BINTA YAHAYA LAWAN

SYNTHESIS AND CHARACTERIZATION OF CHITOSAN MICROPARTICLE AND BLOOD COMPATIBILITY STUDIES

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF APPLIED SCIENCES OF NEAR EAST UNIVERSITY

By
BINTA YAHAYA LAWAN

In Partial Fulfilment of the Requirements for the Degree of Master of Science in Biomedical Engineering

NEU,2017

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

Name, Last name:

Signature:

Date:

ACKNOWLEDGEMENTS

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I am highly indebted to my Parents for their love, support and prayers. I will like to express my gratitude to Musa Ibrahim and Adam Mustapha for their support at various stage of this work. I would also like to express my special gratitude to Craig Adrian Little. My appreciation goes to my colleague.

Lastly, I would like to thank all my friends for their time and support. They all can't be mentioned but I really do appreciate it from the deepest part of my heart.

To my parents….

ABSTRACT

The chitosan microparticles were synthesized by ionic gelation method. Their characterizations were studied by SEM and XRD analysis. The blood compatibility studies were carried out in the NEU Hospital. The prothrombine time (PT %), APTT and INR results were obtained by STAGO STA compact Machine. Results showed that effective blood compatibility exhibiting higher Prothrombine time. Results indicated that chitosan microparticles can be used as a blood compatible material and may be able to prevent blood clotting.

***Keywords*:** Chitosan; Microparticles; Ionic gelation method; Blood compatibility; Prothrombin time

ÖZET

Bu çalışmada, iyonik jelleşme metodu ile kitozan mikroparçacıkları (K-MP) sentezlendi. Kitozan mikroparçacıklarının karakterizasyonu Taramalı Elektron Mikroskopu (TEM) ve X-Işın Analizi (XIA) kullanılarak yapıldı. Mikro parçacıkların TEM analizleri sonucunda çubuk şeklinde sentezlendiği gözlemlendi. X-Işın analiz sonuçları ise kristal yapısının varlığını kanıtladı. Kan uyumluluğu analizleri, YDÜ Hastanesi klinik biyokimya laboratuvarında STAGO STA Compact cihazı kullanılarak yapıldı. Yüksek Protrombin miktarlarının ölçülmesi, kitozan mikrokürelerinin kan uyumluluğunun yüksekliğini etkin şekilde ıspatladı.

Sonuçlar, çubuk şeklindeki kitozan mikrokürelerin kan uyumluluğu gerekli olan biyomedikal cihaz tasarımında kullanımını önerilmektedir.

Anahtar Kelimeler: Kitozan; Kan uyumluluğu; Mikroparçacık; Iyon jellesme metodu; Protrombin zamani

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APTT:	Activated Partial Thrombin Time
PT:	Prothrombin Time
TT:	Thrombin Time
Pka:	Professionally known as (acid dissociation)
Kj:	Kilojoules
Mol:	Mole
TPP:	Sodium Tripolypentaphosphate
DD:	Degree of N-deacetylation
%:	Percentage

CHAPTER ONE
INTRODUCTION

1.1 General Introduction

Chitosan is known as a versatile biopolymer (scaffolds, particles, films, membranes and gels). It is a composed randomly distributed N – acetyl – D – glucosamine (acetylated unit) and β – (1 – 4) – linked D- glucosamine (deacetylated unit) which is a linear polysaccharide and is composed of 2 – amino – 2 – deoxy – D – glucopyranose and N – acetyl – 2 – amino – 2 – deoxy – D – glucopyranose obtained by chitin which is thermochemically deacetylated in the presence of alkali. The shell waste of crabs, lobsters, krills, and shrimps are the main commercial sources of chitin (Dash et al., 2011). β – Chitin is found in squid pens and α – chitin is found in fungal and yeast cells and these are the forms in which chitin occurs.

The degree of N-deacetylation (DD) is the difference between chitin and chitosan and this is known as deacetylated units, 2 – amino – 2- deoxy – D – glucopyranose which is defined generally as the molar ratio. Fully or partially deacetylated chitin compounds when bought together is known collectively as chitosan (Tikhonov et al., 2006). Depending on their DD and MM parameters that can influence its biological and chemical properties significantly, chitosan can be found in batches in great numbers. For instance, chitosan solution viscosity (Kofuji et al., 2005) and chitosan solubility (Schiffman and Schauer, 2007) increases when DD increases and as for polymer crystallinity (Aranaz et al., 2009), when DD decreases it also decreases. Due to the quaternisation of the amine group (pKa 6.3), in dilute acidic solutions (pH < 6.0) chitosan is readily soluble: as there is an increase in the pH above 6, the polymer becomes insoluble and chitosan's amines are deprotonated.

With a unique structure, chitosan is a cationic polyelectrolyte from the chemical point of view. Two types of functional groups are exhibited (hydroxyl and amino) (Agrawal et al., 2009). The non-toxicity and its biocompatibility has already been proven (Chatelet et al., 2001; Rao and Sharma, 1997; Thanou et al., 2001), thereby primarily, the use of chitosan as biomaterial is sustained. According to Rao and Sharma (1997) sited that chitosan is a safety material based on

the acute toxicity test results. Substances such as sodium hydroxide which is an alkaline solution, is used to produce chitosan by treating crustacean shells and shrimps.

In the medical field, Chitosan has been used extensively. It has been widely employed in different forms and for a great number of applications which ranges from industrial areas (e.g dye removal, adsorption of metal ions) to biomedical field (e.g tissue engineering, drug delivery) (Dash et al., 2011; Hritcu et al., 2012; Muzzarelli, 2008; Muzzarelli et al., 2005; Muzzarelli et al., 2012). It is either fully deacetylated or partially chitin. Chitosan is a fully biocompatible natural polymer and also biodegradable. It can be used as an antifungal agent, as an adhesive and as an antibacterial agent.

Due to its biocompatible properties, it has been extensively investigated as a potential drug carrier. Some studies suggested that, in order to increase their bioavailability and reduce their impact on the body, nanoparticles made of other materials should be coated with chitosan. In order to obtain a different physio-mechanical properties, the molecular weight and the degree of deacetylation of chitosan can be modified. ~5.3 x 10 Daltons is the average viscosity molecular weight of chitosan. Nitrogen (7.97%), Carbon (44.11%), and hydrogen (6.84%) are the elemental composition of the chitosan polymer.

Chitosan has some possible biomedical uses and quiet a number of commercial uses also. In medicine, it can be used as a delivery agent (drug delivery into the skin), as an antibacterial agent and in bandages for stopping or reducing of blood. Chitosan biological properties however such as anticoagulant activity (Yang et al., 2008), proliferate and cell adhesion (Chatelet et al., 2001), biocompatibility (Schiffman et al., 1999), and biodegradability (Zhang et al., 2001), are influenced greatly by its parameters. Due to highly non-toxicity for biomedical applications, chitosan of a lower DD is recommended. Imposed by a lot of aspects, biodistribution is an important aspect that concerns the biocompatibility of chitosan, ranging from the formulation type used administration route and polymer characteristics (Schiffman et al., 1999).

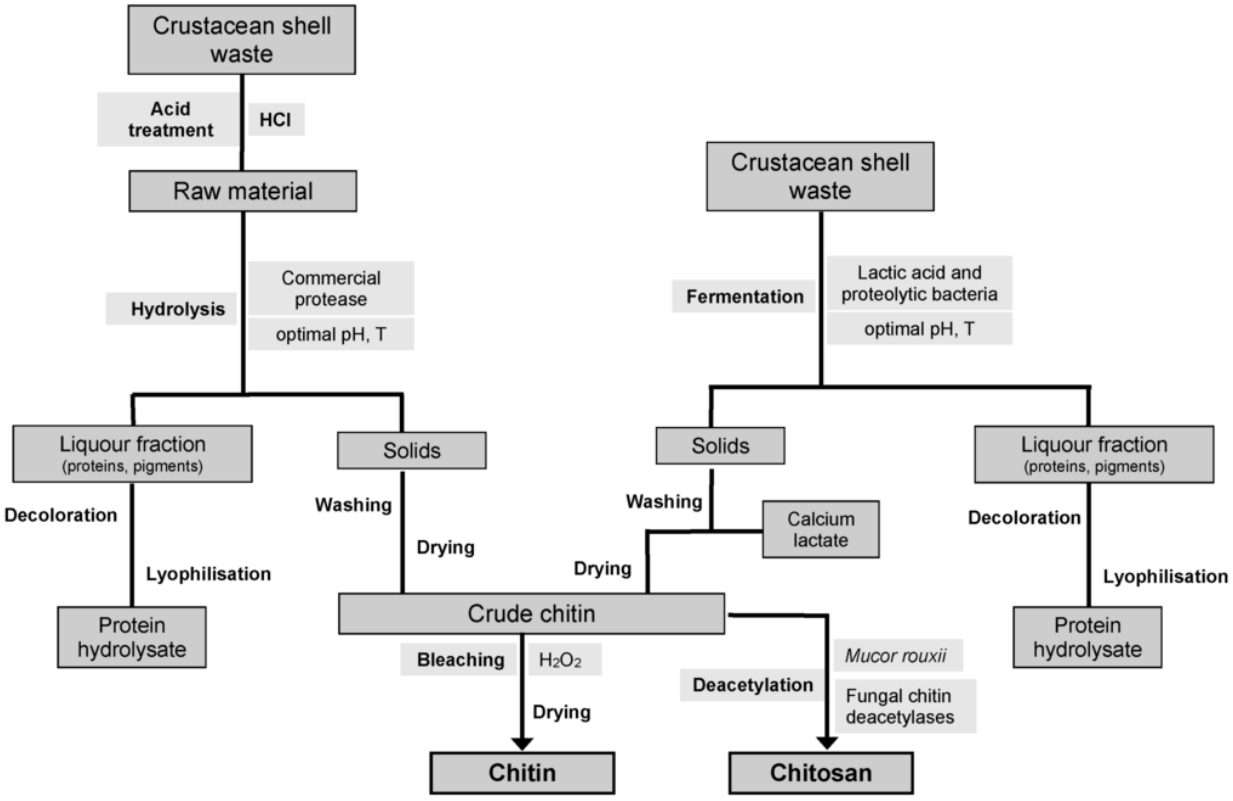

Figure 1.1: Systematic representation of chitin and chitosan preparations (Adapted from Angelica et al., 2016)

In ophthalmology

Immunological compatibility, wettability, gas permeability, optical clarity, sufficient optical correction and mechanical stability are all characters of contact lens which chitosan possesses. Oxygen permeability, tear strength, elongation modulus, and modulus are characteristics of contact lens made from chitosan, thus for ocular bandage lenses development, it is suitable.

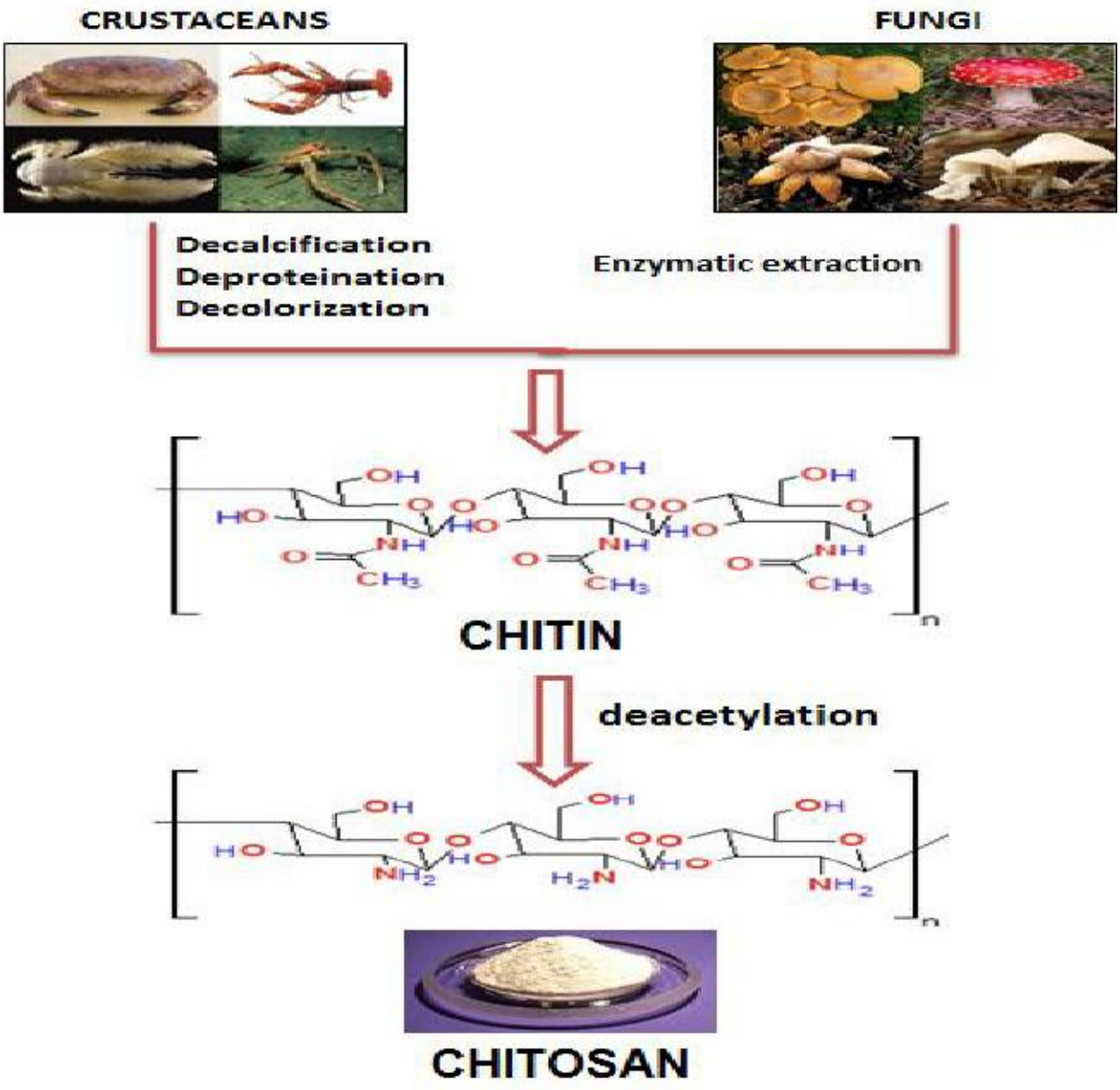

Figure 1.2: Showing extraction of chitin and chitosan
(Adapted from Angelica et al., 2016)

Functionalized-chitosan based system in biomedical application

Nature has been great source of Chitosan, a polysaccharide derived from a chitin, which is the second most abundant polysaccharide in the world after cellulose (Chen et al., 2011). Chitosan presents unique properties such as biocompatibility, biodegradability and its non-toxic nature which confers it wider application in biomedical and pharmaceutical areas such as drug and gene delivery, tissue engineering, wound healing, central nervous system treatment, Cardiovascular applications, antibacterial and microbial activities, and theragnosis (Chen et al., 2011; Balan and Verestiuc, 2014; Ibrahim and El-Zairy, 2015).

Gene and drug delivery

In recent years the use of chitosan as a vehicle of drug delivery has been increasing area of interest to ensure effectiveness and patient compliance. Balan and Verestiuc (2014) underline the newest directions in the field of functionalized carriers based on chitosan as parenteral delivery systems that could overcome the current limitations of clinical use of drug delivery system. Similarly, study conducted by Zhang et al. (2008) has revealed the use of chitosan as a carrier of drug system. In the study, a comprehensive evaluation of pharmacokinetics, tissue distributions, metabolism, excretion, acute toxicity, safety and cell viability of N-octyl-O-sulfate chitosan based nanoparticles was conducted, which showed a streaking result of potential use of this new system of drug carrier. The positive charge on chitosan and negative charge on the cell membrane allow the interaction that leads to reorganization and an opening of the tight junction proteins, explaining the permeation enhancing property of this polysaccharide.

Recent studies revealed that chitosan and its derivatives exhibited antitumor activity in both *in vitro and in vivo* models. Tumor-targeting property of cisplatin-loaded glycol chitosan nanoparticles was demonstrated by Kim et al. (2008). Similarly, Zhu et al. (2011) also showed the ability of chitosan to serve as drug loading vehicle in a modified chitosan with folate. This model could be used for tumor targeting carrier for hydrophobic therapeutics or diagnostic agents. The chemical properties of chitosan allow modification with various side chains to suite the desired target. For instance, Sahul et al. (2011) prepared hydrophobically modified carboxylmethyl chitosan nanoparticles for targeted delivery of paclitaxel with excellent non-cytotoxic property compared with native drug. The main merit of use of chitosan in drug delivery apart from

biocompatibility is they are not hazardous and less expensive.

Tissue engineering

Tissue engineering is a multifaceted technology that involves the use of a combination of cells, engineering materials, and suitable biochemical factors to improve or replace biological functions. This includes a wide range of applications such as repair or replacement of part or whole tissues (Cheung et al., 2015). Chitosan and its derivatives have been explicatively explored as a supporting material used for tissue engineering process (Balan and Verestiuc, 2014). It shared great similarity with gylycosaminoglycans, the components of the liver extra cellular matrix, thus chitosan used as a promising scaffold for hepatocytes cultures. For instance, in a study by Cheung et al. (2015) Chitosan-â-tricalcium phosphate composite exhibited histocompatibility with Beagle mesenchymal stem cells and was devoid of an effect on cellular growth and proliferation. It manifested efficacy in enhancing osteogenesis and vascularization and repair of bone defects in conjunction with mesenchymal stem cells. In other study, chitosan/collagen/heparin matrix with excellent blood compatibility and good hepatocyte compatibility has been suggested as matrix for implantable bioartificial liver (Wang et al., 2005). This reveals the recent application of chitosan-based biomaterial in tissue and cell repair and engineering.

In fluorescent

Fluorescent chitosan nanoparticles are formed by the interaction between chitosan nanoparticles amino groups by which Rhodamine dye get attached to the chitosan nanoparticles. In the near future for the application of cell imaging, prepared fluorescent chitosan nanoprobe could be used. In addition, to improve the chemical and mechanical properties of chitosan, it can be modified by chemical and physical methods.

Healing of wound

The basic features of chitosan and its derivatives in wound healing are its biodegradability, biocompatibility and antimicrobial activity which confer it as excellent biomaterials for wound healing. It has been found that, chitosan accelerate the effect of wound healing where regenerated chitin fibers, films and sponges exhibit an increase in wound healing by over 30 percent (Dutta et al., 2004). Thus, the complex process of wound healing which involves inflammation, granulation

tissue formation, extracellular matrix formation, angionesis and remodeling can be stimulated by chitosan and its derivatives. Cheng et al. (2011) proposed that incorporation of growth factors; antibiotics and antibacterial increase the efficacy of chitosan as excellent biomaterials.

The adhesive-based wound dressing could be applied in surgery to enhance wound healing. The chitosan adhesive shows strong sealing strength as well as not requiring sutures or staples. It can effectively stop bleeding from blood vessels along with air leakage from the lung (Ishihara et al., 2006). Many clinical studies showed a significant result in chitosan in would healing by patients undergoing plastic surgeries, skin grafting and endoscopic sinus surgery (Biagini et al., 1991; Stone et al., 2000; Azad et al., 2004; Valentine et al., 2010). Similarly, due to their versatility, chitosan and chitin are used as diluents in the pharmaceutical industries.

Treatment Cardiovascular Diseases

Ability of chitosan in supporting endothelial cells and to enhance angiogenesis, functionalized chitosan- based formulation has been investigated as cardiovascular scaffolds that able to deliver regenerative cells or bioactive compounds in the treatment (Balan and Verestiuc, 2014). Study by Lu et al. (2009) proved the effectiveness of chitosan-collagen cross linked as a cardiovascular supportive material both *in vitro and in vivo* tests. Similarly, remarkable results showed that chitosan hydrogel could improve myocardial infarction microenvironment, enhance stem cell engraftment, homing in ischemic heart and stimulate heart repair (Balan and Verestiuc, 2014). This agreed with other studies that proved chitosan as excellent biomaterial and could be employed in heart disease treatments (Meng et al., 2009; Pok et al., 2013; Cheung et al., 2015).

Central nervous system treatment

The structural barrier such as blood brain barrier that separates blood and cerebral parenchyma limits the drugs such as anti-Alzheimer, antibiotics, antineoplastic agents and other neuroleptic drugs penetrate deeply into the system. It has been proposed that if this barrier can be effectively been crossed would increase brain absorption of the drugs (Hombach et al., 2009). A preliminary in vitro study using mouse brain endothelial cell showed an efficient uptake of drugs particles across the blood brain barrier. This promising result could be used in employing chitosan coated with nanoparticles or chitosan-based polymers as brain targeting agent.

Nutrition and Food

Lactose intolerance effect occurs in many humans and animals. The whey production showed considerable increase by small supplement of chitosan as feed, which improved change in intestinal microflora as well as in chickens, 2-0.5% • chitosan supplement improved weight gain

Artificial Skin

Chitosan is used for the treatment of fibroplasias and healing of wounds caused by subcutaneous tissue and scapel insertions in skin especially when caused by fire (this is used in situations such as extensive skin loss by patients)

Capturing of water engineering metal from waste water

In heavy metal ions chitosan has a natural selectivity and in acidic medium, N-benzylsulfonate derivates of chitosan were used as sorbents for the metal ions, removal of NI^{2+}, Hg^{2+}, Zn^{2+}, and Cu^{2+} by the adsorption parameters of chitosan has been enhanced, in which on the outer surface of chitosan, metal ions are preferably absorbed.

In textile dye (color removal)

Chitosan has an extremely high affinity and as well have dye of many classes which includes napthal dyes, acid, vat, sulfur, direct, reactive, and disperse and also has a unique molecular structure. Loading thermodynamics enhances adsorption rate and adsorption of dye is significantly caused by the effect of pH, and temperature which shows the favored reaction. In addition, it can also be employed in Photography.

1.2 Chemical Structure of Chitosan

The chemical structure of chitosan has been proposed to have affords from deacylation of chitin and this provides sides for chemical modification (Berger et. al., 2004; Mourya et al., 2010). Based on the source, chitin occurs in two allomorphic forms named α and β, and the third allormorphic structure γ have also been suggested (Averous and Pollet, 2012). The basic difference between the above chemical forms is the presence of tight inter-sheet hydrogen bond.

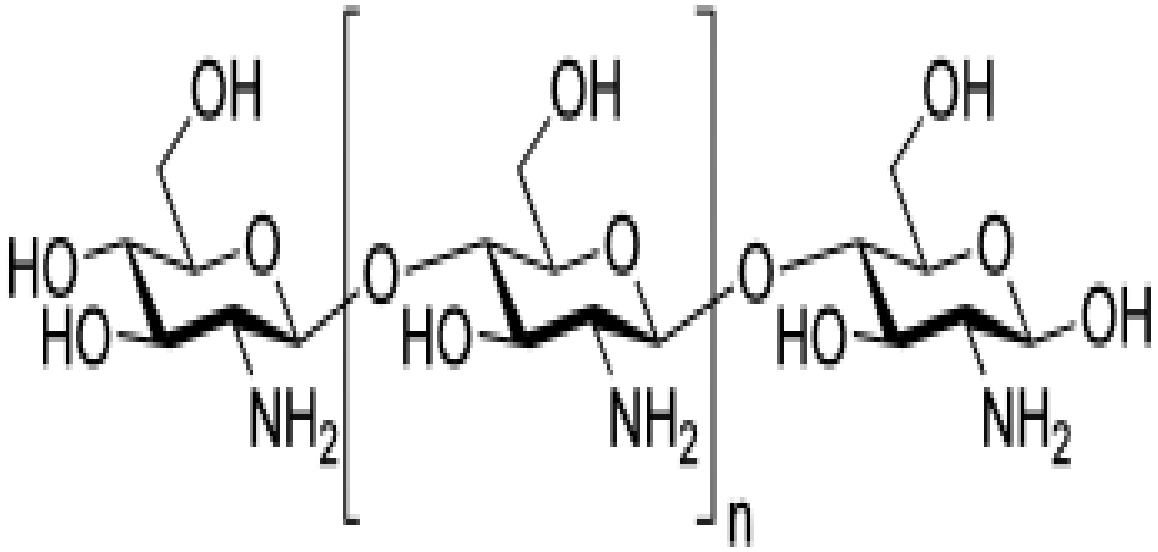

Figure 1.3: The basic structure of chitosan (Majeti and Kumar, 2000)

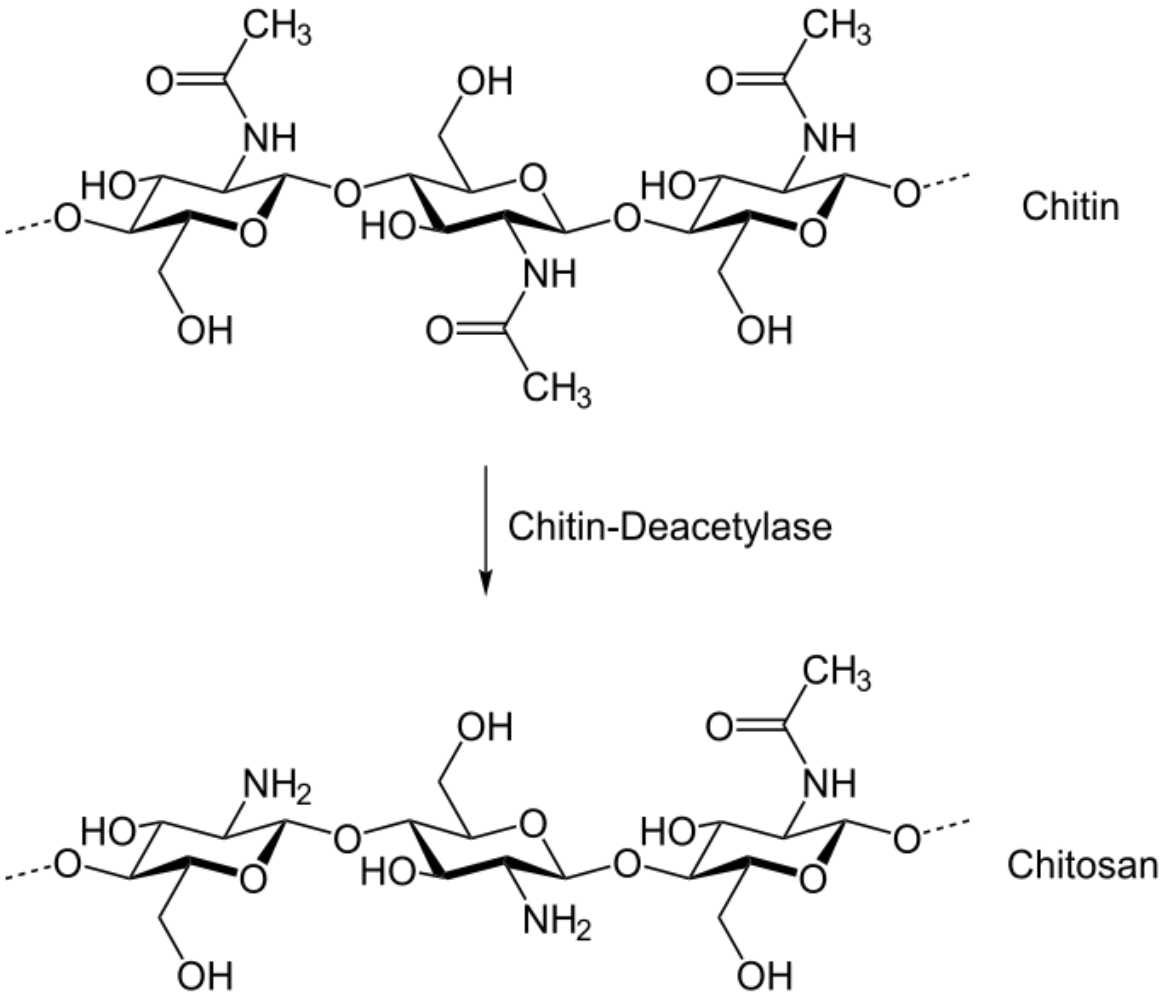

Figure 1.4: Chemical modification of chitin (Majeti and Kumar, 2000)

1.3 Chitosan Non-toxicity

Non toxigenic nature of chitosan has been the unique property that allows use of chitosan in many aspects. Use of chitosan in food industry is well known because it is not toxic for warm-blooded animals. Microcrystalline chitin (MCC) shows good emulsifying properties, superior thickening, and gelling agent for stabilizing foods. It is also used as a dietary fibre in baked foods (Dutta et al., 2014). The nontoxicity of chitosan provides wider application of this natural polymer especially in biomedical sciences such as wound heal, drug delivery, bio-imaging, bioadhesive, gene delivery, tissue engineering (Ikram and Ahmad, 2016).

Its ability to biodegrade into non-toxic residues and the rate of its degradation being highly related to the molecular mass of the polymer and its deacetylation degree and has proved to some extent biocompatibility with physiological medium. All these singular properties make chitosan an outstanding candidate for biomedical applications (Croisier and Jerome, 2013).

1.4 Properties and Characterization of Chitosan

Deacetylation of chitin is a commercial way of the production of chitosan, which in fungi cell wall and crustaceans (such as shrimps and crabs) is the structural element in the exoskeleton. NMR Spectroscopy is used determining the degree of deacetylation, and the commercial chitosan with deacetylation has a range of about 60-100%. Averagely, about 3800 to 20,000 Dalton in the molecular weight of chitosan produced commercially.

Deacetylation of chitin using water as a solvent and as a reagent using sodium hydroxide in excess is one common method for chitosan synthesis. There are two stages in which first order kinetic control reaction takes place. The second step is lower than the first in which the first is the activation energy 25-120^{o}c at 48.76kj/mol is its estimated value (Hammou et al., 2013).

The pKa of chitosan amino group has a value of approximately 6.5, which can lead to a protonation to neutral in acidic solution with a density charge negatively charged surfaces (Dong et al., 2013; Chanoong et al., 2015). Chitosan is biodegradable and biocompatible which across the epithelial surfaces enhances the transportation of polar drugs, for biomedical applications, there are available quantities of purified chitosan.

Trimethylchitosan which is a derivative of chitosan have been used in the delivery of gene which are nonviral. Quaternised chitosan or trimethylchitosan with increased degree of trimethylation increasing the cytotoxicity has been shown to transfect breast cancer cells, approximately trimethylation at about 50%, in gene delivery, the derivative is the most efficient. Oligomeric derivative of 3-6kDa have good gene delivery properties and are relatively nontoxic (Kean et al., 2005). Antitumor activity and immunoadjuvant, fungistatic, central nervous system, osteogenesis (El-Kamel et al., 2007; Roldo et al., 2004), hemostatic, anticholesteremic (Dash et al., 2011; Muzzarelli and Muzzarelli, 2005; Muzzarelli et al., 2012), promoter of wound healing (Fischer et al., 2007; Fischer et al., 2005; Madhumathi et al., 2010; Sudheesh et al., 2010), and spermicidal

are biological properties of chitosan and a biomaterial sustain their use,

1.4.1 Hemocompatibility test of Chitosan

Due to establishing a possible standard for blood material interactions, Hemocompatibility has been studied greatly. Blood compatibility assessment is taken into account due to the fact that there many mechanisms in which blood can respond. Classified into five categories are blood compatibility which are hematology, platelets functions, complement system, blood coagulation, and thrombosis. One of the tests used to evaluate medical device or hemocompatibility of a biomaterial is blood coagulation which is the most used test. Activation of clotting factor cascade is a phenomenon which gives rise to blood coagulation. Activated partial thromboplastin time (APTT) is measured so as to give the estimated value of common coagulation pathway and intrinsic pathway. Prothrombin time (PT) test evaluates coagulation cascade activation of common pathway and extrinsic pathway while fibrin polymerization or thrombin activity is examined by thrombin time (TT). Adsorbed proteins also governs blood compatibility on material surface which is followed by blood exposure (Gorbet et al., 2004). Plasma components and the quantification of cellular of the blood is known as hematology. Hemolysis is the mechanism through which hemoglobin is evaluated. In estimating blood biocompatibility of materials, the reliable and simple method which can be used is the in vitro hemolysis. When it is less than 5%, hemolysis index is regarded as safe (Zhou et al., 2011).

1.4.2 Biodegradability

The successful use of a material in biological application which is an essentially important requirement in chitosan is biodegradability. Chitosan is composed to both enzymatic degradation and chemical degradation in the human body. Colonic bacterial enzymes (Zhang and Neau, 2001), rat cecal, pectinase isozyme (Kittur et al., 2005), leucine amino-peptidase (Rao and Sharma, 1997), and lysozyme (Kean and Thanou, 2009) are enzymes that are able to degrade this polymer when in vitro degradation that test of chitosan was carried out. Eight human chitinases have been identified in recent studies of which (enzymatic activity was revealed in three of them) (Funkhouser and Aronson, 2007) but so far on its derivatives or regarding the effects of these

enzymes on chitosan, there are no literature reports yet.

1.4.3 Chitosan Mucoadhesitivity

Several studies have been conducted to examine the mucoadhesive properties of chitosan (Dhawan et al., 2004; Bizhan et al., 2008; Khameneh et al., 2014). He et al. (1998) evaluated the mucoadhesive properties of chitosan and chitosan microspheres by studying the interaction between mucin and chitosan in aqueous solution by turbidimetric measurements and the measurement of mucin adsorbed on the microspheres.

A strong interaction between chitosan microspheres and mucin was detected. This reveals another important feature of chitosan in absorption of substances using the available charges on the surface, thus it can be used for developing drug delivery systems because of its excellent mucoadhesive properties. Properties such as primary amino groups, electrostatic attraction, hydrogen bonding and hydrophobicity are believe to be responsible for the mucoadhesivity of chitosan (Saqias et al., 2008). In answering why chitosan is mucoadhesive, the authors revealed that change in these properties may influence its mucoadhesivity, for instance, reducing the number of amino groups through their half acetylation results in expansion of chitosan's pH-solubility window up to pH 7.4 but also reduces its capacity to aggregate mucin. Khobragade and Puranik (2015) clearly stated the mucoadhesive property of chitosan and its derivates. The authors described Chitosan structure to possess cationic group, due to this group it has mucoadhesive property.

Chitosan mainly combine with anionic group of mucus i.e sialic acid and sulfonic group substituent. Hence ionic interaction is take place in between cationic primary amino acid group of chitosan with anionic sialic acid group of mucus, mucoadhesion can be achieved. However, the hydrophobic interactions might contribute to its mucoadhesive properties. In comparison with various anionic polymeric excipients such as carbomer, polycarbophil, and hyaluronic acid, however, its mucoadhesive properties are weak. The authors also suggest way to achieve better mucoadhesive properties; polymer should have cohesive properties as that of adhesive. If not then polymer fails to achieve mucoadhesion.

Chitosan has weak cohesive properties but can be improved by formation of complexes with multivalent anionic drug or other anionic excipients. In addition, chitosan possesses excellent

mucoadhesive properties in its swollen state. It can easily adhere to hard and soft tissues. Clinical tests conducted with the use of materials containing chitosan and its derivatives revealed that they do not cause any inflammatory or allergic reactions in the human body. Chitosan is well tolerated by living tissues, including connective, muscle, epithelial, and nervous (Nawrotek et al., 2015).

1.4.4 Chitosan Cytotoxicity

Chitosan and its derivatives have been extensively applied in medical and pharmaceutical fields as promising drug delivery systems. Hence, the safety of this important polymer to cells needs to be studied. Studies on cytotoxicity of chitosans have been previously reported on cultured cells, for example, A549 human alveolar epithelial cells, others used zebra fish model and antitumor effect of paclitaxel-bound nanoparticles in gastric cancer cells (Zaki et al., 2015).

Sabudin et al. (2012) studied the cytotoxicity of silver nanoparticles incorporated in chitosan using Normal Human Dermal Fibroblasts (NHDFs). The result showed chitosan considerable cell cytotoxicity, mainly for water-soluble chitosan paste samples and concluded that the toxicity of the silver nanoparticles is still within the acceptable range for human use. Similarly, different degrees of de-acetylation of chitosan on the cytotoxicity were investigated by (Grobler et al., 2016), where 3T3 fibroblast cells as well as two different human tooth pulp fibroblast cell-lines were used. The study revealed that different cell lines react differently towards different degrees of de-acetylation. The relative survival rates of different cell lines changed at different degrees of de-acetylation. Thus, the cytotoxicity of chitosan is within the limit or showed no toxicity to cells.

1.5 Antimicrobial Activities

The antimicrobial activity of chitosan have been examined against wide range of microorganisms like algae, bacteria, yeasts and fungi in experiments involving *in vivo and in vitro* interactions with chitosan in different forms such solutions, films and composites (Goy et al., 2009). Historically, early research describing the antimicrobial potential of chitin, chitosan, and their derivatives dated from the 1980-1990s (Goy et al., 2016). Generally, in these studies the chitosan is considered to be a bacteriocidal (kills the live bacteria or some fraction therein) or bacteriostatic (hinders the growth of bacteria but does not imply whether or not bacteria are killed), often with

no distinction between activities (Goy et al., 2009; Goy et al., 2016). However, recent study by Benhabiles et al. (2012) indicated the tendency to characterize chitosan as bacteriostatic rather than bactericida, although the exact mechanism is not fully understood and several other factors may contribute to the antibacterial action.

Models have been suggested to elucidate the exact mechanisms of action of chitosan and its derivatives against microorganisms. Goy et al. (2009) proposed the most acceptable being the interaction between positively charged chitin/chitosan molecules and negatively charged microbial cell membranes. In this model the interaction is mediated by the electrostatic forces between the protonated NH^{3+} groups and the negative residues presumably by competing with Ca^{2+} for electronegative sites on the membrane surface. This interaction results in double actions i) by promoting changes in the properties of membrane wall permeability, thus provoke internal osmotic imbalances and consequently inhibit the growth of microorganisms and ii) by the hydrolysis of the peptidoglycans in the microorganism wall, leading to the leakage of intracellular electrolytes such as potassium ions and other low molecular weight proteinaceous constituents (e.g. proteins, nucleic acids, glucose, and lactate dehydrogenase). Thus affect the integrity of the cell wall and osmotic pressure. This mechanism if fully understood would help greatly in controlling some resistant organism and reduce exposure to the use of conventional antibiotics.

Regarding the spectrum of activity, it is somewhat controversial but it is suggested by some authors that chitosan generally showed stronger effects for gram-positive bacteria (e.g. *Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, L. bulgaris,* etc.) than for gram-negative bacteria (e.g. *E. coli, Pseudomonas fluorescens, Salmonella typhymurium, Vibrio parahaemolyticus*, etc.) (Goy et al., 2009). Similarly, Manhwan et al. (2014) indicated the potential use of chitosan as an antibacterial agent. Their studies found that the chitosan reduced pathogenic *Escherichia coli* O157:H7 shedding in cattle by disrupting bacterial cell membrane. Many studies about the antimicrobial characteristics of films made of chitosan and its derivatives have been reported (Xie et al., 2002; Peng et al., 2005; Martins et al., 2014; Sun et al., 2014.). The use of chitosan and its derivatives as a potential antimicrobial agents with wide application on many pathogenic organisms.

1.6 Blood coagulation

Blood coagulation is a complex process that involves activity of red blood cells, white blood cells and plasma. The blood platelets play vital role in blood coagulation. Reduction of protein adsorption is achieved when there is improvement of blood compatibility on a biomaterial surface (Terin and Murat, 2015). The mechanism of blood coagulation is complicated with various factors which are derived from blood proteins (Natalya et al., 2002). Heemskerk et al. (2002) describe the platelet activation and coagulation as complementary, and mutually dependent processes in haemostasis and thrombosis. The authors describe the interaction with many coagulation factors (FI-FXIII), while the coagulation product thrombin as a potent platelet-activating agonist.

There exist two pathways acting as an extracellular signaling cascade; extrinsic and intrinsic factors (Figure 5). Several studies reviewed this important phenomenon of great physiological relevance (Patill, 2002; Natalya et al., 2002; Chu, 2010).

Patill (2002) summarized the mechanisms of both pathways as presented below.

1. Extrinsic Mechanism (Factors involved – III-VII-X-V) for formation of prothrombin activator
    i. It begins with trauma to blood vessel or tissues outside the blood vessel. It releases tissue factor and Tissue phospholipids and clotting process starts.
    ii. The tissue factor complexes with blood clotting factor VII and activates it.
    iii. Activated factor VII in presence of ca++ and tissue phospholipids acts on factor –X and activates it.
    iv. Activated factor X acts on Factor V and activates it.
    v. Activated F-X complexes with tissue phospholipids, Factor-V, ca++ and forms a complex called prothrombin activator.
    vi. Prothrombin activator converts prothrombin in to thrombin under influence of ca++
    vii. Thrombin acts on fibrinogen and converts it in to fibrin monomers
    viii. Fibrin monomers polymerize with other fibrin monomers and form long fibrin threads that form reticulum of the clot.
    ix. At first clot is weak but later on with the help of active fibrin stabilizing factor (F- X III) clot becomes strong.

x. WBCs and RBCs get trapped in to reticulum of the clot

xi. Clots adhere to the damaged surface of the blood vessel and thereby prevents the blood loss.

xii. Clot retraction Following clot formation, the volume of the clot decreases , this is called as clot retraction platelets are necessary for clot retraction contain contractile protein Thrombosthenin, which contracts and reduces the volume of the clot. Following this a clear fluid is separated out called as serum.

2. Intrinsic Mechanism begins with injury to blood itself and continues through following steps (F-III, F-XII-F-XI-F-IX-FVIII-F-X-F-V)

i. Trauma to blood alters two important clotting factors in the blood

ii. Factor XII and Platelet Phospholipids i.e. F- III

iii. When F-XII comes in contact with collagen outside the blood vessel, it gets activated and acts as an enzyme for activation of F-XI

iv. Damaged platelets adhere to the wet surface of blood vessel and release platelet phospholipids i.e. F- III.

v. Activated factor XII acts enzymatically on F-XI i.e. Plasma Thromboplastin Antecedent (PTA –Factor) and activates it.

vi. Activated factor XI acts enzymatically on F- IX i.e. Christmas factor and activates it (ca++ are necessary)

vii. Factor IX activates F-VIII (Anti Haemophilic Factor)

viii. Activated F- IX, F-VIII and platelet phospholipids, activate factor-X.

ix. Activated Factor X acts enzymatically on Factor V (Proaccelarin) and activates it, (ca++ are nessessory).

x. Activated F-V, activated X, Platelet phospholipids and ca++ form a complex called prothrombin activator Prothrombin activator converts prothrombin in to thrombin under influence of ca++

xi. Thrombin acts on fibrinogen and converts it in to fibrin monomers

xii. Fibrin monomers polymerize with other fibrin monomers and form long fibrin threads that form reticulum of the clot. 12)At first clot is weak but later on with the help of active fibrin stabilizing factor (F- X III) clot becomes strong.

xiii. WBCs and RBCs get trapped in to reticulum of the clot

xiv. Clots adhere to the damaged surface of the blood vessel.

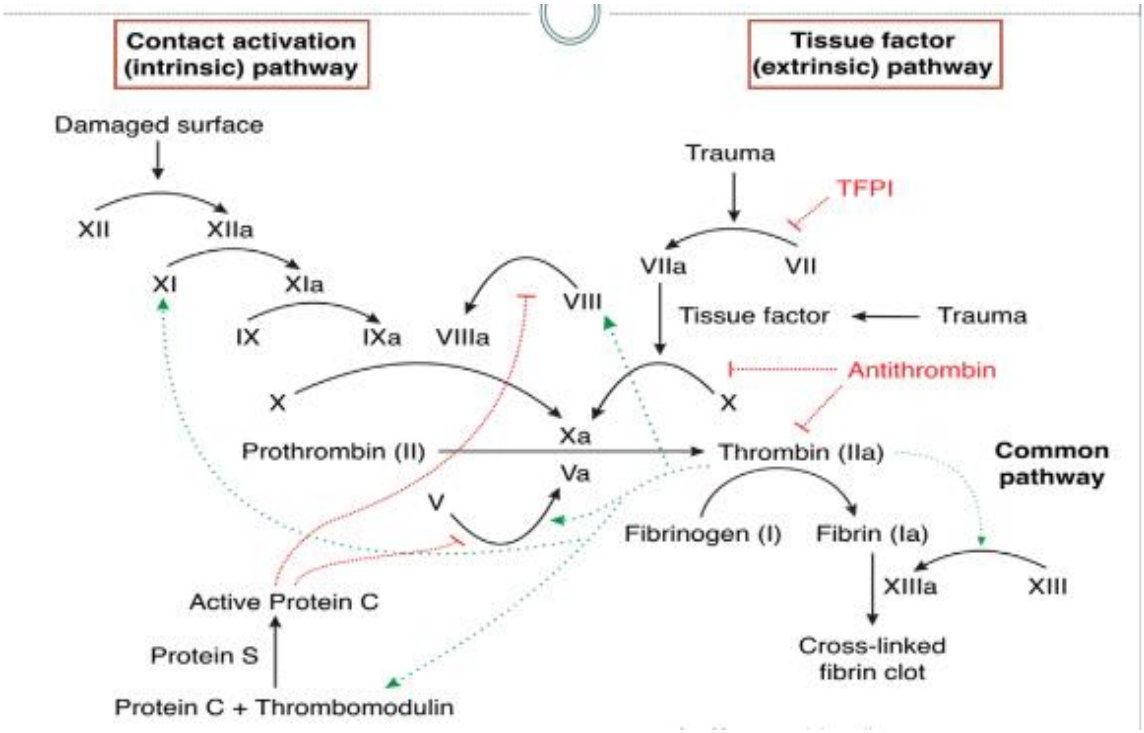

Figure 1.5: Systematic description of blood coagulation pathways (Adapted from Chu, 2010)

1.7 Aim of the Study

The aim of this thesis is to determine the blood compatibility of chitosan microparticles. This is because chitosan is widely investigated for applications in regenerative medicine and in drug delivery Systems. It is a natural origin of biocompatible polymer.

1.8 Objectives of the Study

The objectives of this research work are:

1. Improve Blood Compatibility (Nano/Microparticles)
2. Determining the sensitivity of Chitosan in the Blood
3. Determining the biomedical material versatilely when in contact with bloodstream
4. Chitosan based formulations of Commercialization and clinical application of hemocompatible

CHAPTER TWO
MATERIALS AND METHODS

This work was conducted at the Near East University at the hematology unit, from September to October 2016. The chemicals used in this study are of high standard and purity. All glass wares which were used were properly sterilized by washing and dried in a hot air oven. TPP was purchased from SIGMA-ALDRICH. During the whole experimental process distilled water was used.

2.1 Materials

Materials used are as follow
Acetic acid
Chitosan
Stago US (STAcompact hemostasis system equipment machine) connected to a computer to enable the reading of results
Ethanol
Blood samples
TPP (Sodium Tripolypentaphosphate)

2.2 Sample Collection

Sodium tripolypentaphosphate (TPP) and chitosan were prepared in the laboratory. The brand is Sigma-Aldrich. The viscosity was about 200cps, the deacetylation degree was > 85% with a molecular weight of about 190,000-375,000 as indicated on the container.

2.2.1 Preparation of Chitosan Beads

Two grams of Chitosan powder was dissolved in 1.50 ml of acetic acid. The chitosan was weighed by using the sensitive balance machine. After weighing the chitosan was then put in a beaker and 1.50 ml of acetic acid was added. The mixture was stirred with a magnetic bar in the solution. The

solution was then placed on the magnetic stirrer at a temperature of about 60^0c and at set speed of 40 rpm. With the use of a syringe needle (small syringe needle in order to create nano particles) the chitosan was dropped gently into the TPP solution which instantaneously formed gelled spheres.

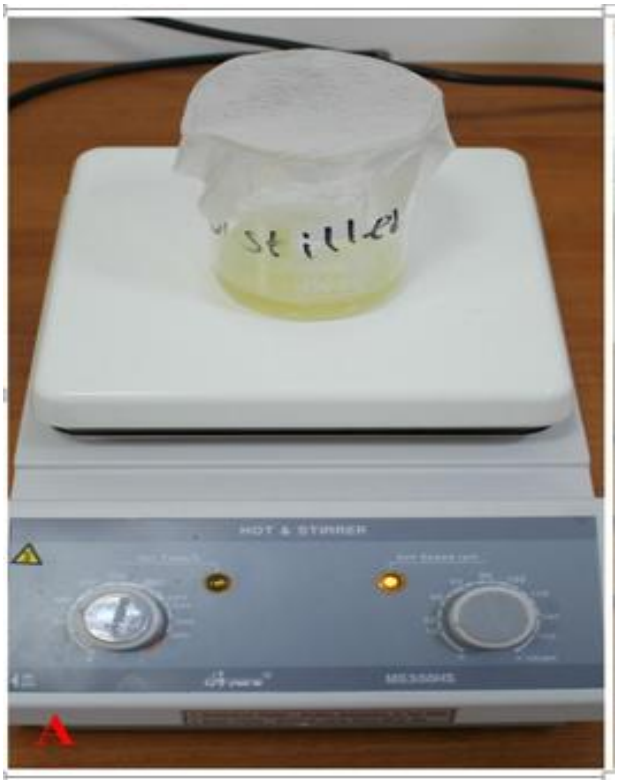

Figure 2.1: Preparation of TPP Solution in Biomedical Engineering Near East University

Below is a sample of gel formed chitosan before drying.

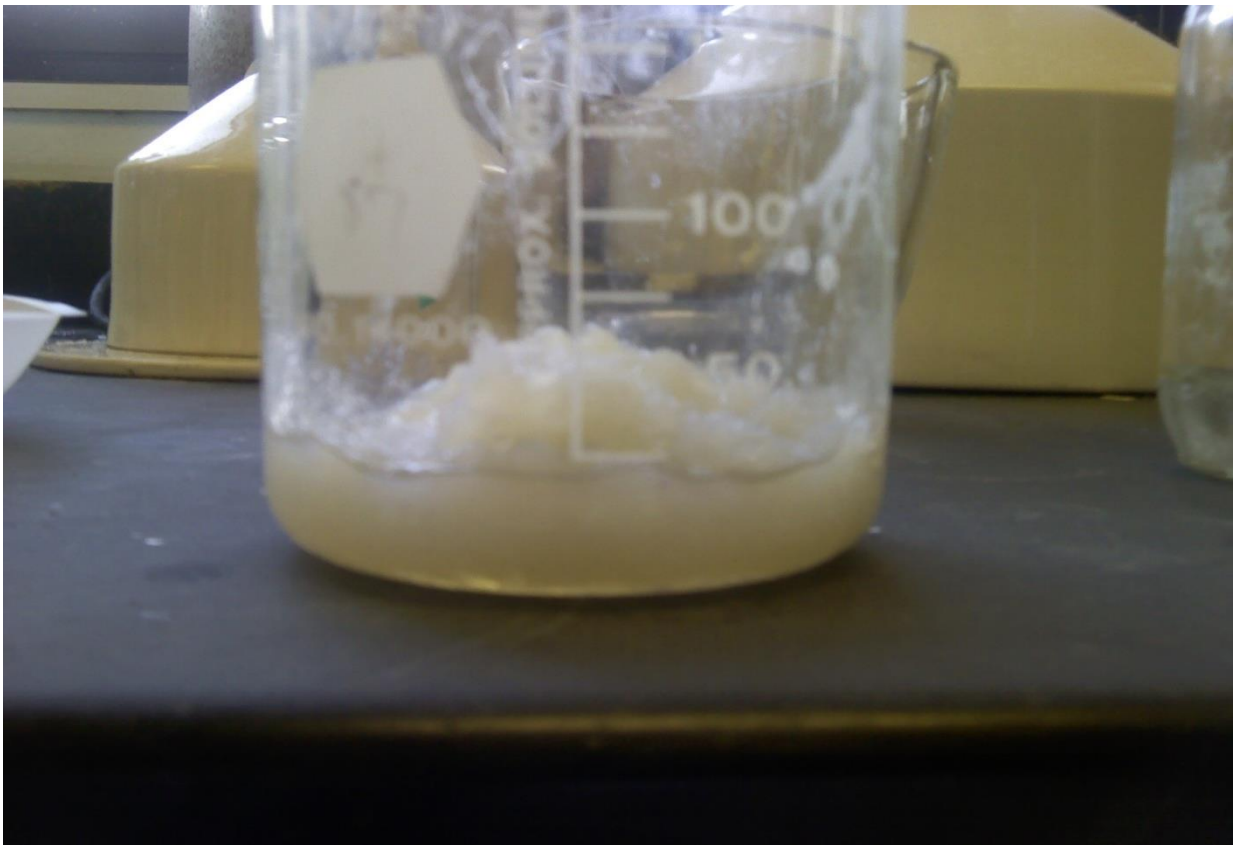

Figure 2.2: Gel like chitosan

Figure 2.3: Sample of dried chitosan particles at room temperature

2.2.2 Preparation of Sodium Tripolypentaphosphate

In the preparation of TPP aqueous solution 0.1% ($^{w}/_{v}$), the powder was dissolved in distilled water. The droplet of the chitosan stood in the solution over-night. The chitosan droplet was then filtered using filtering paper was let too air dry under room temperature.

2.3 Morphological Chitosan Characterisation

The morphologies of the dried beads, the cross-sectional and surface were examined using Field Emission Microscopy (FEM) and Scanning Electron Microscopy (SEM).The air dried chitosan was grinded and stored in the refrigerator in test tubes Tao et al. (2013). The chitosan were sterilized.

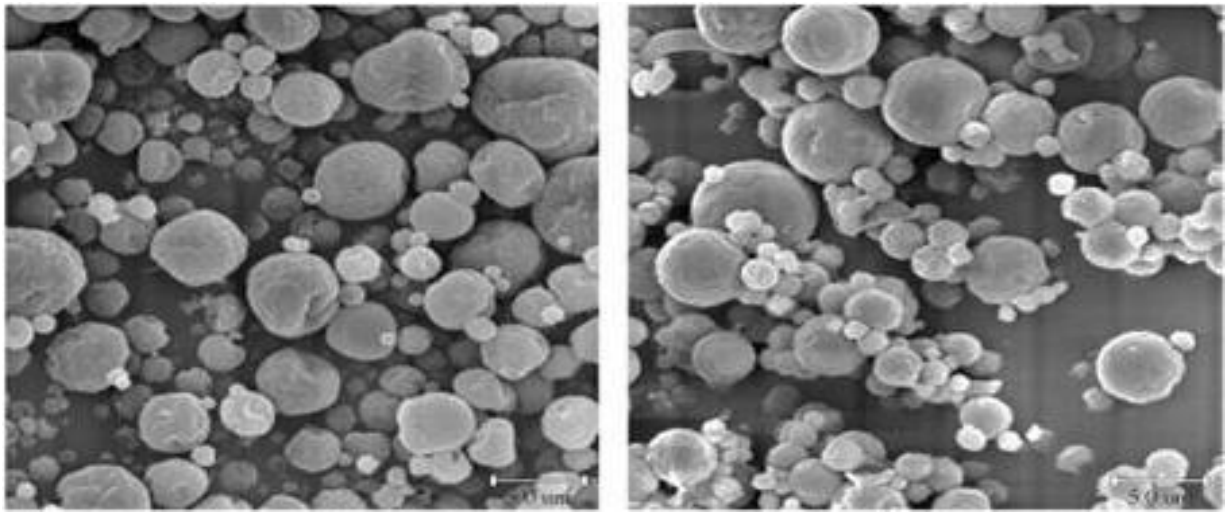

Figure 2.4: Chitosan particles viewed under electron microscope (SEM)
(Adapted from Tao et al., 2013)

Five different samples were measured from one of the test tubes containing the chitosan with the weight of 0.06 grams each. Then blood samples were taken from five different patients. The chitosan of 0.06g were put into each test tubes containing the blood and were centrifuged for 5 mins within 10 mins after sampling. The Prothrombin time (PT), Activated partial thromboplastin (APTT) time and also the international normalized ratio was also taken before the chitosan was poured in.

2.4 Activated Partial Prothromboplastin Time (Aptt or Pt)

A test which is medically carried out that characterizes blood coagulation which is also known as clotting is called the APTT test. It is used with heparin to monitor the treatment effect. It is also used in detecting blood clotting abnormalities.

2.4.1 Partial Thromboplastin Time

It is used by two means of conservatives' series of known biomedical reaction which are the common coagulation pathways and the intrinsic which is now referred to as contact activation pathway. It also measures the speed at which blood flows overall.

In determining how quickly blood clotting takes place, it is used in conjunction with another measure which is known as the (PT) prothrombin time. The prothrombin time, with the aid of extrinsic pathway measures the speed of clotting. Tissue factor pathway is also known as extrinsic pathway.

2.5 Method

On an automated instrument at 37^{o}C partial thromboplastin time is analyzed. From the mixture due to the absence of tissue factor, the test is termed “Partial”. In the test tube containing citrate or oxalate, which binds calcium in a sample (they are molecules which act as an anticoagulant) blood is drawn into the test tube. The blood is mixed gently, then to separate plasma from blood cells it is centrifuged (the most commonly used method for blood plasma is Partial thromboplastin test). Placed into a measuring test tube is a sample of the plasma extracted from the test tube. Then mixed into the plasma sample is an excess of calcium. Finally, an activator is added such as (ellagic acid, kaolin, silica, and celite) in order to activate the intrinsic pathway of coagulation.

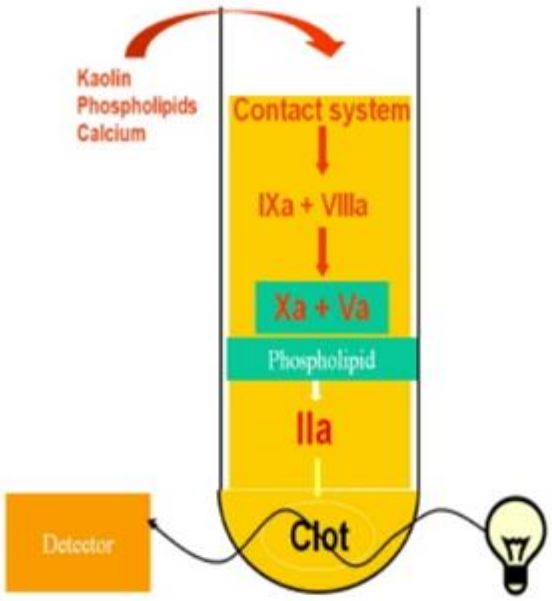

Figure 2.5: Schematic representation of Activated Partial Thrombin Time (Adapted from Perry and Todd, 2013)

CHAPTER THREE
RESULTS AND DISCUSSIONS

In vitro blood compatibility tests of chitosan as a biomaterial were performed using blood clotting test and scanning electronic microscope. Blood compatibility and biodegradability of chitosan were tested in some studies (Lee et al., 1995; Yang and Lin 2002; Lee et al., 2004; Yong et al., 2007).In all the studies, a chemical modification of chitosan were made, this is attributed to the striking characteristic of chitosan as a good biomaterial that allows chemical changes in its structure and molecule.

In this study five (5) samples were taken randomly from healthy individuals and analyzed without addition of chitosan and the result is presented on table 1. In the initial samples, the PT showed a general pattern of coagulation of 12 to 21 seconds, this connotes a significance record of the samples. World health organization recommend standardization of anticoagulants monitoring because different laboratories used indices in expressing result of these tests.

The clotting cascade which comprised of Prothrombin (PT), which evaluate the integrity of extrinsic factors as well as factors common both systems and partial thromboplastin Time (PTT), which determines the integrity of intrinsic factors and the common pathways. Similarly, in all the tests run for the samples without adding chitosan showed similar responds tables 1, 3 and 5. Activated partial Thromboplastin Time (APTT) test a good indicator of monitoring coagulation disorders and to monitor patients taking an anticoagulants drugs also investigated in this study.

Strong positive charge in chitosan play vital role in interaction with other substances. Interestingly, the charges improve the crossing-linking of chitosan with other organic compounds. Fernandes et al. (2011) describe that the activity of blood could be influence by environmental factors and the electrical charges. The authors suggested that intermolecular forces are involved in interaction of blood with any substance which would determine its compatibility. This suggests that the cross-linking of chitosan with blood is determined by the conformity of the charges present and improve the biological activities.

The size of chitosan also play important in binding capacity to substance as evidenced in study conducted by (Tao et al., 2013). This is also evidenced by this study, where blood became compatible with nanoparticles of chitosan in record time and believed to be contributed by the size of chitosan. The interaction of chitosan and its derivatives has received attention of recent, for instance, the formation of an ethylenic double bond in the chitosan–glutaraldehyde interaction (Li et al., 2013).

Table 3.1: Sample taken from five random patients without the addition of chitosan

S/N	Blood sample	PT %	PT sec	APTT	INR
1.	Sample 1	15.7	15.7	37.7	1.30
2.	Sample 2	40.0	21.45	26.0	1.95
3.	Sample 3	107	12.45	24.0	0.96
4.	Sample 4	58.0	17.1	37.6	1.46
5.	Sample 5	129	11.5	28.0	0.87

After Sampling, each sample was observed orderly from the 1st to the 5th according to the timing of 10 mins, 20 mins, 45 mins, and 90 mins. PT, APTT, and INR test was taken from all the samples. Running these test took about 20 mins. STAGO STA compact (Brand name) Machine was used.

Table 3.2: Samples after addition of chitosan

Samples	**Δt (Minutes)**	**PT%**	**PTsec**	**APTT**	**INR**
1.	10	7	86.2	39.8	11.93
	20	5	120.1	96.5	18.37
	45	5	120.1	58.1	18.37
	90	5	120.1	67.2	18.36
2.	10	7	85.9	167.9	11.88
	20	5	120.1	207.2	18.37
	45	5	120.1	300.1	18.37
	90	5	120.1	300.1	18.36
3.	10	18	39.3	300.1	4.30
	20	No plasma	No plasma	No plasma	No plasma
	45	-	-	-	-
	90	-	-	-	-
4.	10	5	120.1	300.1	18.37
	20	10	65.3	71.9	8.32
	45	5	120.1	134.8	18.73
	90	5	120.1	140.2	18.81

The second experiment was carried out by recording the normal values of each blood samples before the test was carried out. The table below shows the value of the blood values.

Table 3.3: Sample taken from five random patients without the addition of chitosan

S/N	Blood sample	PT %	PT sec	APTT	INR
1.	Sample 1	88	13.6	40.6	1.08
2.	Sample 2	37	21.8	49.7	2.12
3.	Sample 3	63	16.2	40.4	1.36
4.	Sample 4	37	22.6	38.0	2.09
5.	Sample 5	91	13.4	37.7	1.06

After Sampling, each sample was observed orderly from the 1st to the 5th according to the timing of 10 mins, 20 mins, 45 mins, and 90 mins. PT, APTT, and INR test was taken from all the samples.

Table 3.4: Second run test with chitosan

Samples	**Δt Minutes**	**PT%**	**PTsec**	**APTT**	**INR**
1.	10	83	14.0	47.9	1.12
	20	72	1.12	51.8	1.19
	45	68	15.5	54.6	1.28
	90	63	16.6	59.1	1.36
2.	10	36	23.3	44.6	2.18
	20	35	23.3	45.9	2.23
	45	31	25.6	46.1	2.46
	90	30	26.2	48.1	2.54
3.	10	59	16.9	37.9	2.28
	20	58	17.0	38.9	2.29
	45	52	18.2	41.1	2.58
	90	51	18.4	43.7	2.47
4.	10	34	24.1	41.6	2.28
	20	34	24.2	44.1	2.29
	45	30	26.2	51.7	2.58
	90	31	25.7	54.4	2.47
5.	10	82	14.1	35.3	1.13
	20	83	14.0	37.1	1.12
	45	74	14.9	37.8	1.12
	90	67	15.7	41.6	1.30

The third experiment was carried out by recording the normal values of each blood samples before the test was carried out. The table below shows the value of the blood values.

Table 3.5: Third run test without chitosan

S/N	Blood sample	PT %	PT sec	APTT	INR
6.	Sample 1	105	12.5	30	0.97
7.	Sample 2	62	16.4	31.9	1.38
8.	Sample 3	105	12.5	23	0.97
9.	Sample 4	88	13.6	31	1.08
10.	Sample 5	87	13.7	32	1.09

Table 3.6: Third run test with chitosan

Samples	**Δt (Minutes)**	**PT%**	**PTsec**	**APTT**	**INR**
1.	10	76	14.6	59.0	1.60
	20	61	16.5	102	1.39
	45	58	17	98.2	1.45
	90	57	17.2	94.5	1.47
2.	10	51	18.4	42.3	1.60
	20	49	18.8	52.3	1.65
	45	48	19	51.5	1.67
	90	44	20.3	59.7	1.82
3.	10	109	12.3	31.0	0.95
	20	97	13.0	34.2	1.02
	45	94	13.2	35.0	1.04
	90	78	14.4	36.3	1.17
4.	10	32	24.9	67.0	2.38
	20	26	29.1	113.8	2.91
	45	23	31.8	75.3	3.26
	90	25	29.9	300.1	3.01
5.	10	98	12.9	37.4	1.09
	20	92	13.3	32.5	1.05
	45	95	13.1	44.9	13.1
	90	83	14.0	44.8	14.0

Studies searching for a compatible biomaterial with living tissues has been a hallmark in bioengineering and biomedical science. In this study, chitosan exhibited a striking ability of dissolving in blood in record time and interestingly no coagulation observed, thus a great advantage in employed in treatment of wounds. Natalya et al. (2002) and Chu (2010) studied blood compatibility of modified chitosan, the results showed ability of the biomaterial to completely dissolve in the blood, and this is in agreement with study where the chitosan became compatible within the blood materials.

Mixture of foreign substances with blood may clot the blood in vitro and may provoke strong immune responds in vivo, thus inflammatory reactions and other immune disorders can occur. Thus, with the results shown in this study, the chitosan will not be considered as a foreign substance by the body immune system, which blood contains many immune cells. Tables 2, 4 and 6, showed results of intrinsic and extrinsic factors, PT and APTT respectively.

Though there are some variability among the different samples which could be attributed individual's blood nature and might involve deficiency of clotting factors like vitamin K. In addition, time play an important role in the compatibility process, in overall, it indicated that increase in time may enhance total dissolving of the chitosan in all the indicators (PT and APTT).

This study significantly found that chitosan can be compatible in living tissue, thus can be employ in bioengineering. The slight increase in both PT and APTT in this work is attributed to time factor in both tests and can play role in its biological activity. Previously, other studies also confirmed biocompatibility of chitosan and its derivatives with tissue. Romani et al. (2013) recognized that the effect of surface materials on erythrocytes aggregation and platelet adhesion and activation as the major parameter in hemocompatibility studies. This is also in agreement with the present studies and other studies (Chen et al., 2009; Xiong et al., 2011; Balan and Verestiuc, 2014). Based on the parameters used to study blood compatibility of chitosan as biomaterial indicated a promising application in bioengineering due its versatility.

Chitosan often not used as single component but mixed with other polymers to enhance compatibility or increase lifetime. Crosslink was often obtained using well-known glutaraldehyde, or m- and p-phthalaldehydes (Rinaudo, 2010). In the study, genepin used as a natural cross-linker by an amide linkage to allow convenient biomedical applications application. In preparation of

chitosan nanoparticles different cross-linkers are used based on their molecular weight and their toxicity. Banerjee et al. (2002) described preparation of chitosan nanoparticles cross-liked using reverse micelles as the media and their physicochemical characterization as well as biodistribution. The authors employed the used of glutaraldehyde and degree of the cross-linking was observed and suggested for a particular degree of cross-linking, the particle size has been determined at infinite dilution of the particle solution. Sodium TPP is considered a good cross link agent for it non toxicity by some researchers (K'deeva et al., 2009; Ponnuraj et al., 2015).

The reaction of cross-linking is to obtain different heterogenous biomolecules for different applications. Owing its nature and structure, chitosan medication by bifunctional cross-linking can be achieved to enhanced activity. The properties of polymers can be modified by using several cross-linking methods (physical and chemical methods). Sionkowska et al. (2014) differentiate between physical and chemical cross-linking, in which physical cross-linking methods, one can use UV radiation, temperature, and gamma radiation and it considered cheap and easy to perform. However, the main disadvantage of this method is the difficulty in controlling the cross-linking process, which means that it is very hard to obtain desirable degrees of cross-linking. In the other hand, Chemical cross-linking methods are based on the chemical reactions which take place after adding the chemical compound as a cross-linking agent to the polymer. Chemicals such as glutaraldehyde or formaldehyde are used as cross-linking agents. But the authors suggested that such chemicals increase the toxicity and make unpleasant for biomaterials for contact with living bodies, hence natural cross-linking agents such as tannic acid are preferred.

CHAPTER FOUR
CONCLUSIONS AND RECOMMENDATION

4.1 Conclusion

Due to its remarkable combination of mechanical, biological and physicochemical properties that highlights its applications extensively, for life science chitosan is a very interesting biomaterial. In different therapeutic systems it can be manufactures and it is readily available: membrane, scaffolds, hydrogels, or nano/microparticles. In biomedical field, chitosan exhibits suitable properties. In blood stream its use is limited, since blood coagulation and surface induced thrombosis is promoted. Strong positive charge in chitosan play vital role in interaction with other substances. Interestingly, the charges improve the crossing-linking of chitosan with other organic compounds.

The reaction of cross-linking is to obtain different heterogenous biomolecules for different applications. Owing its nature and structure, chitosan medication by bifunctional cross-linking can be achieved to enhanced activity. The properties of polymers can be modified by using several cross-linking methods (physical and chemical methods). It showed that the cross-linking of chitosan with blood is determined by the conformity of the charges present and improves the biological activities. The size of chitosan also plays an important role in binding capacity to substance. Chitosan blood interaction have been studied over the years and several strategies are proposed to modulate the interactions.

The result of this work showed that when chitosan was mixed with blood, there was no sign or form of blood clotting. With the result, there was biocompatibility. The result showed that chitosan can be used as a safe biomaterial. Some of these proposed strategies are the association of hemocompatible compounds with chitosan while some are based on the chemical modifications of biopolymer.

So far there has been a lot of progress and researches pertaining higher hemocompatibility and a great number of chitosan have been developed and also in a rapid state. Nevertheless when it comes to the contact with blood, chitosan based system behavior is not quite know. Blood interaction of

biomaterial surfaces are becoming more complex as new therapeutic systems are emerging. For successful recognition of hemocompatibility materials (serves as a derivative for chitosan) and chitosan additional studies are required because most of these formulations are still at the lower level (Laboratory level).

4.2 Recommendations

Series of blood compatibility test was carried out in these studies that evaluated phenomenas such as hematology and thrombosis. Working with chitosan and blood (hematology) which could lead to a giant breakthrough in this research; the world of microparticles with excellent numerous biomaterials will help gain more awareness and acceptance of biomedical engineering and as well as supporting needy individuals or individual in need will tend to benefit good and effective health care.

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