3.2.2 Upper EU limit of maximum tolerated dose (MTD) of intravenously (IV) injected chitosan nanoparticles

The maximum tolerated dose (MTD) of uncoated chitosan-siRNA nanoparticles in mouse is 2.5mg/kg, but in order to increase the siRNA dose and target specificity, the chitosan-siRNA nanoparticles were coated with Hyaluronic acid (HA), which rendered the nanoparticles less toxic and enabled the administration of 8mg/kg HA-CS-siRNA nanoparticles dose in the mouse model at an N: P: C ratio of 2: 1: 1.5 (where C in N:P:C ratio stands for the carboxylic group of HA); this dose translates to 8.85 mg/kg of injected chitosan in mouse. The 8mg/kg dose of HA CS-siRNA nanoparticles translates to individual dose of 15.27 mg/kg hyaluronic acid (HA), 8.85 mg/kg chitosan and 8mg/kg siRNA which dictates the injection dose in animals. As 8 mg/kg nanoparticle dose is MTD and may be designated a No Observed Adverse Effect Level (NOAEL) in mouse model, the human equivalent dose (HED) is derived by dividing the NOAEL with a conversion factor of 12.3 according to the US FDA recommendations. So, the 8.85 mg/kg chitosan dose in mouse is divided by 12.3 to obtain the HED. The compliance of 8 mg/kg CS-siRNA nanoparticles to the endotoxin limit of 5 EU/kg per human parenteral dose is shown by the following calculation:

Dose of chitosan administration derived from 8mg/kg nanoparticle dose in mouse= 8.85 mg/kg

Conversion of the chitosan dose of 8.85 mg/kg in mouse to the human equivalent dose (HED) by dividing with a factor of 12.3 recommended by the FDA:

$$\frac{8.85\ mg/kg}{12.3} = 0.72\ mg/kg$$

For a 70kg human subject, total injected amount of chitosan= $70kg * \frac{0.72mg}{kg} = 50.4\ mg$

The endotoxin level of chitosan determined by the gel-clot assay= ≤300 EU/g or ≤0.3 EU/mg

To calculate the amount of endotoxin in EU of 50.4 mg injected chitosan, the chitosan endotoxin upper limit value obtained by gel clot was multiplied= $50.4mg * 0.3\frac{EU}{mg} = 15.12\ EU$ for entire human body weight

Calculating the concentration of endotoxin per kg body weight of human for comparison with the acceptable endotoxin limit: $\frac{15.12\ EU}{70\ kg} = 0.216\ EU/kg$.

The chitosan endotoxin value of ≤0.216 EU/kg is well below the endotoxin limit set at 5 EU/kg for the human parenteral drugs by the USP, also the EU value of 8mg/kg siRNA used for injection translates to <0.0325 EU/kg derived using the ≤0.05 EU/mg value obtained from the supplier. The EU value of 15.27 mg/kg HA used for the CS-siRNA polyplex coating for targeted delivery to CD44 expressing cells and reduced toxicity is ≤0.0012 EU/kg derived from the ≤0.001 EU/mg value obtained from the certificate of analysis of 1000 kDa Hyaluronic acid. The additive EU value of 8mg/kg HA coated CS-siRNA nanoparticles is <0.245 EU/kg, indicating that even at highest dose the nanoparticle will not induce immune-toxicity initiated by endotoxins in human subjects.

4.3.2.3 EU limit of intra-articular chitosan dose administered in horses

The chitosan scaffolds used in cartilage repair are injected intra-articularly in the horses at a conc. of 1%w/w at a total volume of 3mL. This intra-articular dose injected in the horse joints totals 30mg chitosan. The chitosan dose calculated per kg body weight of 300kg horses is 30mg/300kg= 0.1mg/kg. The endotoxin level for the intra-articular joints in horses used by (Carregaro, Freitas, Ribeiro, Xavier, & Doria, 2014) and (J.L Lucia, 2014) which shows minimal inflammation, is 0.1 EU/kg; this limit is used for the harmonization of the chitosan dose used by our group.

Chitosan dose used for the horse intra-articular injection = 30mg

The endotoxin level of chitosan determined by the gel-clot assay= ≤300 EU/g or ≤0.3 EU/mg

To calculate the EU conc. of 30 mg injected chitosan, multiply with the endotoxin upper limit value of chitosan obtained by gel clot = $30mg * \frac{0.3EU}{mg} = 9\ EU$ for entire body weight of horse.

Calculating the concentration of endotoxin per kg of horse body weight for comparison with the endotoxin limit determined for harmonization: $\frac{9\ EU}{300\ kg} = 0.03\ \text{EU/kg}$

The chitosan endotoxin value of ≤0.03 EU/kg is lower than the endotoxin limit harmonized at 0.1 EU/kg for the horses, indicating that the chitosan dose used for intra-articular injection shouldn't induce an inflammatory response in the joints in horses. However, the ≤0.03 EU/kg value is close to the harmonized 0.1 EU/kg limit, indicating that if the chitosan dose is increased in further studies, it may lead to joint effusion in 300kg average weighed horses.

4.3.3 Rationale of using sensitive kinetic assays for chitosan EU determination

As evident from the calculation of endotoxin in chitosan doses, the maximum allowable endotoxin limit of chitosan depends on a lot of factors, such as: weight of the animal/human, dose modification according to LD50 in different animals, route of administration and difference in EU conc. of different chitosan batches based on handling and processing. These factors may rapidly change and may result in the endotoxin conc. of chitosan to surmount the acceptable endotoxin limit, which may lead to the rejection and withdrawal of the chitosan therapeutics from the marketplace. Additionally, the endotoxin level of the chitosan therapeutics is always affected by that of excipients that may have high endotoxin content; hence, the determination of absolute endotoxin content of chitosan is a strict requirement. Furthermore, the EU limit for the intra-articular administration is not harmonized by the USP; hence, it is very risky to use an estimated value to calculate the endotoxin content of the chitosan injected in the horses. Similarly, humans are very sensitive to varying ranges of endotoxin, eliciting immune response in the presence of even 1pg [0.01 EU/mL] extra endotoxin than the approved level coming in contact with blood proteins or cerebrospinal fluids.

The current specification of our chitosan product is 100 EU/g, which could not be specifically quantified by the semi-quantitative gel-clot assay, as the lowest estimated value possible from gel-clot is ≤300 EU/g. The resolution of EU values between less than 300 EU/g and equal to 300 EU/g will determine the clinical applicability of the chitosan. The EU conc. calculated for intra-articular chitosan dose in horses is close to the harmonized EU limit hence in order to reduce the risk of overriding the acceptable endotoxin limit by using a semi-quantified estimate of endotoxin value generated by gel-clot assay; it is certainly rational to quantify the absolute endotoxin conc.

of the chitosan by using more sensitive methods. The remarkably sensitive assays approved for the accurate endotoxin measurement by USP are the two other formats of the LAL assay:

1) Turbidimetric LAL assay: It functions on the same principle of formation of insoluble coagulin protein from the soluble coagulogen by activated clotting enzyme after coming in contact with the endotoxin. It is a quantitative assay in which a standard curve is plotted with a given range of endotoxin and the sample endotoxin conc. is interpolated from the generated linear equation. The error of quantification in all the LAL assays is 2-fold, and is agreed upon by the USP as an inherent variation. This assay can detect endotoxin at a sensitivity of 0.005 EU/mL, which is 6 fold higher than a gel clot assay.
2) Chromogenic LAL assay: This method has an equivalent sensitivity to turbidimetric LAL but the difference between turbidimetric and chromogenic assay is that the precursor of coagulogen (the substance which produces turbidity) is linked to a chromogenic substrate called Gly-Arg-p-Nitroanilide (pNA). When the enzyme meets endotoxin, the activated cascade cleaves the Gly-Arg and p-Nitroanilide bonds and the released p-Nitroanilide produces a yellow color, which is directly correlated to the quantity of endotoxin present in the sample. The intensity of the chromogenic product is measured by the absorbance with a spectrophotometer. Analogous to the turbidimetric assay, the unknown endotoxin conc. is calculated by deriving from the standard linear equation, and have an error rate of 2-fold in quantification.

4.3.4 Turbidimetric LAL assay

4.3.4.1 Raw data

The first step towards computing EU conc. of test samples is obtaining Time vs. O.D raw data, which represents the assay characteristics of the general LAL reaction with endotoxin over time. The reaction profile, demonstrates three critical properties of LAL gelation in the presence of LPS (Lipopolysaccharide /endotoxin):

a) The increase in optical density followed by the coagulation due to increase in the coagulin proteins.
b) The rate of O.D increase affected by the concentration of LPS.

c) The clotting reaction is a sigmoid curve characterized by a plateau, an exponential phase and a final plateau phase. (Hurley, 1995)

The phase of a LAL reaction in a turbidimetric assay is demonstrated graphically for each standard concentration and negative control in **Figure 4-9** below:

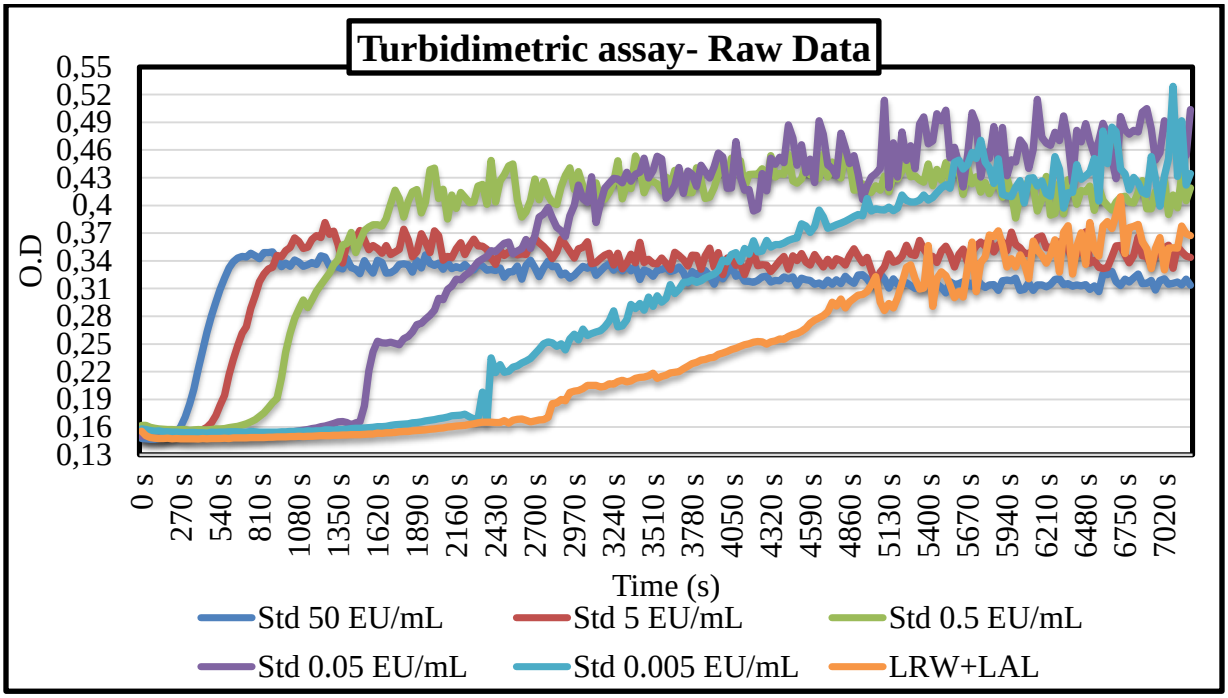


Figure 4-9 : Graphical representation of characteristics of standard calibrators and negative control in turbidimetric LAL assay

As observed in the graph, even the negative control (LRW+LAL) is activated and reaches the equivalent O.D as 0.005 EU/mL after certain time. This phenomenon is exhibited because of the automatic induction of LAL enzyme cascade when it reaches favorable temperature (37°C) in the presence of no or very small endotoxin that may be present in the LRW. The presence of excess enzyme per microliter of lysate supplied by the vendor also plays an important role in development of turbidity in the absence of endotoxin.

Each standard concentration and negative control (LRW+LAL) curve show uneven and bumpy profile of O.D against time. However, in each calibrator, this disturbance occurs after reaching their respective onset times i.e. after crossing the threshold O.D, hence it does not affect the endotoxin quantification by the assay. This pattern is observed because of determination of turbidity by the physical blocking of transmitted light, due to this property the particle size,

number of clotted particles and the reflected and refracted light all affect the measurement of absorbance to various levels.

4.3.4.2 Generating standard curve from raw data:

The standard curve of the assay is obtained by plotting the log conc. vs. log onset time. Onset time is dictated by the rate of protein clot formation, and the time it took the sample to reach a threshold O.D of 0.1. The standard curve is formed using a known range of control standard endotoxin dilutions in LRW. The onset times of each endotoxin conc. is plotted on a graph using linear regression, and the linear equation obtained is used for determining the test sample endotoxin conc. as a function of x using the value of their onset times. A typical representation of the standard curve obtained from analysis of turbidimetric data is shown in

Figure 4-10 below:

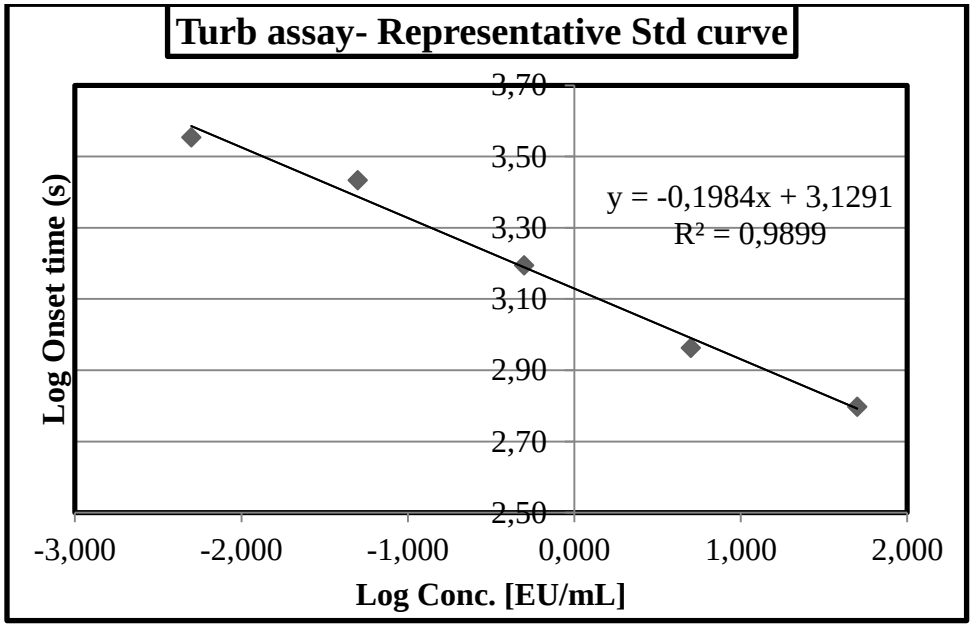


Figure 4-10 : Typical representative linear regression standard curve of turbidimetric LAL assay. Log endotoxin conc. (EU/mL) is plotted on x-axis and log Onset time (secs) is plotted on y-axis

The EU values of the test samples presented in the **section 4.3.6** are interpolated from the linear equations of such standard curves, which may vary inter-experimentally and/or intra-experimentally. The %CV or relative standard deviation (RSD) of the slope, intercept and correlation coefficient of all the acquired standard curves are shown in **section 4.3.4.3** to allow the reader to understand the inherent variability of the standard curves.

4.3.4.3 Determination of optimal repeatable standard curve range for Turbidimetric assay

The repeatability of the standard curve is an important parameter to obtain repeatable results. Based on the performance of the assay in terms of the production of raw data, it is influenced by different CSE and LAL lot, instrument and manual variability, the likelihood of obtaining repeatable standard curve is low. Keeping in mind these factors, there are acceptable limits of Percentage Coefficient of Variation (%CV), which is a measure of variation of certain parameters, that are set out by the lysate vendors for the slope and intercept. For a particular lot of the turbidimetric LAL lysate, the acceptable of a %CV of the slope=10% and %CV of intercept= 3% issued by ACCI.

The primary steps in establishing an assay is to test various ranges of CSE concentrations for constructing the standard curve. Ideally, the standard range with least variability must be chosen to determine the EU concentration of chitosan test samples; however, in this thesis all the tested standard curve ranges were used for determination of chitosan EU conc. A high range of standard curve was used initially i.e. 50-0.005 EU/mL based on the certificate of compliance issued by the supplier (Associates of Cape Cod) suggested for use of this range in a plate reader and because of its widespread use by another eminent supplier Lonza (Lonza, 2014). The ranges of the standard curve tested are systematically shown from **Table 4-2** to **Table 4-4**.

a) Repeatability of 50-0.005 EU/mL range

Table 4-2 : The repeatability of the standard range of 50-0.005 EU/mL with N=6 repetitions is depicted as a function of %RSD= $\frac{|Mean|}{SD} * 100$ (%CV) of slope and intercept and R^2 obtained from linear regression equations. Acceptable Slope %CV= 10%, intercept =3%

Tested range (50-0.005 EU/mL) (N=6)	Slope	Intercept	R^2 correlation coefficient of standard curve
Mean	-0.2041	3.0138	Mean = 0.9946 (SD= $\pm$0.0047 ; RSD= 0.4727)
St Dev	0.0388	0.0737	
%RSD (%CV)	19.0143	2.4457	

The %CV of intercept is below the acceptable limit, but the slope variation is 2 times higher than the acceptable limit, because the repeatability of this range is affected by the onset time of the highest EU conc. of the calibrators used for the curve. The highest standard calibrator of 50 EU/mL reaches the threshold O.D very quickly due to rapid gel formation after addition to LAL enzyme and produces very low onset time around 300 secs, which is too soon to record the whole

reaction in the reader and difficult to replicate. With a 2-fold high %CV value than acceptable limit, another range of standard curve was tested based on the recommendation from a major LAL supplier Charles River Laboratories (CRL) to test a four-log of assay sensitivity (log 0.005*4) standard curve from 10-0.005 EU/mL that may be suitable for the in-process and raw materials testing (CRL, 2006).

b) Repeatability of 10-0.01 EU/mL standard range

Table 4-3 : The repeatability of the standard range of 10-0.01 EU/mL with N=3 repetitions is depicted as a function of %RSD= $\frac{|Mean|}{SD} * 100$ (%CV) of slope, intercept and R^2 obtained from linear regression equations. Acceptable Slope %CV= 10%, intercept =3%.

Tested range (10-0.01 EU/mL) (N=3)	Slope	Intercept	R^2 correlation coefficient of standard curve
Mean	-0.1725	3.0080	Mean= 0.9938 (SD= $\pm$0.00901 ; RSD = 0.9071)
St Dev	0.0601	0.0685	
%RSD (%CV)	34.88	2.278	

Another standard range tested was 10-0.01 EU/mL, consecutive to the previous 50-0.005 EU/mL range; the %CV of the intercept was again under the acceptable limit, but the variability of the slope was even higher than the previous range, thus rejecting the usability of this range for testing chitosan EU conc. The highest conc. of calibrator yields very low onset time, which is not achieved consistently between experiments due to its rapid reaction with enzyme just after addition; due to quick clot formation it reaches the threshold O.D rapidly, and produces low onset time. This disparity creates high variability and thus high %CV of slope. With a difference of 34% from the acceptable limit, yet another range of standard curve with low conc. of starting calibrator was tested.

c) Repeatability of 5-0.005 EU/mL range

Table 4-4 : The repeatability of the standard range of 5-0.005 EU/mL with N=6 repetitions is depicted as a function of %RSD= $\frac{|Mean|}{SD} * 100$ (%CV) of slope, intercept and R^2 obtained from linear regression equations. Acceptable Slope %CV= 10%, intercept =3%

Tested range (5-0.005 EU/mL) (N=6)	Slope	Intercept	R^2 correlation coefficient of standard curve
Mean	-0.1941	3.1168	Mean= 0.9941 (SD= $\pm$0.0063 ; RSD= 0.6307)
St Dev	0.0199	0.1340	

%RSD (%CV)	10.2432	4.2999	

Due to problems in repeatability by using high concentration of standard calibrators, the concentration of the highest standard calibrator was reduced to 5 EU/mL, although the range was prepared using the same 10-fold dilutions and spans 2 logs rather than 4 logs above the assay sensitivity. The variation in slope was at the acceptable limit, but the variation in intercept was 1.4 times higher than the acceptable limit, the effect of 1% intercept variation resonates to 17% variation in the final EU conc. calculations (data not shown). Thus this standard range was classified to still have high concentration for calibrators to produce variation.

Due to high variability in standard curve ranges, there were burning questions as to what technical parameters may be contributing to the variation apart from high EU conc. The variability of the different standard curve ranges was suspected to have arisen from the use of plate reader instead of the tube reader which have lower sensitivity and block heating of sample inhibiting efficient LRW+LAL reaction; the repeatability may be affected by the rate of evaporation, difference in solution pathlengths and difference in temperature of the reagent and samples at the time of mixing. All the parameters were optimized to identify the controlling factors and obtain the repeatable standard curve even with the use of high endotoxin concentration range.

4.3.5 Optimization of parameters to obtain repeatable standard curves

Due to the use of plate reader instead of tube reader, there were several parameters, which had to be optimized to obtain repeatable standard curves. The externally controllable factors like rate of evaporation in the plates, pathlength of reactants and effect of temperature on reactant pathlengths and standard curves were tested to investigate the underlying causes of standard curve variability particularly in ranges with high endotoxin conc. The effect of these parameters on standard curve variability is shown in this section.

4.3.5.1 Use of plate reader for LAL assay

For all the LAL assays (except gel-clot), the plate reader was used because of its comparable sensitivity with the tube reader (Limit of detection: 0.005 EU/mL vs. 0.001 EU/mL). Some functional differences in the instruments were:

Parameters	Plate reader	Tube reader
Limit of detection (Sensitivity)	0.005 EU/mL	0.001 EU/mL
Sample Heating	Complete plate in a block manner	Individual tube heating
Running time	Fixed	Flexible
Pathlength	Variable	Fixed
Number of samples tested	Large	Moderate
Cost effectiveness	High	Medium

Typically, LAL assay is performed in a tube reader, which provides slightly more sensitivity over plate reader because of individual tube heating and flexible measurement time depending on the development of LAL turbidity. The plate reader heats the samples in a non-specific block manner vs. individual heating in tube reader and has a fixed time of measurement compared to flexible measurement time to reach the onset time of sample with lowest EU conc.; but the plate reader has an advantage of testing more samples per plate at a given time.

The difference in measurement of absorbance between tube reader and the plate reader originates from the orientation of absorbance beam. In the tube reader, the light is absorbed by the cuvette in a horizontal direction and in a vertical direction by the well of a 96 well plate in a plate reader. This is the basis of difference in pathlength of a plate reader and a tube reader and the pathlength in each well can fluctuate even by a pipetting error of $\pm$10% apart from the factors like extinction coefficient (**ε**) and concentration of the solution (**c**) as explained in section 4.2.5.1 **Equation 4-1**. Other factors like evaporation and water/solution expansion may also play a major role in measurement of sample absorbance in a plate reader, hence the influence of these varying parameters on the LAL standard curves and endotoxin quantification was observed as shown in the sections below.

4.3.5.1.1 Evaporation in 96 well plates: Middle and inner wells experience lowest evaporation

To observe pathlength as well as evaporation, absorbance of water is measured at 975nm because water shows maximum peak at 975nm by absorbing maximum light, the relative value obtained after subtracting with background absorbance at 900nm is used as an indicator of evaporation. For each well, the A975-A900 value of water may differ depending on the volume of water present. The decrease in the absorbance of water at 975nm is a representative of increasing evaporation (Multiskan, 2010). According to **Figure 4-11A & B and Table 4-5,** the outer wells (A1, A12, H1 and H12) experienced the highest rate of evaporation with the constant

reduction in A975-A900 value, as the A975-A900 started decreasing from t=1200 seconds and constantly decreased with lowest slope at t=7200s with Δ O.D = 0.0029 compared to rest of the well types. The inner and side wells also showed slight evaporation with Δ O.D of 0.0005 and 0.0011 respectively having a small impact on the pathlength at ΔPL= 0.0024 cm and 0.0057 cm respectively (**Table 4-5**). However, the slope of middle wells kept increasing from t=2400 s to the highest among all well types at the end of the run. The Δ O.D value (t_{exp}-t_{7200}) of the middle wells only shows increase in the negative y-axis, because of increased surface tension and water expansion which overpowered the evaporation due to the sentinel effect of water molecules from other wells; this expansion effect leads to highest A975 value at the endpoint (t=7200s).

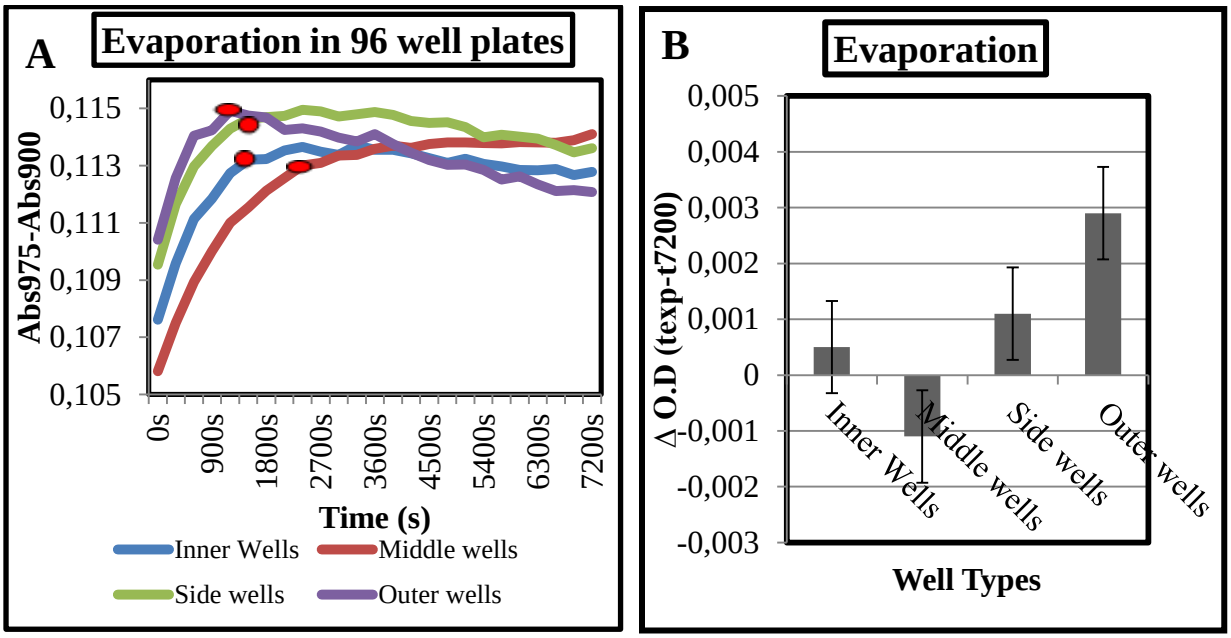


Figure 4-11 : The A975-A900 profile of all the well types are demonstrated, the slope of the middle wells increases and that of outer wells decreases with time, the red dots denote the time of reaching exponential phase (A) the ΔO.D values calculated by subtracting the time at which the A975-A900 reaches the exponential phase by the end point (t_{exp}-$t_{=7200s}$) which is an indicator of evaporation on positive y-axis and volume expansion on negative y-axis, the graph shows highest evaporation for outer wells and volume expansion for middle wells (B).

When the evaporation gradient Δ O.D (t_{exp}-t_{7200}) is compared to the change in pathlength Δ Pathlength (t_{exp}-t_{7200}) in **Table 4-5**, it was observed that inner wells are least affected by change in pathlength and affected very slightly by smaller rate of evaporation. The middle wells,

although experiencing no evaporation, experience higher change in pathlength compared to other wells due to more expansion of water than evaporation, which creates a gap in pathlength values between exponential phase and t=7200s (**Figure 4-11 A**). The results were corroborated by the %CV (Coefficient of Variation) values of A975-A900 in **Table 4-6** showing the highest variation in middle well; the lowest variation in the side and outer wells is attributed to high evaporation and a slight increase in A975 values from t=0s to t=7200s compared to inner and middle wells, %CV of a sample well is calculated by dividing the mean O.D at all the time points with its standard deviation and conversion into percentage.

Table 4-5 : The evaporation quotient of each well type is illustrated by subtracting the highest A975-A900 value at the exponential phase i.e. t_{exp} with that of endpoint value (t=7200s); the time to reach exponential phase for each well type is different showing the effect of well positions on reaction profile; and the Δ O.D (t_{exp}-t_{7200}) results are corroborated by the change in pathlength data between t_{exp} and $t=_{7200s}$ for all well types.

Well type	Time to reach exponential phase (t_{exp})	A975-A900 at t_{exp}	A975-A900 at t=7200s	Δ O.D (t_{exp}-t_{7200})	Δ Pathlength (cm) (t_{exp}-t_{7200}/ K-factor)
Inner Wells	1500s	0.1132	0.1127	0.0005	0.0024
Middle wells	2400s	0.1130	0. 1141	-0.0011	-0.0061
Side wells	1500s	0.1147	0.1136	0.0011	0.0057
Outer wells	1200s	0.1149	0.1120	0.0029	0.0158

Table 4-6 : Percentage coefficient of variation (%CV) of each well type calculated by dividing the mean of all time points (from t=0s to t=7200s) with their standard deviation. The well positions with minimum and maximum %CV in A975-A900 are listed.

Experiment	Minimum %CV (A975-A900)	Well position	Maximum %CV (A975-A900)	Well position
N=1	0.74	D1- side well	1.85	E9- middle well
N=2	1.08	H1- outer well	2.15	D9- middle well
N=3	0.79	H1- outer well	2.42	D9- middle well

The total lost volume from evaporation in the 96 well plates calculated from the difference in weighed mass of the plate+ lid before and after 2 hours' assay at 37˚C was 477µL on average which accounts for the total loss of 3.5% (**Table 4-7**), mainly contributed by the outer wells and in part by the side and inner wells.

Table 4-7 : Volume of evaporated water in the 96 well plates.

Average Initial LRW volume (mL)	Average Volume evaporated (mL)	Average Final volume (mL)	Percent Water loss (%)
14.18	0.477	13.70	3.5%

The outer and side wells were not used in the next part of the studies in order to reduce the variability in the results caused by evaporation. Only the inner and middle wells (B2-G11) were filled with samples and outer and side wells were used as sentinel wells filled with water to limit evaporation.

4.3.5.2 Effect of pathlength on standard curves

Since pathlength affects the absorbance values and in turn the onset time of each endotoxin conc., the pathlength needs to be consistent in each assay, in a plate reader due to the absorbance of light in vertical direction, the height of the liquid determines the pathlength. Hence a $\pm$10% pipetting error can increase/reduce the pathlength to 10%, which can increase/decrease the onset time and in turn affect the repeatability of the standard curve between the assays. Because the manual pipetting error is unavoidable, the absorbance values measured on a microplate, can be normalized to correspond to absorbance values measured in a cuvette by performing pathlength correction (ThermoSci, 2012).

One of the challenges in quantifying the endotoxin concentration of a test article apart from avoiding interference was to obtain a repeatable and reproducible standard curve. Many factors are responsible for producing variability in the standard curve such as lot-to-lot variability in LAL enzyme and CSE, difference in conc. of buffers, processing methods and materials and equipment used. All the factors lead to variability, which skews the endotoxin quantification leading to untrustworthy results. The most important parameter of the standard curve, which affects the endotoxin quantification significantly, is the intercept; even 1% variance in the y-intercept can lead to 17% difference in endotoxin conc. determination (data not shown). Hence, it is important to obtain a repeatable standard curve for proper endotoxin quantification.

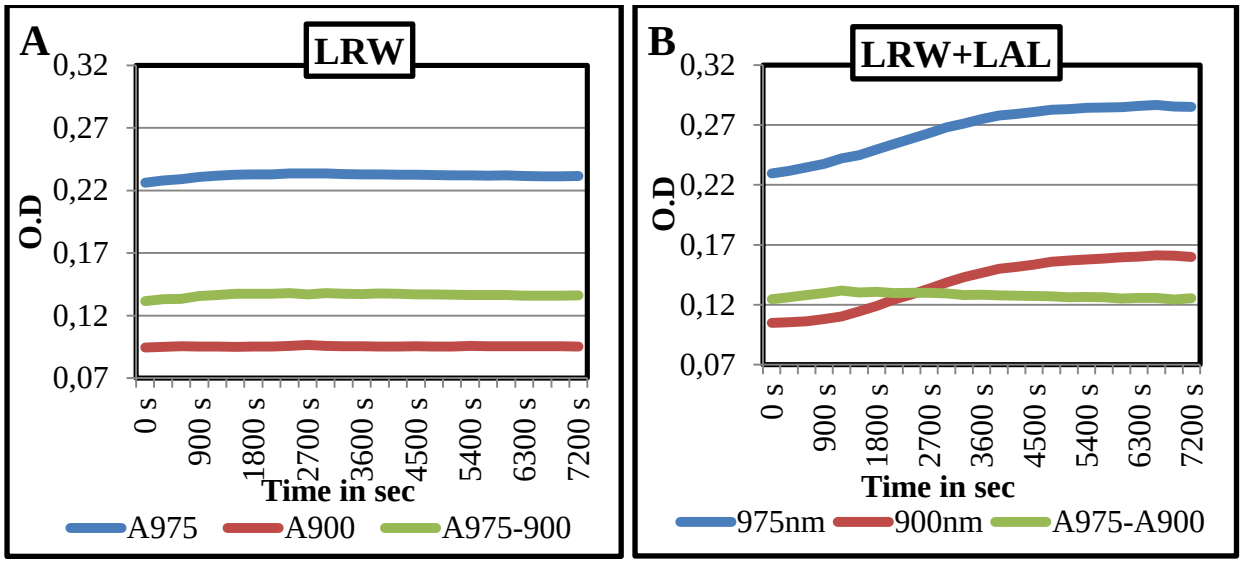
4.3.5.2.1 Determining the pathlength of LRW+LAL used for correcting sample absorbance

The K-factor of the reactants calculated according to **Equation 4-3** and pathlength of different reactants calculated according to **Equation 4-2** used in optimization of LAL assay is shown in **Table 4-8** and **Figure 4-12**. The difference in the pathlength of the reactants can be observed due

to difference in their peak absorbance at 975nm, which is reflected in their K-factors. The pathlength of each reactant remains consistent according to **Figure 4-12** with just 8% decrease in LRW+LAL pathlength than LRW (**Table 4-9**), but the absorbance of LRW+LAL at both 975nm and 900nm increases with time due to enzyme cascade reaction of LAL with endotoxin present in LRW; this reaction could be compared with LAL only, in which enzyme cascade is seen to a lesser extent.

Table 4-8 : K-factor of the reactants used in LAL assay calculated using **Equation 4-3**. LRW+LAL K-factor were used for pathlength correction of CSE samples used as standard calibrators.

Reactant	K-factor
LRW	0.179
LRW+LAL	0.186
LAL	0.185



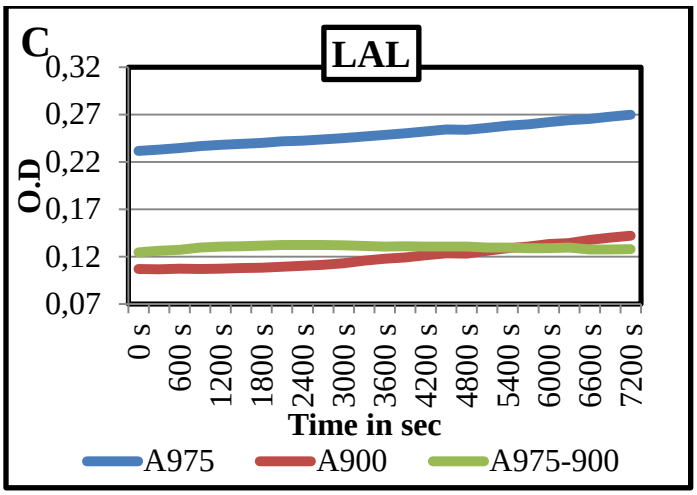


Figure 4-12 : The absorbance profiles at 975nm and 900nm of LRW (A) LRW+LAL (B) and LAL (C). The A975-A900nm value at each time point was used for calculating pathlength by dividing with the K-factor of respective reactant.

According to **Table 4-9**, the calculated maximum and minimum pathlength values are lowest for LRW+LAL and highest for LRW. Since, LRW is used as a diluent for each sample and LAL enzyme is added on top for reaction, LRW+LAL is selected as a reactant for pathlength correction.

Table 4-9 : The maximum and minimum pathlength of each reactant is shown. The %CV shows variability in pathlength of each reactant over time.

Reactant	Max PL	Time to reach Max PL (s)	Min PL	%CV
LRW	0.772	2400s	0.736	1.131
LRW+LAL	0.711	1200s	0.670	1.597
LAL	0.716	2100s	0.674	1.518

4.3.5.3 Effect of temperature of reactant addition on Pathlength

The treatment condition at 37˚C means that the LRW and LAL enzyme were incubated at 37˚C prior to beginning the recording of absorbance at 975 and 900 nm; similarly, the LRW and LAL enzyme were prepared at room temperature for RT condition and then subjected to absorbance readings in the Tecan infinite spectrophotometer set at 37˚C. The pathlength of LRW+LAL is higher at 37˚C than at room temperature (RT) because the volume expansion of the LRW+LAL is higher at 37˚C than at RT. In addition, the molecules of LAL enzyme and small endotoxin

amount in LRW react rapidly with each other after pre-conditioning at optimal reaction temperature of 37˚C. Thus, in the consecutive experiment, the LAL enzyme and the samples were pre-conditioned at 37˚C before absorbance measurement.

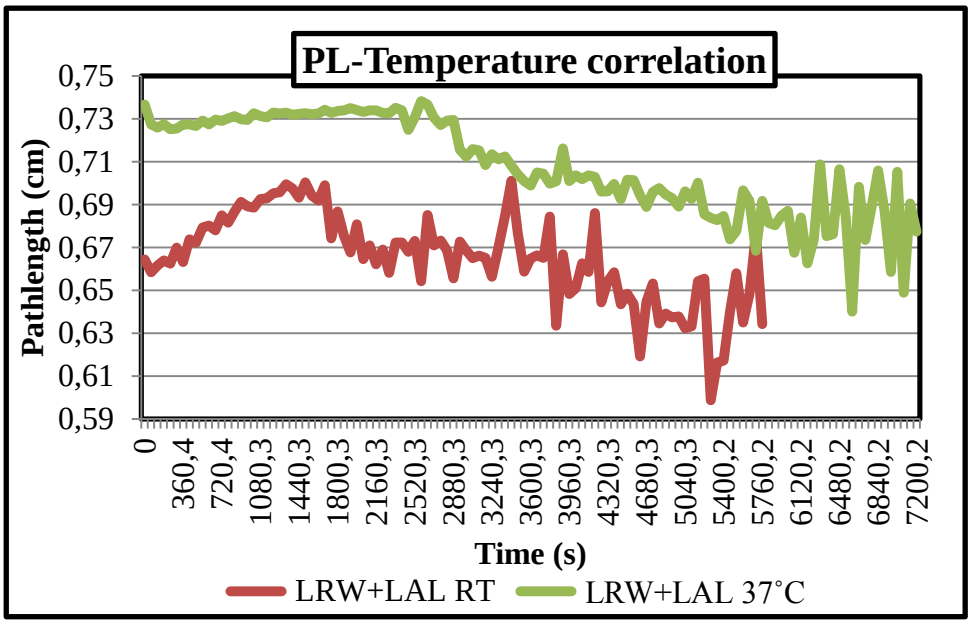


Figure 4-13 : Effect of temperature on the pathlength of LRW+LAL. The difference in the temperature of reactants at the time of addition affects the reaction profile and the pathlength.

4.3.5.4 The repeatability of the standard curves in the plate reader after applying optimized parameters

After identifying all the controlling parameters affecting the repeatability of the standard curves, the experiment was performed by applying the optimal instruments such as using the inner and middle wells, testing with 250µL sample +LAL volume, correcting the sample pathlength with that of LRW+LAL and pre-conditioning the enzyme and the sample at 37˚C before absorbance measurement. The standard curves shown in **Figure 4-14** are produced by diluting the CSE between the range of 50-0.005 EU/mL, with 20, 10 and 1 EU/mL used as intermediate points. The log of onset time and log of calibrator concentrations was plotted in a regression curve. The repeatability of the standard curve depends on the three parameters- slope, intercept and correlation coefficient (R^2). As seen in **Figure 4-14** and **Table 4-10**, there is very small variation between the slopes and intercept values of all the three standard curves. The %CV

of slope and intercept calculated by applying the formula Mean/SD*100 is within the acceptable limit of 10% and 3% respectively set out by the LAL supplier (ACCI).

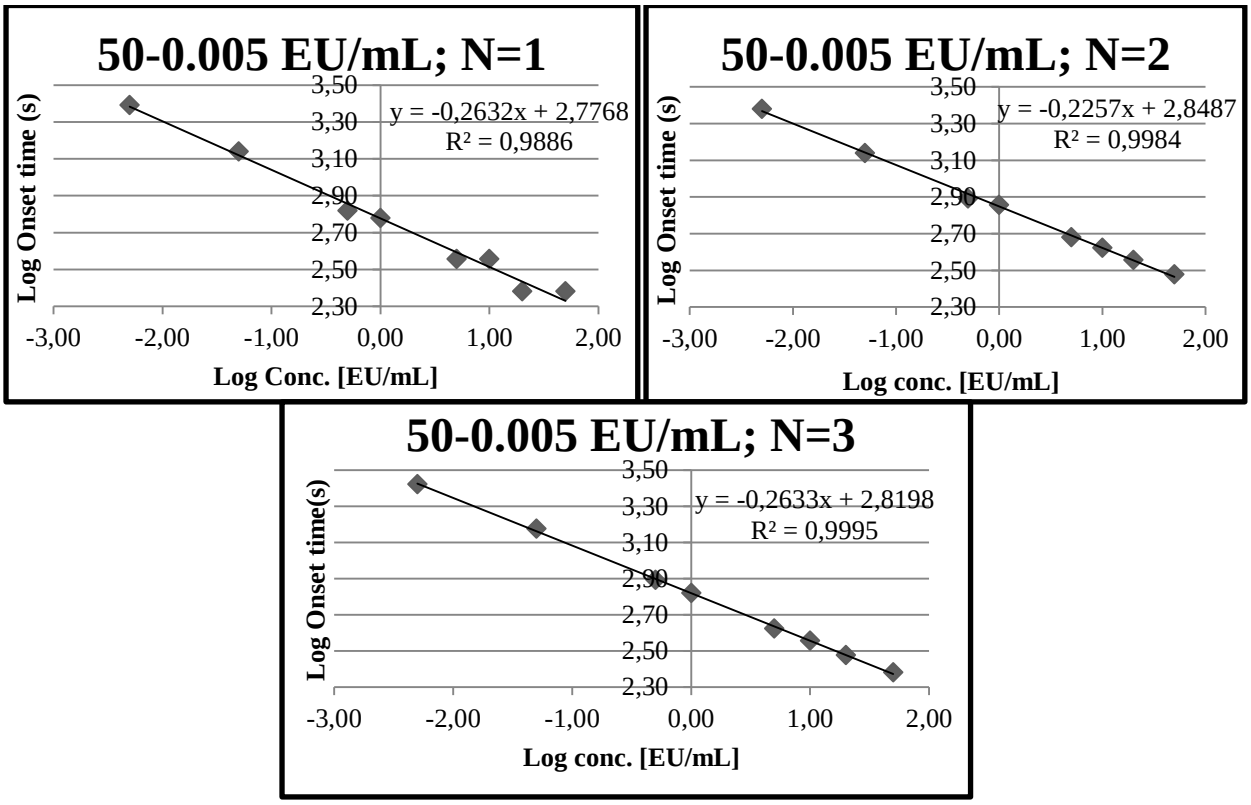


Figure 4-14 : Standard curves generated from turbidimetric LAL assay prepared after applying the optimized parameters like use of inner wells to prevent evaporation, application of LRW+LAL pathlength and pre-conditioning of enzyme and samples at 37°C to obtain repeatable standard curve. The CSE samples used are in the range of 50-0.005 EU/mL prepared at 10-fold dilutions with the intermediate conc. of 20, 10 and 1 EU/mL.

The %CV of slope at 8.65% for N=3 standards is at least 2 times lower than the 19% variability of the same range found before applying the optimal parameters in **Table 4-2**. This indicates that the repeatability of the standard curves after the optimization of parameters has improved significantly.

Table 4-10 : The variability in the slope, intercept and R^2 after pre-conditioning of samples and LAL enzyme at 37˚C shows the repeatability of the 50-0.005 EU/mL standard curve range performed N=3 times in the plate reader.

Treatment conditions	%CV Slope	%CV Intercept	%CV R^2 of standard

			curves
37˚C (N=3)	8.65	1.29	0.60

4.3.6 Post-optimization of parameters: Repeatable standard curve ranges for Turbidimetric LAL assay

4.3.6.1 Use of smaller curve range of 0.16-0.005 EU/mL to obtain repeatable standard curve and match expected endotoxin values in chitosan

Table 4-11 : The repeatability of the standard range of 0.16-0.005 EU/mL with N=3 repetitions is depicted as a function of RSD= $\frac{|Mean|}{SD} * 100$ (%CV) of slope, intercept and R^2 obtained from linear regression equations. Acceptable Slope %CV= 10%, intercept =3%

Tested range (0.16-0.005 EU/mL) (N=3)	**Slope**	**Intercept**	**R^2 correlation coefficient of standard curve**
Mean	-0.253	2.891	Mean= 0.990 (SD= $\pm$0.007; RSD= 0.713)
St Dev	0.015	0.022	
%RSD (%CV)	5.880	0.755	

A small range of standard curve was used to match the expected endotoxin value of chitosan estimated at $\leq$0.03 EU/mL at 1:100 dilutions by gel-clot LAL assay; although all the ranges utilized and detailed in section 4.3.4.3 enclosed this value ($\leq$0.03 EU/mL) within the range for quantification, the high rate of variability allowed large margin of error in quantification. However, the values quantified by using one standard curve range cannot be discarded because the values will be an estimation of absolute conc. of endotoxin present in chitosan.

The repeatability of standard range with low concentration of calibrators at 2-fold difference was very significant; the %CV of slope and intercept was well within the respective acceptable limits. The small conc. of the highest calibrator allows more reaction time, thus making it easier to capture the entire reaction profile and obtain an accurate onset time. This modification is perhaps the most important one in consistently obtaining a repeatable standard curve range. However, this range will be adapted for testing in future quantification of endotoxin in chitosan samples for comparison with the gel-clot estimated EU value.

The variability in the slope of various standard curve ranges tested altogether is about 2-fold higher and that of intercept is closer to the acceptable limit. It was important to compute the variability between the entire curve ranges because the chitosan samples were tested for EU conc.

at different standard ranges in parallel to the determination of the optimal curve range, the evaluated EU conc. of chitosans before and after pre-treatments are computed using the linear equation from several standard curves and reported in Chapter 5.

4.3.7 Conclusion

The parameters affecting the repeatability of the standard curves, especially with ranges including high EU concentrations i.e. 50-0.005 EU/mL, were identified and applied for the improvement of repeatability, which included:

a) The use of inner and middle wells in the 96 well plate to prevent evaporation

b) Correcting the pathlength of the sample and that with appropriate reactant (LRW+LAL) to reduce variability in onset times and resulting slope and intercept. And;

c) Pre-heating the LAL enzyme and the samples at 37˚C before mixing for efficient catalysis of reaction cascade.

These parameters act as a guideline for the future assays to obtain repeatable standard curves. However, the use of small curve range (0.16-0.005 EU/mL) always guarantees high repeatability of the standard curves.

4.4 Discussion

Determination of endotoxin concentration of chitosan is very important to regulate the dosing for future clinical applications of chitosan nanoparticles and scaffolds. The scaffold dose application in the intra-articular injection in horses is specifically sensitive to fluctuating levels of endotoxin, as the acceptable harmonized limit of injectable endotoxin is very low. The estimated EU value at $\leq$0.3EU/mg generated by gel-clot LAL that can also be accepted in case of discrepancy between the other two kinetic LAL methods, could be used for simulation of endotoxin content of currently injected in vivo dose, but for the future applications to avoid the underestimation or over estimation of endotoxin content, the absolute determination of chitosan endotoxin is required. The use of sensitive kinetic turbidimetric LAL assay will allow the absolute endotoxin quantification, but the problem of variation in standard curve deters the significance of quantified values. The range of variation is 50-200% complemented by variations from other technical sources. After recognizing and optimizing the sources/parameters causing variations such as

evaporation in 96 well plates (**Table 4-7**), difference in pathlengths of reactants (**Figure 4-12**) and effect of pre-conditioning of reagents at optimal temperature on assay performance (**Figure 4-13**); we were able to prove our first hypothesis that a repeatable range of standard curve with minimum allowable variation is determined after examination and optimization of all parameters prone to variation (**Table 4-10**). Our second hypothesis is proved by adopting a small range of standard curve matching the gel-clot estimated endotoxin value of chitosan that is repeatable after N=3 repetitions as shown in **Table 4-11** when tested by application of optimized parameters. Since, the endotoxin conc. of chitosan reported in this thesis are quantified from large standard curve ranges, the small range of standard curve is recommended for future quantification of chitosan endotoxin, and must be established for regular use in endotoxin quantification protocols.

CHAPTER 5 QUANTIFICATION OF CHITOSAN ENDOTOXIN; IDENTIFICATION OF INTERFERENCES AND METHODS TO REDUCE CHITOSAN INTERFERENCE WITH ENDOTOXIN

5.1 Introduction

The major inspiration behind the commencement of this thesis was to quantify the endotoxin conc. in different batches of chitosan, in the form of raw materials and nanoparticles and detect the interference caused by the chitosan to endotoxin determination. As a rule, for products involving downstream processing, the quality control protocol must test the raw material and the processed chitosan used in the preparation of CS-siRNA nanoparticles and chitosan scaffolds for intravenous and intra-articular animal injections respectively for endotoxin contamination. A diagram demonstrating the workflow of chitosan preparation identifying the processing points and the types of chitosan produced which should be tested for endotoxin contamination is shown below (**Figure 5-1**).

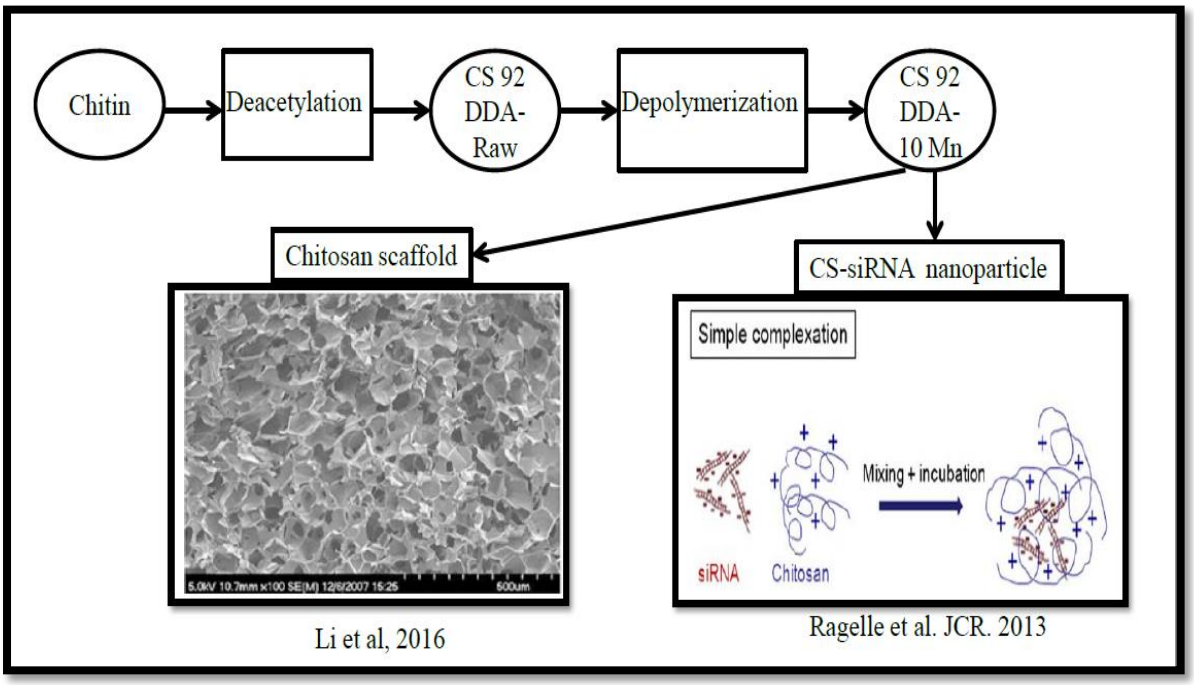


Figure 5-1 : Typical flowchart of processing of raw chitosan for production of specified DDA, then depolymerisation of raw chitosan to low molecular weight chitosan and then to final

nanoparticle and scaffold formulation. Figures adapted from (Liu et al., 2015; Ragelle et al., 2013)

The quantification of absolute conc. of endotoxin in chitosan raw materials i.e. CS 92-10 and CS 92-Raw is detailed in this section by turbidimetric LAL assay according to specifications in BET Chapter 85 of USP. It required the use of positive product controls (PPCs) or Inhibition/Enhancement controls (IEC) to detect the interference by the chitosan or test reagent during the LAL assay (USP, 2005). Different types of interference caused by chitosan during the quantification of endotoxin have been identified, and the removal of enhancement interference by dilution technique is covered. All the chitosan samples and dilutions were tested with different standard curve ranges with turbidimetric LAL assay. Different types of Pre-treatments to reduce the inhibition interference of chitosan such as NaOH, chitosanase and nitrous acid treatments performed before LAL assay are tested for their potential to remove the bound endotoxin from glucosamine chains. Detection of inhibition interference by chitosan towards endotoxin is identified by the use of chitosan challenge sample spiked with a known amount of endotoxin (CSE). Due to the superior accuracy of chromogenic LAL assay in detection of interference by the challenge sample than turbidimetric LAL and reduced interference by chitosan precipitation, the chromogenic LAL assay will be used regularly for chitosan endotoxin determination. Finally, due to deleterious effect of chemical reagents on the endotoxin, the effect of chitosan rehydration/ dissolving agents on endotoxin integrity have been tested.

5.2 Materials and Methods

5.2.1 Quantitative measurement of chitosan endotoxin conc. by kinetic LAL assay according to USP 85

The turbidimetric LAL assay is typically used for the quantification of endotoxin for the desired sensitivity of 0.005 EU/mL. The assay depends on the formation of turbidity in the presence of endotoxin, which is directly proportional to the conc. of endotoxin. Similarly, the sensitivity of the kinetic chromogenic LAL assay is 0.005 EU/mL, and the amount of endotoxin in a sample is a function of development of chromogenic substrate after cleavage of enzyme. The absorbance over time profile of each sample can be observed in kinetic format. In this thesis,

typically turbidimetric LAL assay is used for standard curve repeatability test and simultaneous endotoxin quantification unless otherwise stated.

5.2.1.1 Procedure of endotoxin determination by LAL assay

A standard curve range is prepared by diluting control standard endotoxin (CSE) of known potency eg, 1000 EU/mL for specific lot of enzyme, this potency is determined against a reference standard endotoxin (RSE) prepared by US Pharmacoepia (USP) and changes for each CSE lot due to batch of bacteria and preparation methods. The standard curve range was prepared from CSE and used for determination of unknown chitosan EU conc. For each test chitosan was dissolved in HCl at the HCl: glucosamine ratio of 1:1 overnight at room temperature, unless otherwise stated. The chitosan test sample, show interference in the detection of endotoxin, either by obstructing enzyme activity or binding with endotoxin, this interference can be reduced by the diluting the sample several folds and establishing the minimal dilution at which endotoxin can be detected and interference is eliminated. This method is called Maximum Valid Dilution (MVD) and is recommended by USP, but typically, this method is used for the samples with fixed endotoxin limit and an established dose of administration, which is not the case with chitosan samples. For a controlled pharmaceutical product, the MVD is calculated using the formula below:

Equation 5-1 : $$MVD = \frac{Endotoxin\ Limit * sample\ conc.}{assay\ sensitivity\ (\lambda)}$$

Where $\text{Endotoxin limit (EL)} = \frac{\text{Maximum endotoxin limit allowed per dose}}{\text{Maximum product dose administered per kg body weight per hour}}$ Eg ; $\frac{5\frac{EU}{kg}}{0.5\frac{mg}{kg}/h} =$ 10 EU/mg

Sample conc. = the stock concentration of the sample prepared for endotoxin detection. Eg, 10mg/mL

Assay sensitivity or λ= Sensitivity of the assay used in the determination of sample endotoxin i.e. 0.005 EU/mL.

The MVD of a test sample prepared at 10mg/mL is calculated at 20,000 [(10 EU/mg* 10mg/mL)/0.005 EU/mL]

Samples like chitosan for which no information of clinically applicable dose in humans is available, the interferences are eliminated by testing several dilutions and determining the minimal dilution at which precise measurement of endotoxin is achieved and any possible interference can be eliminated.

5.2.1.1.1 Significance of Positive product control (PPC) or Inhibition/Enhancement control (IEC)

For each dilution of chitosan test or challenge sample (in section 5.3.3) prepared during the assay, a known endotoxin amount was spiked that was near or equal to the middle of the standard curve. This control is called positive product control (PPC) or Inhibition/Enhancement control (IEC) because it helps in detecting the interference produced by the test sample during the assay; it was prepared in parallel to all the test sample dilutions. The acceptable recovery of endotoxin from the PPCs is calculated as

Equation 5-2 : $PPC\ recovery\ at\ a\ certain\ dilution = \frac{PPC\ EU\ conc. - Test\ sample\ EU\ conc.}{Conc.of\ endotoxin\ spike} * 100$

If at any dilution, the recovery of endotoxin from the positive product control was between acceptable recovery range of 50-200%, then the test sample was considered free of interference, the dilution of optimal recovery could be attributed equivalent to the maximum valid dilution, and the absolute endotoxin conc. of the test sample was reported after adjusting for dilution factor. The process of selecting the MVD, reporting sample EU conc. and role of PPC in the assay is explicitly represented in **Figure 5-2** below:

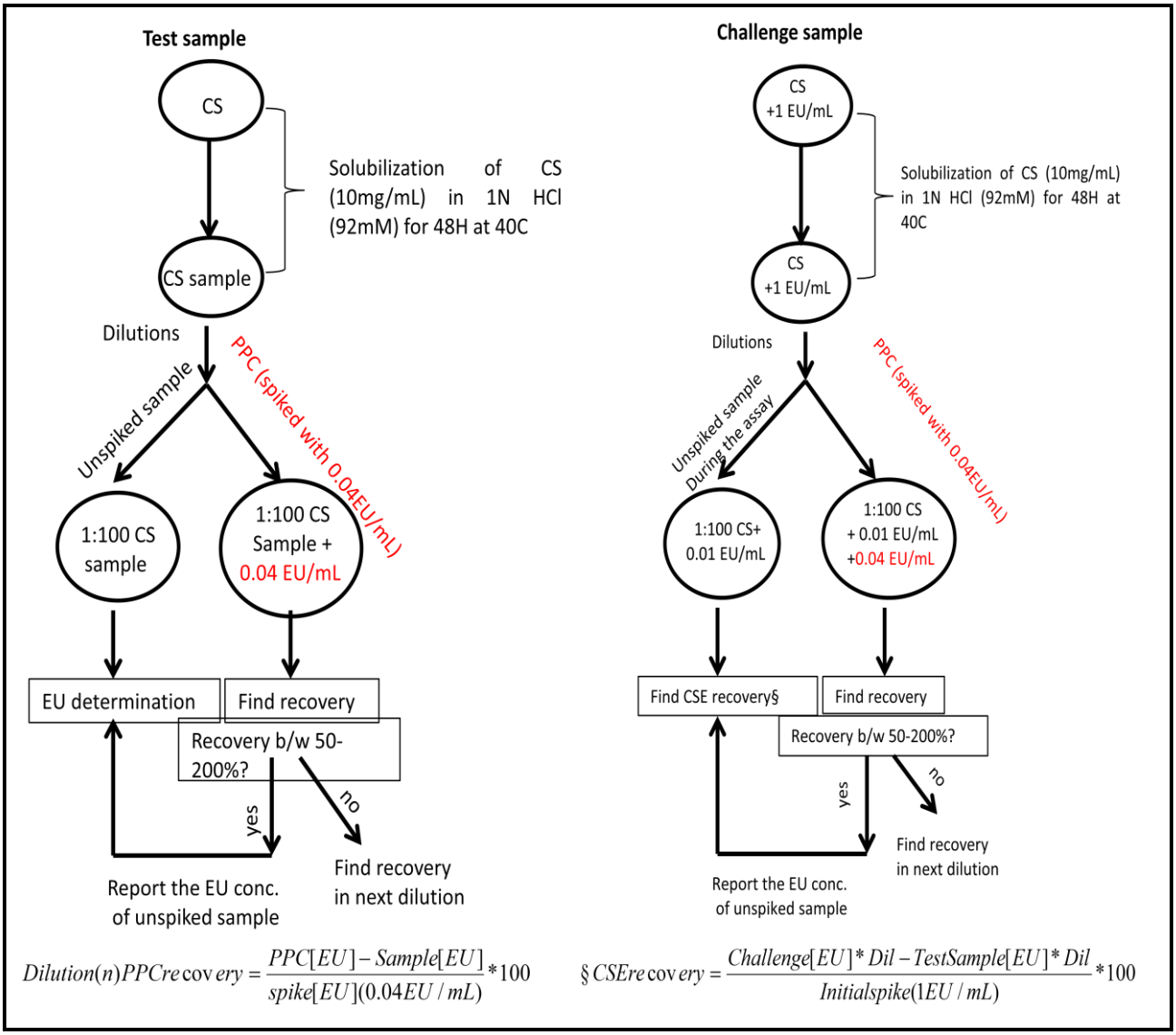


Figure 5-2 : The diagram illustrates the concept of PPC and its role in determining sample EU concentration at corresponding dilution

This interference was recognized in the assay by the deviation from the acceptable 50-200% PPC recovery range. If the endotoxin recovery in PPC was above 200% it indicates enhancement interference and below 50% indicates inhibition interference.

5.2.1.1.2 Importance of a challenge sample

If for a test sample, the treatment conditions such as reagents, time and temperature of incubation are changed then according to the USP; the sample must be spiked with a known amount of endotoxin and tested in addition to the test sample for understanding the effect of the

treatment on the endotoxin integrity; this sample also served to understand the extent of interference to the endotoxin by the chitosan sample. This sample is called a "Challenge sample". Eg; if the treatment of chitosan is changed from HCl to 0.2% acetic acid, then during rehydration/ dissolution in parallel to the test sample, a challenge sample of chitosan with a known endotoxin spike (1 EU/mL) must also be tested to observe the affect of 0.2% acetic acid on the recovery of known conc. of endotoxin from chitosan. Even though the Inhibition/Enhancement controls (IEC) aka PPCs are present for detection of interference by the sample to endotoxin during the assay, the period of incubation of endotoxin with chitosan is less than 1 hour, which may not be an accurate indicator of inhibition interference. To determine the magnitude of interference of chitosan towards endotoxin, the CSE recovery of the challenge sample at any dilution is calculated using the **Equation 5-3**:

Equation 5-3 : $$\%\ CSE\ recovery = \frac{Challenge\ EU\ conc.*Dil - Test\ sample\ EU\ conc.*Dil}{Conc.of\ endotoxin\ spike} * 100$$

The percent CSE recovery was compared with the 100% initial spike conc. and the value will help determine the extent of interference demonstrated by the chitosan towards the endotoxin under the tested treatment conditions and incubation times/ temperature. The acceptable recovery is within 50-200%.

5.2.2 Pre-treatment of chitosan for removal of interference

To reduce the electrostatic interaction between chitosan and endotoxin- the basis of inhibition interference by the chitosan samples, several pre-treatment methods were tested targeting the electrostatic binding by charge neutralization and secondly by reducing the size of chitosan monomers to limit the interaction with large molecular weight E. coli LPS or its aggregates. The pre-treatments performed are described below:

5.2.2.1 NaOH treatment of chitosan

The charge neutralization of chitosan by the alkaline reagent was aimed to reduce the electrostatic charge and release the endotoxin bound to the chitosan in the alkaline solution. This method has not been used elsewhere, and is accustomed for chitosan samples. For NaOH treatment, the chitosan 92-10 was dissolved in HCl at HCl: glucosamine ratio of 1:1 at a conc. of 10mg/mL, the challenge CS 92-10 sample at 10mg/mL was spiked at 1 EU/mL and tested

alongside the test CS 92-10 sample to observe the CSE recovery and effectiveness of treatment to remove chitosan bound endotoxin. Both the samples were treated with 1N NaOH by adding 80 ±10µL in 1mL sample solution. The pH was also measured and instant precipitation was observed in both the sample. The samples were then centrifuged at 10000xg for 10 mins and the supernatant obtained was used for endotoxin quantification by dilution in turbidimetric LAL assay within the detection range of 50-0.005 EU/mL. The EU content of 0.08N NaOH was also determined, which was found to be below the range of detection.

5.2.2.2 Chitosanase treatment

The chitosanase enzyme from Streptomyces griseus -a gram positive bacterium was typically utilized to digest the chitosan glucosamine chains and reduce them into small monomers; the aim of the test was to reduce the interaction of typically large molecular weight LPS or its aggregates with chitosan by reducing the size of glucosamine chains. However, before performing this test, the endotoxin content of the enzyme itself was determined, as a part of quality control process, because of its origin from a biological source that involved multiple culturing, extractions and processing steps. The 100U/mL enzyme reconstituted in 50% glycerol was tested at 1:50 dilution twice by turbidimetric LAL assay within the detection range of 50-0.005 EU/mL, because of estimation of high endotoxin contamination. The reconstitution reagent i.e. 50% glycerol was also tested for endotoxin contamination, which was found to be at a very low range.

5.2.2.3 Nitrous acid (HONO) treatment

With high endotoxin contamination in the chitosanase enzyme, a chemical treatment method with similar principle as enzymatic digestion was utilized that depolymerizes the chitosan and result in the production of small sized monomers leading to reduced interaction with high MW LPS or its aggregates. This is a commonly used method for reducing high molecular weight chitosans into low molecular weight, but due to harsh acidic and alkaline treatment is considered as the last resource to reduce interference in chitosan. Hence, the effect of this treatment was observed on the integrity of endotoxin by treating the endotoxin (CSE) spiked in LAL reagent water (LRW) at high conc. i.e. 50 EU/mL, because of high probability of reduction in endotoxin content due to acid hydrolysis. The acid hydrolysis occurs under highly acidic conditions at moderate temperature, which is supplied in this treatment at 50°C for 3 hours. The nitrous acid (HONO)

has a pKa of 3, which is maintained under acidic conditions supplied by HCl. The nitrous acid is activated in the presence of HCl, hence HCl conc. is constantly maintained 28mM higher than the conc. of HONO used for any glucosamine: HONO ratio aka $R_{NH2/\ HONO}$ used in depolymerisation.

Three different conc. of HONO+ HCl were used to treat 50 EU/mL CSE spiked LRW to obtain one conc. suitable for endotoxin recovery, 1st conc. equivalent to the glucosamine: HONO ratio ($R_{NH2/\ HONO}$=0.5) used the depolymerisation of chitosan with 55.91mM HONO+ 83.91mM HCl, 2nd conc. with high HONO+ HCl conc. at R=0.2 with 139.7mM HONO+ 167.7mM HCl and 3rd conc. with low HONO+ HCl conc. at R=0.9 with 31.06mM HONO+59.06mM HCl; one CSE spiked LRW sample was treated only with 83.91mM HCl equivalent to R=0.5 without HONO to observe the effect of HCl on endotoxin integrity at conc. used for depolymerisation of chitosan. As HONO was activated in the presence of HCl, HCl conc. was always 28mM higher than HONO to attain excess hydrogen ions for efficient depolymerisation reaction. The treatment was repeated three times (N=3) for high robustness in the data. The turbidimetric LAL assay was performed after the completion of 3 hours' reaction quantified within the range of 50-0.005 EU/mL. The only pH adjustment was at the end of the assay by addition of LAL enzyme reconstituted in glucashield buffer.

5.2.2.3.1 Chromogenic LAL: Repeatability of 0.16-0.005 EU/mL standard range

Small range of standard curve such as turbidimetric was used for comparability and to test the EU conc. of chitosan. Chromogenic assay was found to be more sensitive to quantify EU conc. of chitosan without the elements like interference with LAL proteins impeding the detection due to different assay chemistry. It could easily detect interference of endotoxin in chitosan challenge sample, giving reliable results compared to turbidimetric assay. The initial results of chitosan EU determination and interference detection were performed with turbidimetric assay while the chromogenic assay was used for determination of optimal reagent for chitosan rehydration.

The standard calibrators were prepared by diluting the CSE stock of 100 EU/mL 5-folds to obtain an intermediate solution of 20 EU/mL, serially another 3.2 and 0.32 EU/mL intermediate solutions were prepared to finally begin the preparation of 0.16 EU/mL, 0.08 EU/mL, 0.04 EU/mL, 0.02 EU/mL, 0.01 EU/mL and 0.005 EU/mL standard calibrators. All the samples were pipetted in triplicate wells of a 96 well plate and inserted in an incubating plate reader at 37°C for 20 mins before beginning the reaction. The pyrochrome lysate (LAL enzyme for chromogenic

assay) was reconstituted in glucashield buffer for endotoxin specificity and incubated in a water bath set at 37°C for approximately 20-30 mins. The temperature equilibrated LAL enzyme was pipetted on top of the acclimatized samples for reaction to start rapidly; 5 mins within the pipetting of enzyme, the reaction was allowed to run for 2 hours at 405nm at the absorbance recording interval of 1 min 25 sec, the absorbance at 975nm and 900nm were also measured to correct for pathlength in each well. After run completion the onset time was automatically calculated by the Magellan software and the standard curve was plotted by log onset time vs log (calibrator) conc. A straight line passing through all the regression points represents the linearity, which was within the USP acceptable limit of $\geq$0.98 for all the three repeated standard curves; the %RSD (%CV) for slope and the intercept was compared with the issued acceptable limit of 10% and 3% respectively by the ACCI.

5.2.2.4 Determination of optimal chitosan rehydration method for maximum endotoxin recovery

Due to the observed interference by 83.91mM HCl in endotoxin detection, and the probability of low pH unbuffered after the addition of glucashield buffer diluted in LAL enzyme, the USP method was used which recommends buffering of (chitosan) samples treated with 92mM HCl by β (1-3)-D -glucan blocking buffer commercially named glucashield buffer (from ACCI) to optimize the pH for efficient endotoxin detection. Since low pH by HCl was a problem, another reagent used by ACCI to treat and rehydrate chitosan samples was also tested after buffering to elucidate the effect of different rehydration/ chitosan dissolving agents on the endotoxin recovery due to their possible endotoxin releasing mechanisms. The high conc. of endotoxin used for spiking LRW samples in previous tests was also reduced from 50 EU/mL to 1 EU/mL, to increase the accuracy of detection. Both the CSE spiked LRW samples and challenge chitosan samples were tested in this study.

To choose the optimal method for rehydration of chitosan powder, to enable it to repel endotoxin and release it from its surface, a study was designed using different standard methods utilized by the USP for chitosan monograph and the Associates of Cape Cod (ACCI). The USP recommends dissolving chitosan in 92mM HCl at a concentration of 10mg/mL at 40°C for 48 hours before endotoxin detection by LAL assay. The ACCI is a contract research organization that specializes in endotoxin detection and a leader from past 40 years in the field. For chitosan,

they utilize rehydration method by 0.2% acetic acid at a conc. of 10 mg/mL at 37°C for 1 hour. The USP and ACCI methods lead us to investigate the role of time of incubation and temperature of the treatment in reducing/enhancing the recovery of endotoxin from the samples.

The study plan shown from **Table 5-1** to **Table 5-2** was devised systematically to identify and eliminate a reagent that has the maximum affect in degrading the endotoxin present in free form and that bound to the chitosan. For this purpose, a CSE sample prepared by spiking LRW with 1 EU/mL was treated with either 92mM HCl or 0.2% acetic acid at different temperatures for varying amount of times; the time and temperature variables are an integral element in determining endotoxin recovery from CSE samples. In order to determine interference of chitosan towards endotoxin, the chitosan 92-10 sample was spiked with 1 EU/mL CSE to form challenge sample, which was treated with either 92 mM HCl or 0.2% acetic acid at standard time and temperature recommended for respective methods and tested with chromogenic LAL assay for endotoxin recovery. The samples were diluted at 1:100 and at 1:150 by 1:50 dilution in LRW and then subsequently by diluting 1:2 and 1:3 times with glucashield buffer respectively for pH adjustment. For each sample dilution, a positive product control was prepared by adding a CSE spike of 0.04 EU/mL to detect interference by the sample reactants during the assay. The samples were quantified for endotoxin under the range of 0.16-0.005 EU/mL. The assay was validated by ensuring the recovery within 75-125% of a CSE spiked LRW sample prepared during the assay.

Table 5-1 : Table of samples used for USP treatment (92mM HCl for 48h at 40°C) and the objective for using each sample is stated

Sample treatment before LAL assay				**Processing during LAL assay**	
Samples	**Time of incubation**	**Temperature of incubation**	**Objective**	**Dilutions (in glucan inhibiting buffer)**	**Positive product controls (PPCs)**
Challenge sample= 10mg/mL Chitosan 92-10+ 1EU/mL CSE+ 92mM HCl + LRW	48 hours	40˚C	Interaction of endotoxin with Chitosan in the presence of HCl at 40˚C	1:100 1:150	1 :100 CS Chall +0.04 EU/mL 1 :150 CS Chall +0.04 EU/mL

CSE spike= 1 EU/mL CSE+ 92mM HCl + LRW	48 hours	40˚C	Action of HCl on endotoxin over a long period of time at 40˚C	1:100 1:150	1 :100 CSE Spike +0.04 EU/mL 1 :150 CSE Spike +0.04 EU/mL
CSE spike= 1 EU/mL CSE+ 92mM HCl + LRW	1 hour	40˚C	Action of HCl on endotoxin over a short period of time at 40˚C	1:100 1:150	1 :100 CSE Spike +0.04 EU/mL 1 :150 CSE Spike +0.04 EU/mL
CSE spike= 1 EU/mL CSE+ 92mM HCl + LRW	1 hour	RT	Action of HCl on endotoxin without temperature stimulus over a short period of time	1:100 1:150	1 :100 CSE Spike +0.04 EU/mL 1 :150 CSE Spike +0.04 EU/mL
CSE spike – 0.04 EU/mL CSE+ LRW	N/A	N/A	CSE control in LAL assay- Assay Validation	No Dil	Assay Validation

Table 5-2 : Table of samples used for ACCI treatment (0.2% acetic acid for 1 h at 37°C) and the objective for using each sample is stated

Sample treatment before LAL assay				Processing during LAL assay	
Samples	**Time of incubation**	**Temperature of incubation**	**Objective**	**Dilutions (in glucan inhibiting buffer)**	**Positive product controls (PPCs)**
Challenge sample= Chitosan+ 1EU/mL+ 0.2% Ac. A+ LRW	1 hour	37˚C	Chitosan+ endotoxin interaction in the presence of 0.2% Ac. A	1:100 1:150	1 :100 CS Chall +0.04 EU/mL 1 :150 CS Chall +0.04 EU/mL
CSE spike= 1 EU/mL CSE+ 0.2% Ac. A+ LRW	1 hour	37˚C	Action of 0.2% Ac. A on endotoxin at 37˚C	1:100 1:150	1 :100 CSE Spike +0.04 EU/mL 1 :150 CSE Spike +0.04 EU/mL
CSE spike= 1 EU/mL CSE+ 0.2% Ac. A+ LRW	1 hour	RT	Action of 0.2% Ac. A on endotoxin without temperature stimulus	1:100 1:150	1 :100 CSE Spike +0.04 EU/mL 1 :150 CSE Spike +0.04 EU/mL
CSE spike – 0.04 EU/mL CSE+ LRW	N/A	N/A	CSE control in LAL assay- Assay Validation	No Dil	Assay Validation

The outcome of the study to determine optimal method between the USP and ACCI for rehydration of chitosan and selection of one of them is detailed in the results section.

5.3 Results

5.3.1 Determination of Chitosan EU conc. by Turbidimetric assay according to USP 85

5.3.1.1 Detection of enhancement interference at higher conc. of chitosan during endotoxin determination of chitosan 92DDA- 10KDa MW used in animal injection

Table 5-3 : Exhibition of enhancement interference by the chitosan 92DDA-10kDa MW due to precipitation after reaching a neutral pH of 6-8 by addition of glucashield buffer. The precipitate interferes in the quantification of endotoxin, thus over estimating its content in the chitosan.

Test conc. of Chitosan 92DDA-10kDa MW (mg/mL)	Dilution adjusted Absolute EU conc. (EU/mg)	Percentage PPC recovery (%)	Used standard curve range (N=1)
1	4.31	13956.80	10-0.01 EU/mL
0.25	2.257	872.36	10-0.01 EU/mL

The CS 92-10 was dissolved in HCl, typically with HCl: glucosamine molar ratio of 1:1 at room temperature overnight at a conc. of 10mg/mL. The initial concentrations used for quantification of endotoxin were 1mg/mL and 0.25 mg/mL; both the concentrations were tested with the same standard curve range of 10-0.01 EU/mL, and were deemed as “High” because of their interfering property with the LAL assay detected by the more than 200% PPC recovery of 0.5 EU/mL spiked sample dilution. This phenomenon is seen in turbidimetric LAL assay, when LAL enzyme is reconstituted in glucashield buffer (added to remove β (1-3) D-glucan activation of LAL), as high conc. of chitosan produces precipitation after buffering to the optimal reaction pH 6-8, which results in production of turbidity equivalent to that produced during the reaction of LAL enzyme with the endotoxin.

The high concentration of chitosan and most importantly a high PPC recovery are an indicator of enhancement interference in this sample, the formation of precipitation after addition of glucashield buffer is also observed visually for chitosan 92-10. The enhancement interference is a classic example of the obtaining a false positive result leading to rejection of the end-product going into the clinical trials.

5.3.1.2 Detection of enhancement interference at higher chitosan conc. during endotoxin determination of CS Raw material used for the production of CS 92-10.

Table 5-4 : Exhibition of enhancement interference by the chitosan 92DDA-Raw due to precipitation after reaching a neutral pH of 6-8 by addition of glucashield buffer. The precipitate interferes in the quantification of endotoxin, thus over estimating its content in the chitosan.

Test conc. of Chitosan 92DDA-Raw material (mg/mL)	Dilution adjusted Absolute EU conc. (EU/mg)	Percentage PPC recovery (%)	Used standard curve range (N=1)
0.25	0.156	272.73	10-0.01 EU/mL

The source of chitosan 92-10 production i.e. the raw material acquired from Marinard was also tested for endotoxin content (**Figure 5-1**), as a standard quality control measure. The raw material of CS at 92 DDA was dissolved in HCl in a similar manner as CS 92-10; with HCl: glucosamine ratio of 1:1 overnight at room temperature with final stock conc. of 10mg/mL. Although, no definitive data is present claiming that 1 mg/mL of CS 92-Raw is the "high" concentration producing interference, the data shown in **Table 5-4** demonstrates that the minimum concentration of CS 92-Raw needed to produce enhancement interference is 0.25mg/mL after neutralization of pH by the glucashield buffer used for reconstitution of turbidimetric LAL assay. The chitosan sample stock was diluted 40 times during the assay to obtain 0.25 mg/mL for testing; duplicate sample at 0.25mg/mL was spiked with 0.5 EU/mL to prepare PPC for interference detection. This conc. was tested with 10-0.01 EU/mL standard range; and the interference was detected by more than 200% recovery of the PPC spike than the expected value.

5.3.1.3 Further dilution of sample for reduction of interference by precipitation and determination of optimal dilution range

5.3.1.3.1 CS 92DDA-10kDa MW

As explained in the materials and methods and recommended by USP, the best method to remove interference: enhancement or inhibition, is by the dilution of sample manifolds, until the interference disappears and absolute EU conc. can be detected; samples with well established dose regime in humans are diluted until the product's maximum valid dilution, which is calculated using **Equation 5-1**. For chitosan several dilutions of 10mg/mL stock were prepared

and tested to find a dilution which removed the observed enhancement interference and lead to determination of absolute endotoxin conc. The results of turbidimetric LAL test for CS 92DDA-10kDaMW dissolved at 10mg/mL in HCl at an HCl: glucosamine ratios of 1:1 at room temperature overnight tested between the detection ranges of 10-0.01 EU/mL are shown in the **Table 5-5** below:

Table 5-5 : Reduction of enhancement interference from CS92-10 after dilution in LRW. All the dilutions demonstrate good PPC recovery within the acceptable range of 50-200%. Higher dilutions reduce the endotoxin content beyond the range of detection and sensitivity. The values are obtained from two separate experiments performed with 10-0.01 EU/mL standard range.

Dilution factor of 10mg/mL CS 92DDA-10kDa MW sample	**Test Conc. corresponding to the sample dilution (mg/mL)**	**Calculated EU conc. at test dilution (EU/mL)**	**Reported value per mg of CS (EU/mg)** $\frac{EU}{mg} value = \frac{Dil.factor * \frac{EU}{mL} value}{conc. 10mg/mL}$	**Percentage PPC recovery (%)**	**Used standard curve range (N=2)**
80	0.125	0.023	0.182	185.83	10-0.01 EU/mL
1000	0.01	0.0008	0.08	142.25	
10000	0.001	0.0003	0.33	63.86	

After dilution of 10mg/mL CS 92-10 stock up to 1:80, the enhancement interference disappeared and EU value of the chitosan at each dilution was obtained. The range of dilution to obtain uninhibited endotoxin recovery is very high for CS 92-10 i.e. until 1:10000. Although, the sample endotoxin at 1:1000 and 1:10000 dilutions are at a minimal level beyond the sensitivity of the assay, so the values are extrapolated by the linear equation of the standard curve. The inconsistency in the EU/mg values is the result of extrapolation and hence only the endotoxin value at 1:80 could be reported due to its quantification within the range of detection.

In the next part of the study, the dilutions were reduced from 1:1000 to 1:200 to quantify the sample EU values within the range of assay sensitivity. For this quantification a higher standard curve range at 50-0.005 EU/mL was used, due to the quantification of high conc. of chitosan at 0.5mg/mL which was still preliminarily assumed to have high EU conc. and was showing interference with the PPC recovery at 1008% with quantified value of 3.665 EU/mg; this interference producing chitosan conc. is not reported in the **Table 5-6**. Subsequent dilutions from 1:80 to 1:200 of the 10mg/mL chitosan stock were predicted to be within the range of detection

of the standard curve. The reported endotoxin values at each dilution and EU per mg values calculated from the EU/mL values are disclosed in the **Table 5-6** below:

Table 5-6 : Testing CS 92-10 at narrow dilution range to quantify endotoxin within the range of detection of 50-0.005 EU/mL standard curve. The dilution ranges from 1:80- 1:200 is found optimal for absolute endotoxin quantification and reduction of enhancement interference. The PPC recovery is under the acceptable limit of 50-200%.

Dilution factor of 10mg/mL CS 92DDA-10kDa MW sample	**Test Conc. corresponding to the sample dilution (mg/mL)**	**Calculated EU conc. at test dilution (EU/mL)**	**Reported value per mg of CS (EU/mg)** $\frac{EU}{mg} value = \frac{Dil.factor * \frac{EU}{mL} value}{conc. 10mg/mL}$	**Percentage PPC recovery (%)**	**Used standard curve range (N=1)**
80	0.125	0.027	0.217	168.32	50-0.005 EU/mL
100	0.1	0.067	0.669	179.93	
200	0.05	0.024	0.484	138.30	

As evident, all the chitosan 92-10 dilutions quantify the endotoxin values under the limit of detection of standard curve i.e. within 0.005 EU/mL, which reiterates the applicability of these dilutions for future testing. The EU/mg values are calculated for each dilution factor and the PPC recoveries are within the acceptable 50-200% USP limit indicating removal of enhancement interference at the narrow dilution range.

As 1:80 dilution of 10mg/mL stock is tested in both the standard curve ranges, and it is the first dilution to remove interference, the absolute endotoxin conc. of the CS 92-10 could be calculated at this dilution. The mean EU/mg values from **Table 5-5** and **Table 5-6** reported as absolute EU value of CS 92-10 is 0.1995 EU/mg. This value is below the estimated value of 0.3 EU/mg obtained from the gel-clot assay. This shows the utility of more sensitive kinetic LAL assay in determining the absolute EU conc. of the chitosan samples and also emphasizes the high throughput ability of testing multiple sample dilutions in the plate at the same time.

5.3.1.3.2 CS 92 DDA- Raw

The raw material of the 92 DDA chitosan was also diluted several folds to remove the enhancement interference per recommendation by USP. The stock was prepared at 10mg/mL in HCl at an HCl: glucosamine ratio of 1:1 overnight at room temperature. Analogous to CS 92-10, the CS 92-Raw was also highly diluted to bring the chitosan concentration very low in order to

remove interference. The interpolated EU/mL values at each dilution calculated from the linear equations of 10-0.01 EU/mL standard range, and calculated EU values per mg of chitosan are shown **Table 5-7** below:

Table 5-7 : Reduction of enhancement interference after higher dilution of CS92-Raw in LRW. All the dilutions demonstrate good PPC recovery within the acceptable range of 50-200%. The highest dilution at 1:1000 surpasses the limit of assay sensitivity, making the dilution ineffective for EU quantification. The values are obtained from two separate experiments performed with 10-0.01 EU/mL standard range.

Dilution factor of 10mg/mL CS 92DDA-Raw sample	**Test Conc. corresponding to the sample dilution (mg/mL)**	**Calculated EU conc. at test dilution (EU/mL)**	**Reported value per mg of CS (EU/mg)** $\frac{EU}{mg} value = \frac{Dil.factor * \frac{EU}{mL} value}{conc. 10mg/mL}$	**Percentage PPC recovery (%)**	**Used standard curve range (N=2)**
80	0.125	0.050	0.400	167.33	10-0.01 EU/mL
160	0.0625	0.020	0.318	66.20	
1000	0.01	0.0005	0.05	50.32	

The disappearance of enhancement interference is observed after the chitosan was diluted below the critical interfering concentration of 0.25 mg/mL, signifying that 0.25mg/mL chitosan is high enough to initiate precipitation at neutral pH and the role of dilution in significantly reducing the interference in LAL assay. The chitosan shows optimal recovery of PPC for all the dilution ranges from 1:80 to 1:1000, but the quantified endotoxin conc. at 1:1000 dilution (0.01 mg/mL) is beyond the limit of sensitivity of the assay i.e. 0.005 EU/mL, which suggests the narrowing of chitosan dilution for accurate endotoxin quantification.

The chitosan dilution range was narrowed down for precise quantification of absolute EU value of CS 92-Raw, which was quantified in parallel with CS 92-10 using the same standard curve range of 50-0.005 EU/mL. The sample stock at 10mg/mL was diluted at a range of 1:80 to 1:200 producing 0.125mg/mL to 0.05 mg/mL chitosan, which was estimated to have endotoxin content within the detection range of 50-0.005 EU/mL standard curves. The results are shown in **Table 5-8** below:

Table 5-8 : Testing CS 92-Raw at narrow dilution range to quantify endotoxin within the detection range of 50-0.005 EU/mL standard curve. This dilution range is found optimal for

absolute endotoxin quantification and reduction of enhancement interference. The PPC recovery is under the acceptable limit of 50-200%.

Dilution factor of 10mg/mL CS 92DDA-Raw sample	**Test Conc. corresponding to the sample dilution (mg/mL)**	**Calculated EU conc. at test dilution (EU/mL)**	**Reported value per mg of CS (EU/mg)** $\frac{EU}{mg} value = \frac{Dil.factor * \frac{EU}{mL} value}{conc. 10 mg/mL}$	**Percentage PPC recovery (%)**	**Used standard curve range (N=1)**
80	0.125	0.048	0.385	99.92	50-0.005 EU/mL
100	0.1	0.025	0.248	85.79	
200	0.05	0.023	0.467	73.57	

Similar to CS 92-10, the endotoxin content of CS 92-Raw at all the dilutions in narrow range are within the detection range of the standard curve, this is suggestive of the utility of this range even for CS 92-Raw in future. For the calculation of absolute EU value of CS 92-Raw, the mean EU/mg value computed from **Table 5-7** and **Table 5-8** at 1:80 dilutions will be reported due to its repetition in both the tests, and also due to the low variation in its value in both the repetitions. The average absolute EU conc. of CS 92-Raw is calculated at 0.392 EU/mg, this value is higher than the estimated EU value of 0.3 EU/mg obtained from gel-clot assay. The possible reasoning is that the raw materials obtained from indigenous sources such as crustaceans and processed from chitin are always contaminated with high amount of endotoxins. Due to ubiquitous presence of endotoxin it is difficult to get rid of the contamination; however due to alkaline treatment during the production of low molecular weight CS 92-10, the endotoxin in CS raw material degrades which results in a cleaner final product.

The absolute quantification of EU conc. of CS 92-Raw material and CS 92-10 validates the chitosan production process followed by our group without the introduction of endotoxin, as the endotoxin contamination only decreases from high to low rendering the final product (CS 92-10) safe for use in animal studies and future pre-clinical and clinical use.

5.3.1.4 Detection of inhibition interference in the chitosan challenge sample

The chitosan 92-10 sample and challenge CS 92-10 sample was dissolved in HCl at HCl: glucosamine ratio of 1:1 to prepare 1mg/mL solution each and the challenge sample was fortified with 1 EU/mL CSE to keep equal CS: endotoxin ratio of 1:1. Both the samples were treated overnight in HCl at room temperature. The samples were then tested by turbidimetric LAL assay

at multiple dilutions at a standard curve range of 50-0.005 EU/mL, which was used due to testing of challenge sample with high EU conc. The dilution below 1:50 was not tested due to very low CS conc. of 0.01mg/mL that may exceed the limit of detection similar to 1:1000 dilutions of 10mg/mL CS 92-10 as observed in **Table 5-5**. The EU conc. of CS 92-10, Challenge CS 92-10 sample and the recovery of endotoxin spike from challenge sample are shown in the **Table 5-9** below:

Table 5-9 : Extent of interference to endotoxin by chitosan binding is exposed by the challenge sample, the binding occurred during the dissolving process and lead to the reduction in the endotoxin content available for detection; the detectable endotoxin is termed as "free endotoxin" and the undetectable endotoxin is termed as (chitosan) "bound endotoxin".

Dilution factor of 1mg/mL CS 92DDA-10kDa and Challenge CS 92-10 samples	**CS Conc. corresponding to the sample dilution (mg/mL)**	**Expected CSE conc. of Challenge CS 92-10 corresponding to sample dilution [EU/mL]**	**Calculated EU conc. of challenge CS 92-10 sample at test dilution (EU/mL)**	**Dilution adjusted EU conc. of challenge CS 92-10 sample (EU/mL)**	**Dilution adjusted EU conc. of CS 92-10 sample (EU/mL)**	**CSE percentage recovery (%) from challenge sample**
10	0.1	0.1	0.029	0.289	0.111	17.863
50	0.02	0.02	0.017	0.849	0.552	29.676

The results shown above provide an interesting insight in to the interaction of chitosan samples with endotoxin; the chitosan challenge sample exposes the extent of inhibition of endotoxin by the chitosan due to electrostatic interaction. When the quantified EU conc. of CS 92-10 sample was subtracted from that of the challenge sample, the percentage value obtained represented the free endotoxin, which is only in the range of 17-29%. This indicates that chitosan has a high affinity of binding towards endotoxin and from the 1 EU/mL CSE spike 70-80% endotoxin was bound to 1mg/mL chitosan and only a small amount was available for detection. This creates a significant degree of uncertainty in accepting the absolute EU conc. of the chitosan 92-10 and CS 92-Raw samples quantified in sections 5.3.1.3.1 and 5.3.1.3.2. It could be hypothesized that the chitosan interacts with the native endotoxin present in the environment and does not allow absolute detection of endotoxin by LAL assay due to concealing of the Lipid A detection site of the endotoxin/LPS. To reduce the interaction of chitosan with endotoxin, several methods were

tested detailed in section below, which targets the electrostatic properties of chitosan by charge neutralization and reduction of chitosan monomers to limit the interaction with endotoxin aggregates or large monomers.

5.3.2 Pre-Treatment of chitosan using different methods for removal of interference

5.3.2.1 NaOH treatment of chitosan

In order to remove the electrostatic interaction between chitosan and endotoxin, the NaOH treatment was performed, initially with a high pH to ensure total charge neutralization. The chitosan 92-10 and 1 EU/mL challenge chitosan samples at 10mg/mL rehydrated in HCl, at an HCl: amine ratio of 1:1 were precipitated in the final conc. of 0.08± 0.01N sodium hydroxide (NaOH) at approximate pH of 12 to neutralize the charge and stabilize electrostatic interaction between chitosan and endotoxin. The supernatant was yielded for endotoxin quantification after precipitation. The supernatant obtained from the challenge sample was tested at no dilution, 1:10 and 1:100 dilutions, in order to keep the endotoxin spike conc. at 1, 0.1 and 0.01 EU/mL i.e. within the limit of detection of 50-0.005 EU/mL range. However, CS 92-10 sample was tested at 1:10 and 1:100 dilutions to reduce the chitosan conc. to 1 and 0.1 mg/mL, to avoid the probability of enhancement interference in the supernatant and mainly to minimize the alkalinity of the NaOH, which might impact the LAL proteins and reaction efficiency. The EU conc. of 0.08N NaOH was tested and found below the limit of detection. The results at the two dilutions of CS 92-10 and challenge samples are shown below in **Table 5-10**:

Table 5-10 : Pre-treatment of CS 92-10 (10mg/mL) sample and Challenge CS 92-10 (10mg/mL + 1 EU/mL) sample with 0.08± 0.01 N NaOH. The supernatant of the charge-neutralized samples after precipitation with NaOH are tested at 1:10 and 1:100 dilutions. The CS 92-10 EU values are reported after confirming the validity by the recovery of PPCs within acceptable limit (50-200%).

Sample	Dilutions	Expected CS Conc. in supernatant corresponding to the sample dilution (mg/mL)	Expected CSE conc. of Challenge CS 92-10 supernatant corresponding to sample	Calculated EU conc. of samples at test dilution (EU/mL)	Dilution adjusted EU conc. [EU/mL] (EU conc. * Dil)	CSE recovery from challenge sample (between 50-

			dilution [EU/mL]			200%)
CS 92-10 challenge supernatant (10mg/mL+ 1 EU/mL) after precipitation with 0.08N NaOH	1:10	1	0.1	0.009	0.091	4.084
	1:100	0.1	0.01	0.009	0.894	63.496
CS supernatant (10mg/mL CS precipitation with 0.08 N NaOH)	1:10	1	Not applicable	0.005	0.0497	Not applicable
	1:100	0.1	Not applicable	0.0026	0.2588	Not applicable

The values of CSE recovery from the challenge sample calculated using **Equation 5-3** at 1:10 dilution does not show acceptable recovery because of probable interference to LAL enzyme by 0.008N NaOH present in the 10-fold diluted solution, but at 1:100 dilutions the 0.08N NaOH seem to be able to release the endotoxin bound to chitosan within the acceptable recovery of 50-200%. However, at this dilution, the quantified EU conc. of CS 92-10 supernatant is below the detection/sensitivity range of the assay, which directs us towards the use of low conc. of NaOH in future to limit the dilution and allow the absolute quantification of endotoxin without inhibition by NaOH. As the 0.0026 EU/mL is an extrapolated value of CS 92-10 supernatant, it is futile to apply it to determine the CSE recovery from the challenge CS 92-10 supernatant sample. In this case only the percent CSE recovery value obtained at 1:10 dilution can be accepted, which indicates either inhibition of LAL proteins by NaOH or unsuccessful removal of bound endotoxin from glucosamine chains of chitosan. 0.1N NaOH i.e. "High conc. NaOH" is also known to degrade the endotoxin in chromatography solutions within very short exposure times of 30 mins to 1 hour (Application Note 18-1124-57), hence the time of exposure of NaOH to endotoxin must also be controlled; In a simple CSE spiked LRW solution treated with 0.08N NaOH for 30 mins, the endotoxin recovery of 1 EU/mL was found at 14% (data not shown), which indicates the phenomenon at work even at 0.08N NaOH conc. i.e. 0.08NNaOH has degrading effect on

endotoxin integrity, thus 0.08N can also be deemed as “High conc.” of NaOH conc. and must be avoided in future.

Concluding from this study, low conc. of NaOH needs to be tested to reduce the inhibition interference of chitosan towards endotoxin, as well as by NaOH to LAL enzyme. Low conc. of NaOH closer to 0.001N or 0.0008N as present in 1:100 dilution of samples is recommended as these conc. does not allow overshot of ionic strength in the samples deleterious for LAL functionality (Lonza technical tips) i.e. they maintain low ionic strength while also working on removing the bound endotoxin from chitosan chains which will enhance the efficiency of the assay and allow absolute quantification of EU values of the CS 92-10 and CS challenge samples respectively.

5.3.2.2 Chitosanase Treatment

Chitosanase enzyme from Streptomyces griseus is typically used for the digestion of high molecular weight chitosans into small monomers, and its activity is defined by the supplier-Sigma Aldrich as “one unit of chitosanase will release 1μmole glucosamine from chitosan per minute at pH 5.0 at 37˚C”; which represented very high efficiency of this enzyme in breaking the glucosamine chains. Due to its origin from a bacteria Streptomyces griseus, it was decided to preliminarily test the endotoxin content of the enzyme. Although, the bacteria are gram-positive, that lacks the lipopolysaccharide (LPS/endotoxin); but other LAL reactive components such as Lipoteichoic acids, endotoxin contamination during cell culture, extraction and processing may activate the LAL cascade and produce false positive results (Rockel et al. 2012). The turbidimetric LAL test was performed twice on 1:50 dilutions of 100 U/mL enzymes under a detection range of 50-0.005 EU/mL, due to high probability of endotoxin contamination.

Table 5-11 : Determination of endotoxin content/ LAL activating potential of chitosanase enzyme produced and processed from S. griseus. The enzyme was tested N=2 times at 1:50 dilutions. The enzyme was found to contain large LAL activating components and/or endotoxins.

Sample (N=2 repetitions)	Dilution factor	Calculated average EU conc. of sample at test dilution (EU/mL)	Dilution adjusted average EU conc. [EU/mL] (EU conc. * Dil)	Average EU conc. Per unit of enzyme (EU/enzyme unit U)	Conclusion

Chitosanase enzyme from S.griseus	50	786.06	39302.83	393.02	High endotoxin contamination in chitosanase

According to the results obtained from the tests, it is confirmed that chitosanase treatment will be ineffective in removing the interference of chitosan with endotoxin, as the treatment itself will introduce high level of contamination, rendering the chitosan non-functional for any in vivo application.

5.3.2.3 Nitrous (HONO) acid treatment

To reduce the size of glucosamine monomers, the HONO treatment was performed. However, due to harsh treatment conditions like use of HCl and HONO which results in highly acidic conditions pH<3 might also be deleterious to endotoxin in the presence of heat at 50˚C for the duration of 3 hours due to the probability of acid hydrolysis (PDA letter, 2013). Hence, the high conc. of endotoxin at 50 EU/mL spiked in LRW was first tested with three conc. of HONO+ HCl before testing chitosan to discover whether these conditions have hydrolytic effect on endotoxins. The turbidimetric LAL assay was performed after the completion of 3 hours' reaction quantified within the range of 50-0.005 EU/mL. The only pH adjustment was at the end of the assay by addition of LAL enzyme reconstituted in glucashield buffer.

Table 5-12 : EU quantification of 50 EU/mL CSE spiked LRW samples treated with three different conc. of HONO+ HCl combination designated as high, medium and low, with medium conc. equivalent to the glucosamine: HONO ratio (R=0.5) used for chitosan depolymerisation. The samples tested without dilution and 1:10 dilution within the detection range of 50-0.005 EU/mL show under acceptable recovery.

Sample	Dilutions	Expected CSE conc. of test sample corresponding to sample dilution [EU/mL]	Calculated average EU conc. of sample at test dilution (EU/mL)	Dilution adjusted average EU conc. [EU/mL] (EU conc. * Dil)	Conclusion
CSE_{HONO} R=0.2	No Dilution	50	No onset time	No onset time	High conc. HONO/HCl inhibits LAL enzyme
	1:10	5	0.133	1.331	High conc. HONO/HCl has a

					degrading effect on endotoxin
$CSE_{HONO\ R=0.5}$	No Dilution	50	No onset time	No onset time	Medium conc. HONO/HCl inhibits LAL enzyme
	1:10	5	0.047	0.468	Medium conc. HONO/HCl has a degrading effect on endotoxin
$CSE_{HONO\ R=0.9}$	No Dilution	50	0.232	0.232	Low conc. HONO/HCl has a degrading effect on endotoxin
	1:10	5	0.174	1.744	Low conc. HONO/HCl has a degrading effect on endotoxin
CSE without HONO	No Dilution	50	No onset time	No onset time	Medium conc. of HCl inhibits LAL enzyme
	1:10	5	0.015	0.147	Medium conc. of HCl had a degrading effect on endotoxin

All the treatment conditions with and without HONO show poor recovery of endotoxin and hence this treatment is deemed deleterious for endotoxin; the absence of recovery from CSE spiked LRW sample treated without HONO points towards greater role of HCl (at 83.91mM) in masking the endotoxin from LAL enzyme or possibly degrading/ conforming the endotoxin to prevent quantification. However, another possible explanation could be that at such low pH the glucashield buffer present at 1:1 dilution in LAL enzyme was unable to bring the pH to the optimum level i.e. 6-8 for efficient reaction, resulting in no onset time in non-diluted samples. The initial pHs of the samples without buffering are shown in the **Table 5-13** below:

Table 5-13 : pH of CSE spiked LRW samples treated with three different conc. of HONO+ HCl and HCl alone. The initial pHs before adjustment are very low, which might reduce LAL functionality when not buffered properly.

Samples	pH (N=3)
$CSE_{HONO\ R=0.2}$	0.48
$CSE_{HONO\ R=0.5}$	0.81
$CSE_{HONO\ R=0.9}$	1.01

CSE **without HONO**	0.80

Although glucashield has a high buffering capacity, it might not be able to bring up the pH of these acidic samples for efficient reaction. However, it is expected to bring the pH of the sample treated with lowest HCl conc. at R=0.9 towards optimum and result in absolute quantification of endotoxin. To eliminate the variation caused by pH a separate test recommended by USP for treatment of chitosan was performed, that involved the use of 92mM HCl, but a buffering step before adding the LAL enzyme (also reconstituted in glucashield buffer) which might result in uninhibited efficient reaction. Another test to increase the sample pH recommended by ACCI was applied that required the use of 0.2% acetic acid to dissolve the samples; CSE spiked LRW and challenge chitosan samples were used for both the test to determine the optimum dissolving agent that exhibit minimum interference with LAL assay and produces optimum endotoxin recovery.

The conc. of CSE spike might also affect the recovery of endotoxin from HONO treated samples, 50 EU/mL CSE is very high to be detected by the linear range of 50-0.005 EU/mL, as the spike conc. is detectable at or near the high conc. calibrators. However, the evidence of low recovery at 1:10 dilution, points towards the action of HCl/HONO in inhibiting endotoxin quantification. Hence, a lower CSE conc. will be used to spike LRW and chitosan for testing the effect of dissolving/ rehydration reagent on endotoxin recovery. The determination of optimal chitosan dissolving/ rehydration reagents was performed using chromogenic format of LAL assay.

5.3.2.4 Superiority of chromogenic LAL assay in detection of interference

The functioning of chromogenic LAL assay is based on the measurement of the absorbance of a colorimetric substrate called p-nitroanilide cleaved from the clotting enzyme after activation by endotoxin. Because p-nitroanilide only absorbs light at 405nm, instead of refracting it to cause uneven absorbance curve as in the case of turbidimetric assay; the time vs. O.D curve of each standard calibrator resolves at high range of absorbance, without disturbances and portray very smooth profile.

Chromogenic LAL was found to have higher accuracy and reduced error in detection of known and unknown amount of endotoxin. A well-resolved absorbance profile of each standard calibrator showed that there were fewer disturbances in the measurement of absorbance as observed in **Figure 5-3** during the chromogen development compared to the turbidity

development by coagulogen chain formation. This also proved that chromogenic assay was well suited for quantifying the chitosan EU conc. due to reduced probability of enhancement interference by precipitation- a major problem during quantification with turbidimetric LAL assay as well as for the accurate estimation of inhibition interference to endotoxin by chitosan sample.

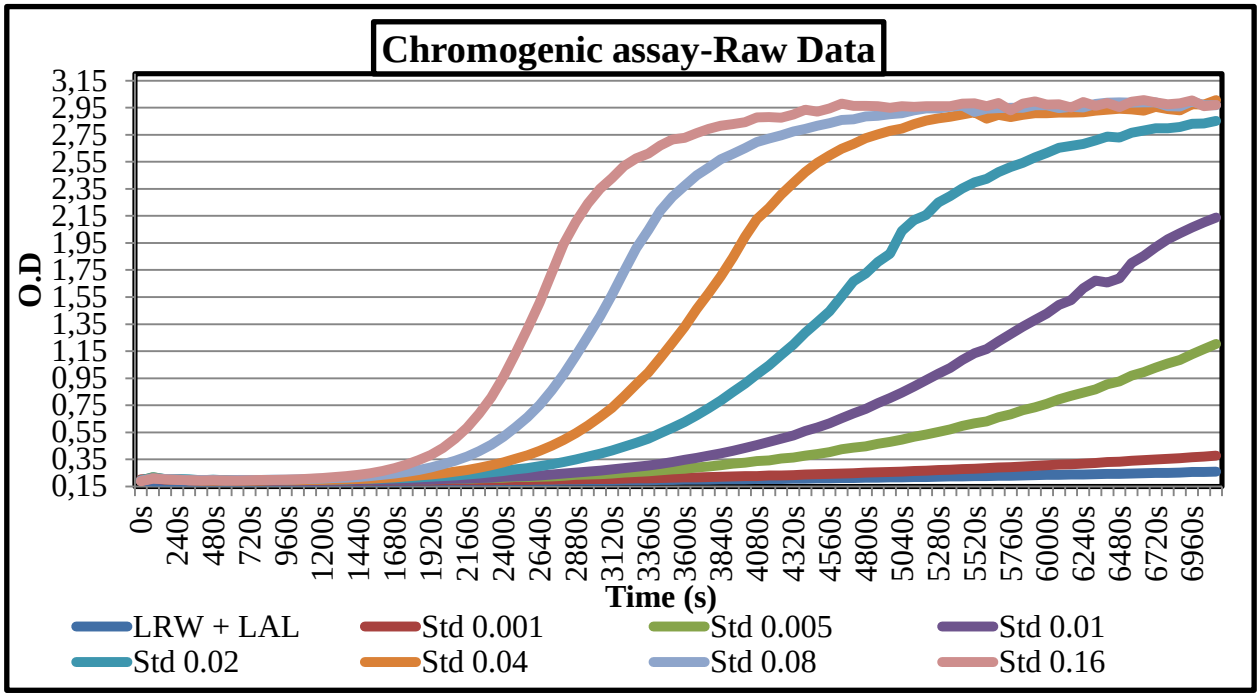


Figure 5-3 : Characteristics of absorbance curves against time of the standard calibrators and negative control in chromogenic LAL assay.

5.3.3 Determination of optimum chitosan dissolving/reconstitution reagent to improve endotoxin recovery and reduce interference

As observed in previous study, HCl which is also a chitosan dissolving reagent leads to low endotoxin recovery in CSE spiked LRW samples at high conc., and will probably be deleterious to the endotoxin present or spiked in chitosan challenge samples. This variability was reckoned to arise by non-reactivity of LAL enzyme in low pH or likely by the deleterious effect of HCl on endotoxin; which factor was contributing more towards low endotoxin recovery (LER) was unknown. In order to differentiate between the two pernicious factors, the LRW and chitosan were “spiked” and “challenged” respectively with CSE and treated with 92mM HCl used for dissolving 10mg/mL chitosan at 40˚C for 48 hours as per USP recommendation, and then

processed for LAL assay, but at each sample dilution the pH was adjusted by the glucashield buffer to ensure reaction efficiency also recommended by the USP. The recovery results obtained from this study will allow us to determine whether HCl is deleterious to endotoxin.

Simultaneously, the CSE spiked LRW and CSE challenged chitosan sample at 10mg/mL were dissolved in 0.2% acetic acid following the treatment by Associates of Cape cod (ACCI) for quantifying chitosan EU conc. The acetic acid has a higher pH than HCl, and might be within optimal pH range after dilution with just LRW, but for precaution, the acetic acid containing samples were buffered with glucashield to ensure complete LAL reaction efficiency. The LAL quantified recovery of both the samples will elucidate the advantage of using acetic acid over HCl and the authenticity of the method used by ACCI- a specialist in endotoxin detection, in quantifying absolute endotoxin content of chitosan.

Comparing the results of the two dissolving/rehydration reagents will allow us to identify a reagent that will not degrade the endotoxin and will facilitate in releasing the endotoxin from the glucosamine chains of chitosan.

5.3.3.1 Small range of standard curve for detection by chromogenic LAL assay in this study

As the amount of spike conc. in LRW was reduced because of the evidence of compromised quantification due to the use of high spike conc., a low range of standard curve at 0.16-0.005 EU/mL was used. The repeatability of the chromogenic standard curve is superior to the turbidimetric assay, as the variation of total N=7 standard curves with 0.16-0.005 EU/mL range was within the acceptable variation limit laid out by ACCI to 10% for slope and 3% for intercept as shown in **Table 5-14**.

Table 5-14 : The repeatability of the standard range of 0.16-0.005 EU/mL for N=7 repetitions is depicted as a function of %RSD= $\frac{|Mean|}{SD} * 100$ (%CV) of slope, intercept and R^2. Acceptable Slope %CV= 10%, intercept =3%

Tested range (0.16-0.005 EU/mL) (N=7)	Slope	Intercept	R^2 correlation coefficient of standard curve
Mean	-0.245	3.052	0.995
St Dev	0.026	0.033	0.005
%RSD (%CV)	10.542	1.067	0.459

The repeatability of 0.16-0.005 EU/mL range was exceptional, with %CV of slope and intercept within the acceptable level of 10% and 3% respectively as shown in **Table 5-14**. This range of standard was used to calculate the endotoxin conc. of the test spiked and challenge chitosan samples treated with different rehydration reagents.

5.3.3.2 Outcome of the endotoxin recovery in challenge chitosan 92-10 and CSE spiked LRW samples treated according to the USP method

The challenge chitosan 92-10 sample at 10mg/mL and the LRW sample were spiked with 1 EU/mL CSE immediately after addition of 92mM HCl and then treated in HCl for 48 hours at 40°C, on the day of testing two other CSE spiked LRW samples at 1 EU/mL were prepared with 92mM HCl but one was incubated at 40°C for 1 hour, and another was kept at room temperature for 1 hour, to also understand the effect of time and temperature of HCl incubation on endotoxin recovery along with direct effect of HCl. During the testing by chromogenic LAL assay, all the samples were diluted at 1:100 by using the USP recommended 1:50 dilution in LRW and 1:2 dilutions in glucashield buffer, one more dilution at 1:150 was tested by diluting 1:50 in LRW and 1:3 times in glucashield buffer to obtain robust results. In this study for each sample dilution, a positive product control (PPC) was introduced to ascertain whether or not the HCl interferes with the endotoxin during the assay after pH adjustment. The PPCs were spiked with 0.04 EU/mL, which was quantified in the middle of the 0.16-0.005 EU/mL standard curve. The quantification results are shown in the **Table 5-15** below:

Table 5-15 : Determination of EU conc. and CSE recovery of challenge chitosan 92-10 and CSE spiked LRW samples treated with 92mM HCl at 48 hours recommended by USP, and 1 EU/mL CSE spiked LRW samples treated with 92mM HCl for 1 hour at 40°C and RT for comparison of duration and temperature of incubation.

Sample and treatment conditions	Dilutions	Expected spike conc. in samples [EU/mL]	Expected Chitosan conc. in challenge sample (mg/m	Calculated EU conc. of sample at test dilution (EU/m	Dilution adjusted average EU conc. [EU/mL] (EU conc. *	% CSE recovery of sample (dilution adjusted sample	EU conc. per gram of chitosan challenge sample	Corresponding PPC recovery at test dilution (Sample	Reported dilution (acceptable PPC recovery betwee

			L)	L)	Dil)	EU conc. /1*100)	[EU/g]	dilution+ 0.04 EU/mL Spike)	n 50-200%)
Challenge CS 92-10 48h 40˚C	1:100	0.01	0.1	0.006	0.640	64.00	64	58.80	1:100
	1:150	0.0066	0.066	0.004 (<0.005)	0.593	59.94	59	51.55	1:150
CSE spiked LRW 48h 40˚C (tested in duplicate average)	1:100	0.01	No CS present	Not available (No onset time)	Not available (No onset time)	Not available (No onset time)	N/A	Not available (No onset time)	Not reported
	1:150	0.0066	No CS present	Not available (No onset time)	Not available (No onset time)	Not available (No onset time)	N/A	Not available (No onset time)	Not reported
CSE spiked LRW 1h 40˚C	1:100	0.01	No CS present	Not available (No onset time)	Not available (No onset time)	Not available (No onset time)	N/A	Not available (No onset time)	Not reported
	1:150	0.0066	No CS present	Not available (No onset time)	Not available (No onset time)	Not available (No onset time)	N/A	Not available (No onset time)	Not reported
CSE control 1h RT	1:100	0.01	No CS present	0.0012 (<0.005)	0.118	11.8%	N/A	80.79	1:100
	1:150	0.0066	No CS present	Not available (No onset time)	Not available (No onset time)	Not available (No onset time)	N/A	Not available (No onset time)	Not reported

The results suggest that the CSE spiked LRW sample treated with 92mM HCl at 40°C for any duration of time does not recover the endotoxin at any dilution either from the sample or from the corresponding PPC re-fortified with 0.04 EU/mL. This is because the rate of change of absorbance in the sample and the PPCs was stagnant, and could not reach the threshold O.D

within the given reaction time of 2 hours, resulting in no onset time. The CSE spike treated with HCl for 1 hour at room temperature also shows 10 times less recovery than the initial spiked conc. of 1 EU/mL, which could be attributed to the deleterious action of HCl on endotoxin as it does not interfere in the endotoxin detection during the assay confirmed by 80% PPC recovery. Conversely, the chitosan challenge sample treated with 92mM HCl for 48 hours at 40°C shows 61% recovery of the 1EU/mL spiked endotoxin on average, thus showing that the same amount of HCl could not affect the endotoxin present along with the chitosan.

The slow rate of change of absorbance in CSE spiked LRW samples resulting in no onset time can be explained as follows: -

a) The high conc. of HCl directly attacks the free endotoxin present in the LRW solution, reducing its recovery by LAL assay. The mechanism of action is explained by the acid hydrolysis of endotoxin by as low as 50mM HCl at high temperature in just less than 30 minutes. The HCl can cause the physiological change in LPS by attacking the eight carbon sugar two-keto-three-deoxyoctonic acid (2.3-KDo) linking the Lipid A to the O-antigen, the separated Lipid A reduces the pyrogenicity of the LPS (Sandle, 2011). This phenomenon is completely observed in the CSE samples cooked in 92mM HCl for 48 hours at 40°C, which proves that longer incubation times can also result in depyrogenization; and partially observed in CSE samples treated with 92mM HCl for 1 hour at ambient temperature, showing that temperature may not be necessary for hydrolysis of LPS and subsequent depyrogenization.

The optimal recovery of spiked endotoxin in challenge chitosan sample shows that chitosan protects the endotoxin against the hydrolyzing effect of HCl, possibly by attracting the negatively charged O-antigen of LPS to the positively charged glucosamine chains protonated by the high conc. of HCl. This interaction may conceal the 2, 3-KDo chains from the hydrolyzing effect of HCl, and may not allow the separation of Lipid A entity while protecting the integrity of LPS and allowing the detection of pyrogen by LAL enzyme.

This observation provides an intriguing insight on the functioning of chitosan and its interaction with endotoxin.

5.3.3.3 Outcome of the endotoxin recovery in chitosan challenge and CSE control samples treated according to the ACCI method

The challenge chitosan 92-10 and LRW samples were spiked with 1 EU/mL CSE immediately after adding 0.2% acetic acid, and incubated at 37°C for 1 hour, simultaneously, 1 EU/mL CSE spiked LRE sample was treated with 0.2% acetic acid at room temperature, to observe the effect of incubation temperature on endotoxin recovery. The samples during the LAL assay were diluted 1:100 and 1:150 times first in LRW, then in glucashield buffer similar to the USP method to reduce LAL inhibition due to low pH. The PPCs were prepared at each dilution for each sample to eliminate the possibility of interference to endotoxin by acetic acid after pH adjustment during the assay. The EU conc. and CSE recovery of each CSE spiked LRW sample and challenge CS 92-10 sample is shown in the table below:

Table 5-16 : Determination of EU conc. and CSE recovery of challenge chitosan 92-10 and CSE spiked LRW samples treated with 0.2% Acetic acid for 1 hour at 37°C recommended by the ACCI, and 1 EU/mL CSE spiked LRW samples treated with 0.2% acetic acid for 1 hour at RT for comparison of duration and temperature of incubation.

Sample and treatment conditions	Dilutions	Expected spike conc. in samples [EU/mL]	Expected Chitosan conc. in challenge sample (mg/mL)	Calculated EU conc. of sample at test dilution (EU/mL)	Dilution adjusted average EU conc. [EU/mL] (EU conc. * Dil)	% CSE recovery of sample (dilution adjusted sample EU conc. /1*100)	EU conc. per gram of chitosan challenge sample [EU/g]	Corresponding PPC recovery at test dilution (Sample dilution+ 0.04 EU/mL Spike)	Reported dilution (acceptable PPC recovery between 50-200%)
Challenge CS 92-10 1h 37˚C	1:100	0.01	0.1	0.011	1.074	107.39	107	100.14	1:100
	1:150	0.0066	0.066	0.005	0.793	79.29	79	88.89	1:150

CSE spiked LRW 1h 37˚C (tested in triplicate average)	1:100	0.01	No CS present	0.0028 (<0.005)	0.286	28.57	N/A	103.3	1:100
	1:150	0.0066	No CS present	0.0013 (<0.005)	0.196	19.62	N/A	15.09	Not reported
CSE spiked LRW 1h RT	1:100	0.01	No CS present	0.0005 (<0.005)	0.046	4.58	N/A	27.25	Not reported
	1:150	0.0066	No CS present	0.0017 (<0.005)	0.249	24.88	N/A	62.93	1:150

The outcome of the ACCI treatment indicate that the challenge chitosan sample with 1 EU/mL spike treated with 0.2% acetic acid for 1 hr at 37˚C show 100% recovery of the endotoxin spike at 1:100 dilutions. The PPCs, which are the interference control fortified with additional 0.04 EU/mL during the assay, are also recovered between acceptable limit of 50-200% at an optimal 100% and 88% level at 1:100 and 1:150 dilutions respectively. The CSE spiked LRW samples treated with 0.2% acetic acid for 1 hr at 37˚C and at room temperature show at least 5-fold reduction from the initial 1EU/mL spike at their respective reported dilutions.

These results show that the treatment with 0.2% acetic acid for a short duration at 37˚C is favourable for the chitosan sample, but deleterious at any temperature for the endotoxin present in water alone. Tirosaga et al explained in 2007 (Tirsoaga, El Hamidi, Perry, Caroff, & Novikov, 2007) that even milder acidic conditions with pH 4.4-4.5 can induce the acid hydrolysis and liberate the Lipid A entity from the LPS. They used sodium acetate salt along with SDS to improve the reaction kinetics to render the LPS less active or inactive for detection. But the reaction can be accelerated by the use of heat (Cole, 1996). In this case, acetic acid easily provides a mild acidic environment to induce LPS hydrolysis in the presence of heat.

The chitosan at 92% DDA and low molecular weight protects the endotoxin from the mild hydrolysis by 0.2% acetic acid. The mechanism of protection may be similar to what is observed in HCl treatment. However, the mild hydrolysis may destroy the endotoxin unbound to chitosan, resulting in inaccurate absolute quantification.

5.3.4 Discussion of this study

The endotoxin determination by 10-0.01 EU/mL standard curve range of kinetic turbidimetric LAL assay, allowed us to identify the two different types of interferences in detection of endotoxin during LAL assay. The enhancement interference of CS 92-10 and CS 92-Raw was observed at high chitosan conc. up to 0.25mg/mL, detected by high PPC recovery; this lead us to conclude that the chitosan interfered with the LAL assay due to precipitation at the neutral pH of reaction. The precipitation interfered with the turbidity measurement characterized by the absorbance leading to high conc. of sample and the PPC recovery. The enhancement interference of chitosan was reduced by diluting the chitosan below the critical conc. of 0.25mg/mL; the chitosan started showing optimal PPC recovery at 50-200% starting from 0.125mg/mL i.e. 1:80 dilution of 10mg/mL chitosan. Tests performed from 1:80 to higher dilutions up to 1:1000 showed that higher dilutions of chitosans reduced the endotoxin below the undetectable level i.e. below the assay sensitivity. An optimal dilution range was determined for both CS 92-10 and CS 92-Raw at 10mg/mL from 1:80-1:200 by further testing, which will be used in future testing as well.

Another type of interference i.e. inhibition interference was recognized which was intuitive to the nature of chitosan interaction with endotoxin by the use of challenge sample. The challenge chitosan sample was prepared in parallel to the test sample, and during rehydration spiked with a known amount of endotoxin to be detected later by LAL assay after rehydration to determine the CSE recovery within the acceptable 50-200%. The CSE recovery of 1mg/mL chitosan 92-10 Challenge sample was found below the acceptable recovery calculated by subtracting the EU value of the test sample, which confirmed the existence of inhibition interference in chitosan samples.

In order to reduce the inhibition interference and release the bound endotoxin from chitosan chains, some pre-treatments were performed with NaOH, chitosanase and nitrous acid. The NaOH treatments was aimed at reducing the electrostatic interaction between chitosan and endotoxin and release the bound endotoxin in the supernatant, the supernatant of 10mg/mL chitosan 92-10 and 1 EU/mL+ 10mg/mL Challenge CS 92-10 samples treated with 0.08N NaOH tested by turbidimetric LAL assay showed very low CSE recovery of the challenge sample at the detectable 1:10 dilution, other dilution at 1:50 presented optimal recovery, but the EU conc. of

test sample was out of the detection limit, thus the recovery at 1:50 dilution wasn`t acceptable. This left us to conclude that the low CSE recovery was either due to interference of LAL enzyme by NaOH or by degradation of endotoxin at high NaOH conc. Consequently, the use of low NaOH conc. at 0.001N is recommended for future application in charge neutralization, removal of bound endotoxin and uninhibited endotoxin detection by LAL.

Another pre-treatment was enzymatic digestion of chitosan monomers in to small chains; the rationale was to reduce the monomer size up to 1kDa which will not bind to the large molecular weighted LPS. However, due to the origin of chitosan digesting chitosanase enzyme from a bacterial source, it was first tested in a pure form endotoxin contamination. The LAL results suggested very high contamination of the enzyme with 393.0 EU per unit of enzyme. This could be due to introduction during processing and enzyme manufacturing or due to the presence of glycans that activates the alternate LAL cascade pathway. Conclusively, the use of chitosanase enzyme was rejected for reduction of chitosan inhibition interference.

The third pre-treatment was with nitrous acid (HONO) which is commonly used for depolymerization of high molecular weight chitosan to lower molecular weight. Due to harsh chemical treatment, the action of HONO which is activated in the presence of high conc. of HCl was first observed on high endotoxin conc. of 50 EU/mL. Three conc. of HONO+ HCl were chosen, for selection of the one with optimal endotoxin recovery and least influence on endotoxin integrity. After the 3 hours' reaction at 50˚C, the results obtained from turbidimetric LAL assay demonstrated extremely low endotoxin recovery at 1:10 dilution and the sample did not even reach the threshold O.D at high CSE conc. suggesting that the nitrous acid treatment is deleterious to endotoxin. An enhanced role of HCl was observed in reducing the endotoxin recovery from the sample without nitrous acid. However, the role of pH in reducing the recovery and high CSE conc. near the highest detectable conc. are also considered to be playing a role in low recovery.

With the detectable role of HCl in degrading endotoxin, which is a commonly used reagent in dissolving chitosan, a mild acid i.e. 0.2% acetic acid commonly used by ACCI for chitosan rehydration was compared with HCl. The procedure utilized by USP for endotoxin determination in chitosan was utilized to reduce the interference with LAL due to pH issues. The role of rehydration/ dissolving reagents on the endotoxin recovery was tested under controlled

conditions i.e. at low CSE conc. for spike and challenge samples, under different temperature at different times and pH adjustment with glucashield buffer as per USP. Meanwhile, the efficacy and accuracy of chromogenic LAL assay was discovered in detecting the sample interferences, thus the subsequent tests were performed with chromogenic LAL assay. The results of the comparison between the rehydration reagents showed the superiority of 0.2% acetic acid in optimal recovery of endotoxin from chitosan challenge sample, but not with CSE spiked LRW samples. In fact, the spiked water sample showed low recovery in both the reagents, but interestingly the chitosan challenge sample showed optimum PPC recovery in both HCl and 0.2% acetic acid showing a protective role of chitosan in preventing endotoxin from degradation. This intriguing insight leads us to apply the reagents with neutral pH to conserve the integrity of endotoxin in spiked water but more importantly, to release the endotoxin bound to chitosan.

The analysis of the pre-treatment results leads us to the further investigation of hypothesis 1 that states that the pre-treatment of chitosan will release endotoxin bound to the positively charged and large glucosamine chains and facilitate absolute quantification of endotoxin. However, the low endotoxin recovery in CSE spiked water samples after HCl treatment during depolymerisation and subsequent test of rehydration reagents allow us to prove our second hypothesis that states that the harsh chemical treatment used in depolymerisation reaction will be deleterious to the endotoxin integrity and reduce detection.

CHAPTER 6 GENERAL DISCUSSION

The subject of endotoxin contamination in biomaterials is a vital yet overlooked aspect. Historically the infections introduced during the surgery have been a leading cause of septic shock death (Schoenberg M. H., 1998) and half of the nosocomial infections are accounted by medical devices (Peter, Guggenbichler, Assadian2, Boeswald3, & Kramer4, 2011). The medical devices include prosthetics, ventilator, intravascular catheter etc. that causes bacteraemia and endotoxemia in the patients and lead to inflammation, multiple organ failure and death.

With the advent of biomedical engineering, several types of biomaterials and nanoparticles are rapidly progressing towards clinical trials and are used or may soon be used in hospitals for treatments. For the prosthetics and scaffolds finding applications in Intra-articular (IA) and nanoparticles through Intravenous (IV) or intrathecal (IT) route, it is necessary to be cognizant of the level of bacterial and specifically endotoxin contamination because there is no confirmatory diagnosis available to detect sepsis. Thus, the US FDA has declared a limit of endotoxin for IV and IT drugs and issued strict regulatory guidelines. It is mandatory to test the level of endotoxin in medical devices, medical implants and the active pharmaceutical ingredients by LAL assay before market release and the contamination level must be under the 5 EU/kg/hour limit for Intravenous drugs and 0.2 EU/kg/hour for Intrathecal drugs (USP, 2005).

From the perspective of nanosafety, many nanoparticles that show toxic/inflammatory properties may actually be contaminated with endotoxins and its effect may synergize with that of the nanomaterials to induce toxicity. Hence, many nanoparticles may get rejected before approval due to unrecognized endotoxin contamination. The endotoxin in nanoparticles can be detected by the LAL assay, but many nanoparticles interfere with different formats of LAL. According to the recent findings by Li and Boraschi, 2016 (Li Y, 2016), many commercially available nanoparticles are contaminated with different levels of endotoxin depending on the variability of cleanliness during their synthesis process. The nanoparticles adsorb the endotoxin on its surface to reduce their surface-free energy. The LPS may bind to hydrophobic surfaces through its lipid

moiety and its phosphate group can bind to positively charged nanoparticles surfaces, which leads us to design methods to reduce interference of endotoxin with nanoparticles.

Because of the widespread application of chitosan in the form of scaffolds and nanoparticles, with projected final application in humans, it was necessary to quantify the endotoxin concentration of the chitosan raw material. However, Davydova et al, cites that chitosan interacts with endotoxin by electrostatic binding, hydrogen bonds and Vander Waals interaction, which also leads to its application in endotoxin removing columns. In order to reduce such strong interaction between chitosan and endotoxin, sodium hydroxide was used to neutralize the chitosan surface charge and liberate the bound endotoxin, but high NaOH conc. proved deleterious to endotoxin. The chitosan also interferes with the LAL proteins consistent with the properties of other nanoparticles to cause interference with LAL enzyme during endotoxin detection. However, this interference was reduced by dilution of chitosan below 0.25mg/mL following the guidelines from USP, which states that the best method to reduce interference is by adopting the maximum valid dilution method. The effect of chitosan rehydration reagents such as hydrochloric acid and 0.2% acetic acid on the endotoxin integrity may also impede the absolute detection of endotoxin in chitosan, which lead us to reduce their concentration during rehydration. The protective effect of chitosan towards endotoxin points us towards verification of the interaction mechanism of chitosan with rough LPS (without O-antigen) proposed by (Arabski et al., 2013) in which the rough-LPS (Ra-LPS) forms inverse micelles with acyl groups pointing outwards that produces higher pyrogenic response than the smooth-LPS. This finding also leads us towards the fact that the harsh chemical treatment by rehydration reagents detaches the O-antigen from the LPS, and leaves only the negatively charged core-sugars, Kdo_2 and phosphate groups of LPS to bind to the chitosan.

This study leads us to provide clear recommendations for future studies and verify the mechanism of interactions between chitosan and endotoxin.

CONCLUSION AND RECOMMENDATIONS

The estimated value of endotoxin conc. in the CS 92-10 and CS 92-Raw samples obtained by gel-clot could be accepted currently for calculating the EU conc. of in vivo doses, as the methods to reduce interference are still under progress. The sensitive kinetic LAL assays suffered from the problems of standard curve repeatability, which has been resolved by optimizing parameters such as identifying the evaporation areas in 96-well plate, correcting the measurement absorbance at 405nm with pathlength, and pre-conditioning the LAL enzyme and samples at 37°C for rapid enzyme reaction. However, the major breakthrough was the use of low range from 0.16-0.005 EU/mL that allowed highly repeatable standard curve.

The chitosan samples show enhancement interference up to 0.25 mg/mL, so in future this conc. must be avoided, a dilution range of 1:80- 1:200 of 10mg/mL chitosan sample was found to be a non-interfering range suitable for calculating absolute EU conc. With the detection of inhibition interference by the challenge sample at these dilutions, the pre-treatments of chitosan 92-10 was performed. NaOH pre-treatment was aimed at neutralizing the charge of the chitosan glucosamine chains and release the endotoxin bound to it in the supernatant of the precipitate. However, due to high conc. of NaOH the endotoxin integrity was compromised and the test was inconclusive. For future experiments, low conc. of NaOH around 0.01 or 0.008N will be used, to have an equilibrium between pH for non-interference with LAL enzyme and to remove the bound endotoxin from chitosan by charge neutralization while saving endotoxin integrity. The chitosanase treatment was intended for enzymatic digestion of chitosan glucosamine chains into monomers; however, due to its origin from a biological source i.e. gram positive bacteria S. griseus and involvement of multiple processing steps, the endotoxin contamination was found at an extremely high level in the enzyme, hence the enzymatic digestion of chitosan was aborted. To cleave the chitosan chains into small monomers, the conventional depolymerisation reaction by nitrous acid was performed initially on 50 EU/mL CSE spiked water samples to observe the

effect of high conc. of HCl in the reaction on endotoxin integrity; the endotoxin recovery was compromised hypothesized to be due to acid hydrolysis of endotoxin or limited enzyme reactivity at low pH or less dilution of 50 EU/mL CSE spike exceeding detection limit. Based on these results, a buffering step was introduced before performing the LAL assay for the samples treated with high conc. of HCl as recommended by the USP. Hence, the chitosan challenge sample and CSE spiked water samples were then tested by the USP method at high HCl conc. (92mM) for 48 hours and 40°C along with the testing of time and temperature variables by treating CSE spiked water sample with HCl at different time (1h) and temperature (RT), the samples were then diluted in LRW and then buffered in glucashield buffer to optimize the pH for efficient enzyme reaction. To compare the effect of HCl on the samples, another rehydration reagent i.e. 0.2% acetic acid was used as utilized by ACCI for their chitosan testing. The chitosan challenge and CSE spiked LRW samples were treated with 0.2% acetic acid for 1 hour at 37°C and CSE spiked water sample was also tested at RT in 0.2% acetic acid to test temperature variable. Overall, it was found that for chitosan challenge sample 0.2% acetic acid was more effective in the recovery of challenge sample endotoxin than HCl, but for CSE spiked water sample, both the reagents were showing deleterious effects ranging from no detectable recovery to very low recovery for HCl and 0.2% acetic acid respectively at all the time and temperature conditions. This lead to the conclusion that both the reagents cause acid hydrolysis of the endotoxin and results in degradation, but also a very interesting observation, that chitosan has a protective effect on the endotoxin, which may make it difficult to remove the endotoxin bound to the chitosan. The endotoxin content of chitosan challenge sample has to be compared with unspiked chitosan test sample to determine the absolute CSE recovery from challenge sample and determine the loss of endotoxin. However, the mild acid hydrolysis may also affect the free/unbound endotoxin present in chitosan challenge solution; hence, a reduction in the rehydration concentration of 0.2% acetic acid is recommended for the chitosan sample as well as CSE spiked water samples. Ideally, a solution with neutral or basic pH (such as glucashield buffer or low conc. NaOH) must be utilized for chitosan rehydration for maximum reduction of electrostatic interaction between chitosan and endotoxin and thus it is recommended for future use with chitosan.

In order to reduce the interference of chitosan towards endotoxin and allow absolute endotoxin quantification in chitosan, there are multiple ways recommended for future testing: -

1) Replacement of magnesium ions in the chitosan solution will release the endotoxins bound to chitosan surface (Tsuchiya, 2013).
2) Treatment of chitosan with a reagent of neutral pH, to reduce protonation and electrostatic interaction with endotoxin.
3) Treatment of chitosan with detergents like Triton-X to break the aggregated inverted cubical structure of LPS in to monomers, for reducing the high pyrogenecity and easy detection with LAL enzyme.
4) A dissociating agent called deoxycholate at 2-3% concentration can be used to reversibly break LPS aggregates bound to chitosan in to monomers. However, LPS monomers below an optimal size do not show biological activity and may go undetected by LAL assay. (Tsuchiya, 2013).

Extraction of LPS bound to chitosan by surface neutralization using NaOH at pH 8 instead of pH 12 and testing the endotoxin in supernatant after precipitate centrifugation must be tried for future investigations.

BIBLIOGRAPHY

ACCI. (Instruction guide). PyrosateI_Limulus Amebocyte Lysate (LAL).

ACCI. (Product Sheet). PyrosateDatasheetPR14-021.

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen Recognition and Innate Immunity. *Cell, 124*(4), 783-801. doi: 10.1016/j.cell.2006.02.015

Alameh, M., Dejesus, D., Jean, M., Darras, V., Thibault, M., Lavertu, M., . . . Merzouki, A. (2012). Low molecular weight chitosan nanoparticulate system at low N:P ratio for nontoxic polynucleotide delivery. [Research Support, Non-U.S. Gov't]. *Int J Nanomedicine, 7*, 1399-1414. doi: 10.2147/IJN.S26571

Andra B. Schromm, K. B., Harald Loppnow,Ulrich Zähringer, Ernst Th. Rietschel, Stephen F. Carroll, Michel H. J. Koch, Shoichi Kusumoto and Ulrich Seydel. (1998). The Charge of Endotoxin Molecules Influences Their Conformation and IL-6-Inducing Capacity. *The Journal of Immunology*(161), 5464-5471.

Annamaria, B. (2012). *Structural characteristics of bacterial endotoxins.* University o PECS.

Arabski, M., Lisowska, H., Lankoff, A., Davydova, V. N., Drulis-Kawa, Z., Augustyniak, D., . . . Kaca, W. (2013). The properties of chitosan complexes with smooth and rough forms of lipopolysaccharides on CHO-K1 cells. [Research Support, Non-U.S. Gov't]. *Carbohydr Polym, 97*(2), 284-292. doi: 10.1016/j.carbpol.2013.05.008

B. Akbar John, K. C. A. J., Y.B. Kamaruzzaman and K. Zaleha. (2010). Mechanism in the Clot Formation of Horseshoe Crab Blood during Bacterial Endotoxin Invasion. *Science Alert*.

Bagheri, M., Keller, S., & Dathe, M. (2011). Interaction of W-substituted analogs of cyclo-RRRWFW with bacterial lipopolysaccharides: the role of the aromatic cluster in antimicrobial activity. *Antimicrob Agents Chemother, 55*(2), 788-797. doi: 10.1128/AAC.01098-10

Bio-One, G. (2008). F073041_AN_UV-Spectroscopy. *Application Note*. Retrieved from

Buschmann, M. D., Merzouki, A., Lavertu, M., Thibault, M., Jean, M., & Darras, V. (2013). Chitosans for delivery of nucleic acids. [Review]. *Adv Drug Deliv Rev, 65*(9), 1234-1270. doi: 10.1016/j.addr.2013.07.005

Carregaro, A. B., Freitas, G. C., Ribeiro, M. H., Xavier, N. V., & Doria, R. G. (2014). Physiological and analgesic effects of continuous-rate infusion of morphine, butorphanol, tramadol or methadone in horses with lipopolysaccharide (LPS)-induced carpal synovitis. [Research Support, Non-U.S. Gov't]. *BMC Vet Res, 10*, 966. doi: 10.1186/s12917-014-0299-z

Cole, Y. W. a. R. B. (1996). <Wang_et_al-1996-Acid and Base Hydrolysis of Lipid A.pdf>. *JOURNAL OF MASS SPECTROMETRY,, 31*, 138-149.

CRL. (2006). qc_en_n_endosafe_times_august_2006. *Endosafe times*. Retrieved from

Dobrovolskaia, M. A., Germolec, D. R., & Weaver, J. L. (2009). Evaluation of nanoparticle immunotoxicity. [Research Support, N.I.H., Extramural]. *Nat Nanotechnol, 4*(7), 411-414. doi: 10.1038/nnano.2009.175

EP. (2005). BACTERIAL ENDOTOXINS *EUROPEAN PHARMACOPOEIA 5.0*.

Fan, F.-Y. (2014). *Journal of Medical and Biological Engineering, 34*(3), 211. doi: 10.5405/jmbe.1516

FDA. (1987). *Guideline on validation of the limulus amebocyte lysate as an end-product test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices*.

FDA. (2005). *Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*.

Garidel, P., Rappolt, M., Schromm, A. B., Howe, J., Lohner, K., Andra, J., . . . Brandenburg, K. (2005). Divalent cations affect chain mobility and aggregate structure of lipopolysaccharide from Salmonella minnesota reflected in a decrease of its biological activity. [Research Support, Non-U.S. Gov't]. *Biochim Biophys Acta, 1715*(2), 122-131. doi: 10.1016/j.bbamem.2005.07.013

GmBH, H. (2013). Endotoxin-Compendium

Haile, L. A., Puig, M., Kelley-Baker, L., & Verthelyi, D. (2015). Detection of innate immune response modulating impurities in therapeutic proteins. [Research Support, U.S. Gov't, P.H.S.]. *PLoS One, 10*(4), e0125078. doi: 10.1371/journal.pone.0125078

Hansen, G. H., Rasmussen, K., Niels-Christiansen, L. L., & Danielsen, E. M. (2009). Lipopolysaccharide-binding protein: localization in secretory granules of Paneth cells in the mouse small intestine. [Research Support, Non-U.S. Gov't]. *Histochem Cell Biol, 131*(6), 727-732. doi: 10.1007/s00418-009-0572-6

Hiroko Kawasaki, T. N., Tatsushi Muta, Sadaaki Iwanaga, Yasuyuki Shimohigashi, and Shun-ichiro Kawabata. (2000). Head-to-Tail Polymerization of Coagulin, a Clottable Protein of the Horseshoe crab. *JBC Papers*

Hoemann, C. D., Sun, J., McKee, M. D., Chevrier, A., Rossomacha, E., Rivard, G. E., . . . Buschmann, M. D. (2007). Chitosan-glycerol phosphate/blood implants elicit hyaline cartilage repair integrated with porous subchondral bone in microdrilled rabbit defects. [Research Support, Non-U.S. Gov't]. *Osteoarthritis Cartilage, 15*(1), 78-89. doi: 10.1016/j.joca.2006.06.015

Hoemann CD, T.-K. N., Chevrier A, Chen G, Lascau-Coman V, Mathieu C, Changoor A, Yaroshinsky A, McCormack RG, Stanish WD6, Buschmann MD. (2015). Chondroinduction Is the Main Cartilage Repair Response to Microfracture and Microfracture With BST-CarGel: Results as Shown by ICRS-II Histological Scoring and a Novel Zonal Collagen Type Scoring Method of Human Clinical Biopsy Specimens. *Am J Sports Med. , 43*(10), 2469-2480.

Hurley, J. C. (1995). Endotoxemia: Methods of Detection and Clinical Correlates. *CLINICAL MICROBIOLOGY REVIEWS*, 268–292.

Iwanaga, S. (2007). Biochemical principle of Limulus test for detecting bacterial endotoxins. *Proc. Jpn. Acad. Ser, 83*.

J.L Lucia, J. A. C., C. E. Arnold and K.N. Winsco. (2014). Influence of an intra-articular lipopolysaccharide challenge on makers of inflammation and cartilage metabolism in

young horses. *American Society of Animal Science, 91*, 2963-2969. doi: 10.2527/jas2012-5981

Jean, M., Alameh, M., De Jesus, D., Thibault, M., Lavertu, M., Darras, V., . . . Merzouki, A. (2012). Chitosan-based therapeutic nanoparticles for combination gene therapy and gene silencing of in vitro cell lines relevant to type 2 diabetes. [Comparative Study

Research Support, Non-U.S. Gov't]. *Eur J Pharm Sci, 45*(1-2), 138-149. doi: 10.1016/j.ejps.2011.10.029

Jin, M. S., & Lee, J. O. (2008). Structures of TLR-ligand complexes. [Research Support, Non-U.S. Gov't

Review]. *Curr Opin Immunol, 20*(4), 414-419. doi: 10.1016/j.coi.2008.06.002

Joiner, T. J., Kraus, P. F., & Thomas C. Kupiec, P. (2002). Comparison of Endotoxin Testing Methods for Pharmaceutical Products. *International Journal of Pharmaceutical Compounding, 6*(6).

Jong, W. H. D., & Borm, P. J. (2008). Stealth liposomes and long circulating nanoparticles: Applications and Hazards. *International Journal of Nanomedicine.*

JP. (2001). Bacterial Endotoxin Test.

Judge, A., & MacLachlan, I. (2008). Overcoming the innate immune response to small interfering RNA. [Review]. *Hum Gene Ther, 19*(2), 111-124. doi: 10.1089/hum.2007.179

Kim, H. M., Park, B. S., Kim, J. I., Kim, S. E., Lee, J., Oh, S. C., . . . Lee, J. O. (2007). Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. [Research Support, Non-U.S. Gov't]. *Cell, 130*(5), 906-917. doi: 10.1016/j.cell.2007.08.002

Klaus Brandenburg, U. S., Andra B. Schromm, Harald Loppnow, Michel H.J. Koch, Ernst Th. Rietschel. (1996). Conformation of lipid A, the endotoxic center of bacterial lipopolysaccharide. *Journal of Endotoxin Research, 3*(3), 173-178.

Lavertu, M., Methot, S., Tran-Khanh, N., & Buschmann, M. D. (2006). High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. [Research Support, Non-U.S. Gov't]. *Biomaterials, 27*(27), 4815-4824. doi: 10.1016/j.biomaterials.2006.04.029

Levin J, B. F. (1964). THE ROLE OF ENDOTOXIN IN THE EXTRACELLULAR COAGULATION OF LIMULUS BLOOD. *Bull Johns Hopkins Hosp.*(115), 265-274.

Li Y, B. D. (2016). Endotoxin contamination: a key element in the interpretation of nanosafety studies. *Nanomedicine, 11*(3).

Liu, Y., Zhang, Y., Dong, P., An, R., Xue, C., Ge, Y., . . . Liang, X. (2015). Digestion of Nucleic Acids Starts in the Stomach. [Research Support, Non-U.S. Gov't]. *Sci Rep, 5*, 11936. doi: 10.1038/srep11936

Lonza. (2014). Manuals Product Instructions_Kinetic-QCL_Product_Insert.

Maeshima, N., & Fernandez, R. C. (2013). Recognition of lipid A variants by the TLR4-MD-2 receptor complex. [Research Support, Non-U.S. Gov't

Review]. *Front Cell Infect Microbiol, 3*, 3. doi: 10.3389/fcimb.2013.00003

Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., . . . Dixit, V. M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't]. *Nature, 440*(7081), 228-232. doi: 10.1038/nature04515

Martinon, F., Petrilli, V., Mayor, A., Tardivel, A., & Tschopp, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. [Research Support, Non-U.S. Gov't]. *Nature, 440*(7081), 237-241. doi: 10.1038/nature04516

McCullough, K. Z. (2010). Variability-in-the-bacterial-endotoxins-test. *MMI Associates*.

Méthot S, C. A., Tran-Khanh N, Hoemann CD, Stanish WD, Restrepo A, Shive MS, Buschmann MD. (2016). Osteochondral Biopsy Analysis Demonstrates That BST-CarGel Treatment Improves Structural and Cellular Characteristics of Cartilage Repair Tissue Compared With Microfracture. *Cartilage, 7*(1), 16-28.

MJ, G. (1997). Performing the LAL gel-clot test in facilities. *LAL Update, 15*(4).

Moghimi, S. M., & Szebeni, J. (2003). Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog Lipid Res, 42*(6), 463-478. doi: 10.1016/s0163-7827(03)00033-x

Molinaro, A., Holst, O., Di Lorenzo, F., Callaghan, M., Nurisso, A., D'Errico, G., . . . Martin-Santamaria, S. (2015). Chemistry of lipid A: at the heart of innate immunity. [Research Support, Non-U.S. Gov't

Review]. *Chemistry, 21*(2), 500-519. doi: 10.1002/chem.201403923

Multiskan. (2010). Turbidometric Growth Curve Measurements and Liquid Evaporation Studies with a Microplate Photometer. *Application Note*. Retrieved from

Neun, B. W., & Dobrovolskaia, M. A. (2011). Detection and quantitative evaluation of endotoxin contamination in nanoparticle formulations by LAL-based assays. [Comparative Study

Evaluation Studies

Research Support, N.I.H., Extramural]. *Methods Mol Biol, 697*, 121-130. doi: 10.1007/978-1-60327-198-1_12

note, A. (2001). Use of sodium hydroxide for cleaning and sanitizing chromatography media and systems.

Ohto, U., Fukase, K., Miyake, K., & Shimizu, T. (2012). Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. [Research Support, Non-U.S. Gov't]. *Proc Natl Acad Sci U S A, 109*(19), 7421-7426. doi: 10.1073/pnas.1201193109

Oktay, E. O., Demiralp, B., Senel, S., Cevdet Akman, A., Eratalay, K., & Akincibay, H. (2010). Effects of platelet-rich plasma and chitosan combination on bone regeneration in experimental rabbit cranial defects. [Comparative Study

Research Support, Non-U.S. Gov't]. *J Oral Implantol, 36*(3), 175-184. doi: 10.1563/AAID-JOI-D-09-00023

Park, B. S., Song, D. H., Kim, H. M., Choi, B. S., Lee, H., & Lee, J. O. (2009). The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. [Research Support, Non-U.S. Gov't]. *Nature, 458*(7242), 1191-1195. doi: 10.1038/nature07830

Peter, J., Guggenbichler, Assadian2, O., Boeswald3, M., & Kramer4, A. (2011). Incidence and clinical implication of nosocomial infections associated with implantable biomaterials – catheters, ventilator-associated pneumonia, urinary tract infections. *GMS Krankenhaushygiene Interdisziplinär, 6*.

Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S., & Beutler, B. (2000). Physical contact between lipopolysaccharide and toll-like receptor 4 revealed by genetic complementation. *Proc Natl Acad Sci U S A, 97*(5), 2163-2167. doi: 10.1073/pnas.040565397

Ragelle, H., Vandermeulen, G., & Preat, V. (2013). Chitosan-based siRNA delivery systems. [Research Support, Non-U.S. Gov't

Review]. *J Control Release, 172*(1), 207-218. doi: 10.1016/j.jconrel.2013.08.005

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science, 31*(7), 603-632. doi: 10.1016/j.progpolymsci.2006.06.001

Ruiz, N., Kahne, D., & Silhavy, T. J. (2009). Transport of lipopolysaccharide across the cell envelope: the long road of discovery. [Research Support, N.I.H., Extramural

Review]. *Nat Rev Microbiol, 7*(9), 677-683. doi: 10.1038/nrmicro2184

Sandle, T. (2011). A Practical Approach to Depyrogenation Studies Using Bacterial Endotoxin. *Journal of GXP Compliance*.

Sandle, T. (2013). Pharmaceutical Product Impurities: Considering Beta Glucans. *American Pharmaceutical Review*.

Schoenberg M. H., W. M., Radermacher P. (1998). Outcome of patients with sepsis and septic shock after ICU treatment. *Langenbeck's Arch Surg, 383*, 44–48.

Schroder, K., & Tschopp, J. (2010). The inflammasomes. [Research Support, Non-U.S. Gov't

Review]. *Cell, 140*(6), 821-832. doi: 10.1016/j.cell.2010.01.040

Shi, W., Li, X., & Ma, H. (2014). Fluorescent probes and nanoparticles for intracellular sensing of pH values. *Methods and Applications in Fluorescence, 2*(4), 042001. doi: 10.1088/2050-6120/2/4/042001

Smulders, S., Kaiser, J. P., Zuin, S., Van Landuyt, K. L., Golanski, L., Vanoirbeek, J., . . . Hoet, P. H. (2012). Contamination of nanoparticles by endotoxin: evaluation of different test methods. [Research Support, Non-U.S. Gov't]. *Part Fibre Toxicol, 9*, 41. doi: 10.1186/1743-8977-9-41

STE-1.4, N. M. (2015). Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by

Kinetic Chromogenic LAL Assay *NCL Method STE-1.4*. Frederick, MD.

Steimle, A., Autenrieth, I. B., & Frick, J. S. (2016). Structure and function: Lipid A modifications in commensals and pathogens. *Int J Med Microbiol, 306*(5), 290-301. doi: 10.1016/j.ijmm.2016.03.001

ThermoSci. (2012). Microplate Based Pathlength Correction Method for Photometric DNA Quantifi cation Assay. *Application Note*. Retrieved from

Thibault, M., Astolfi, M., Tran-Khanh, N., Lavertu, M., Darras, V., Merzouki, A., & Buschmann, M. D. (2011). Excess polycation mediates efficient chitosan-based gene transfer by promoting lysosomal release of the polyplexes. [Evaluation Studies

Research Support, Non-U.S. Gov't]. *Biomaterials, 32*(20), 4639-4646. doi: 10.1016/j.biomaterials.2011.03.010

Thomas G. Koch, L. C. B., Dean H. Betts. (2009). Current and future regenerative medicine — Principles, concepts, and

therapeutic use of stem cell therapy and tissue engineering in equine

medicine. *Can Vet J*, 50.

times_CRL, E. (2006). endosafe_times_CRI_august_2006. Retrieved from

Tirsoaga, A., El Hamidi, A., Perry, M. B., Caroff, M., & Novikov, A. (2007). A rapid, small-scale procedure for the structural characterization of lipid A applied to Citrobacter and Bordetella strains: discovery of a new structural element. *J Lipid Res, 48*(11), 2419-2427. doi: 10.1194/jlr.M700193-JLR200

Tsuchiya, M. (2013). Possible-Mechanism-of-Low-Endotoxin-Recovery-Article. *Microbiology*.

USP. (2005). BACTERIAL ENDOTOXINS TEST. In U. Inc. (Ed.).

V. N. Davydova*, I. M. Y., V. I. Gorbach, I. N. Krasikova, and T. F. Solov'eva. (2000). Interaction of Bacterial Endotoxins with Chitosan. Effect of Endotoxin Structure, Chitosan Molecular Mass, and Ionic Strength of the Solution on the Formation of the Complex. *Biochemistry*.

V. N. Davydova*, I. M. Y., V. I. Gorbach, I. N. Krasikova, and T. F. Solov'eva. (2000). Interaction of Bacterial Endotoxins with Chitosan. Effect of Endotoxin Structure, Chitosan Molecular Mass, and Ionic Strength of the Solution on the Formation of the Complex. *Biochemistry, 65*, 1278-1287.

Vreugdenhil, A. C. E., Snoek, A. M. P., Greve, J. W. M., & Buurman, W. A. (2000). Lipopolysaccharide-Binding Protein Is Vectorially Secreted and Transported by Cultured Intestinal Epithelial Cells and Is Present in the Intestinal Mucus of Mice. *The Journal of Immunology, 165*(8), 4561-4566. doi: 10.4049/jimmunol.165.8.4561

Wang, L., Wang, Q., & Reeves, P. R. (2010). The variation of O antigens in gram-negative bacteria *Subcell Biochem* (2010/07/02 ed., Vol. 53, pp. 123-152).

Wang, X., & Quinn, P. J. (2010). Lipopolysaccharide: Biosynthetic pathway and structure modification. [Research Support, Non-U.S. Gov't

Review]. *Prog Lipid Res, 49*(2), 97-107. doi: 10.1016/j.plipres.2009.06.002

Wang, X., & Quinn, P. J. (2010). Lipopolysaccharide: Biosynthetic pathway and structure modification. *Progress in Lipid Research, 49*(2), 97-107. doi: 10.1016/j.plipres.2009.06.002

Warren, H. S., Fitting, C., Hoff, E., Adib-Conquy, M., Beasley-Topliffe, L., Tesini, B., . . . Cavaillon, J. M. (2010). Resilience to bacterial infection: difference between species could be due to proteins in serum. [Comparative Study

Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't]. *J Infect Dis, 201*(2), 223-232. doi: 10.1086/649557

Williams, K. L. (2001). *Endotoxins: Pyrogens, LAL Testing and Depyrogenation* (2 ed.): Marcel Dekker.

Wistrom, C. A. A. a. A. O. (1998). Critical Aggregation Concentrations of Gram-Negative Bacterial Lipopolysaccharides (LPS). *Biochemical and Biophysical Research Communications 253*.

Wittrup, A., Ai, A., Liu, X., Hamar, P., Trifonova, R., Charisse, K., . . . Lieberman, J. (2015). Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't]. *Nat Biotechnol, 33*(8), 870-876. doi: 10.1038/nbt.3298

Wooten. (2003).

Wright, S. D., Ramos, R.A.,Tobias, P.S.,Ulevitch, R.J.,Mathison, J.C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science, 249*(4975), 1431-1433.