Modified Chitosans for Biomedical Applications

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Submitted to the
Institute of Graduate Studies and Research
in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy
in
Chemistry

Eastern Mediterranean University
May 2013
Gazimağusa, North Cyprus

Approval of the Institute of Graduate Studies and Research

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ABSTRACT

The subject of this thesis is the exploration of the suitability of chitosan and some of its derivatives for some chosen biomedical applications. Chitosan-*graft*-poly (N-vinyl imidazole), Chitosan-tripolyphosphate and ascorbyl chitosan were synthesized and characterized for specific biomedical applications in line with their chemical functionalities.

Chitosan-*graft*-poly (N-vinyl imidazole), Chi-*graft*-PNVI, was synthesized by two methods; via an N-protection route and without N-protection to observe the effect of free amine groups on the antibacterial activity. Both chitosan and Chi-*graft*-PNVI samples demonstrated antibacterial activity against both gram-positive *Staphylococcus epidermidis (S. epidermidis)*, and against gram-negative *Escherichia coli* (*E. coli*). Decreased antibacterial activity was measured with increasing grafting percentage of PNVI. The observed trend can be explained by the fraction of free amine groups present in the samples. PNVI grafting onto chitosan proceeds via both OH-bearing carbons and free amine groups which results in a reduced fraction of free amine groups responsible from the antibacterial effect. When the amine group is protected, grafting proceeds mainly from the hydroxyl bearing carbon atoms. Hence, after deprotection there are higher fraction of free amine groups available for antibacterial activity leading to improved antibacterial activity. New combinations of an aminoglycoside antibiotic, gentamicin with the natural aminopolysaccharide chitosan and Chi-*graft*-PNVI were investigated as antibacterial agents. It was found that a mixture of gentamicin and Chi-*graft*-PNVI exhibited a higher antibacterial activity against both *S. epidermidis* and

against *E. coli* when compared to gentamicin alone. Gentamicin produces an inhibition zone of 8.2±0.2 mm against *S. epidermidis* when undiluted, while the inhibition zone increases to 25.8±0.7 mm in combination with Chi-*graft*-PNVI prepared via N-protection. These combinations have a potential to form a basis to new formulations of gentamicin with improved antibacterial activity and might allow usage of decreased doses of the antibiotic.

Blood contact properties of chitosan and its derivatives were investigated in detail. Chitosan-TPP bioadsorbents and ascorbyl chitosans were prepared. Nonimprinted and Fe^{3+} imprinted chitosan TPP gel beads were prepared via physical gel formation. A method based on *in-situ* crosslinking using ethylene glycol diglycidyl ether, EGDE, was developed to imprint the chitosan-TPP gels with Fe^{3+} without deteriorating the gel beads. The Fe^{3+} imprinted and *in-situ* crosslinked beads proved to be durable and effective adsorbents for Fe^{3+} in solution. The characteristics of the beads as biomedical iron adsorbents were tested by evaluating their serum iron removal capacities from human blood. The preliminary tests carried out showed that Fe^{3+} imprinted beads were more effective in decreasing serum iron in human blood, when compared to the nonimprinted beads. The decrease in serum iron level accompanied a parallel decrease in the haemoglobin level. The calcium level was also affected upon contact with the beads. The Fe^{3+} imprinted beads were less effective than the nonimprinted ones in decreasing the calcium level indicating selectivity towards iron containing species. Blood contact properties of ascorbyl chitosans were investigated by testing for changes in total cholesterol, HDL cholesterol, LDL cholesterol and triglyceride levels in addition to total calcium analysis, complete blood count analysis and prothrombin time determination upon contact in human blood with ascorbyl chitosans. The lipid lowering activity

increased with ascorbyl substitution. The inherent non specific adsorption capability of chitosan due to its chelating power with several different functional groups was exhibited by ascorbyl chitosans as well. This behaviour was exemplified in a simultaneous decrease in the total iron values of the volunteers together with lower lipid levels. Furthermore, ascorbyl chitosans were observed to have less hemocompatibility but increased anticoagulant activity when compared to chitosan alone. Ascorbyl chitosans performed better blood contact properties as biomaterials compared to ascorbic acid alone but poorer when compared to chitosan alone.

In summary, the suitability of chitosan-based materials was examined in different biomedical domains. Our results highlight the importance of this particular polymer in biotechnology and biomedical applications.

Keywords: Chitosan, N-vinyl imidazole, antibacterial activity, tripolyphosphate, Fe^{3+} adsorption, imprinting, blood contact chitosan, cholesterol lowering activity, vitamin C (ascorbic acid), biomedical applications.

ÖZ

Bu tezde kitosan ve türevlerinin biyomedikal uygulamalarda uygulanabilirliği incelendi. Kitosan-*aşı*-poli (N-vinil imidazol), kitosan-tripolifosfat ve askorbil kitosanlar sentezlenip kimyasal işlevleri doğrultusunda özel biyomedikal uygulamalarda kullanımı ile ilgili olarak karaterize edilmeye çalışıldı.

Kitosan-*aşı*-poli (N-vinil imidazol), serbest aminlerin antibakteriyel aktiviteye etkisini anlamak için serbest amin gruplarını korumalı ve korumasız olmak üzere iki farklı metodla sentezlendi. Hem kitosanın hem de amin korumalı olarak sentezlenen kitosan- *aşı*-poli(N-vinil imidazol) örnekleri gram-pozitif *Stapilokokkus epidermidis (S. epidermidis)*, ve gram-negatif *Escherichia coli* (*E. coli*) ye karşın antibakteriyal etki gösterdiği saptandı. Aşılanan PNVI yüzdesi arttıkça antibakterial aktivitede azalma olduğu gözlendi. Gözlenen bu eğilim örneklerdeki mevcut serbest amin grupları ile açıklanabilir. PNVI nin kitosana aşılanması antibakterial aktivite için gerekli fonksiyonel gruplardan OH taşıyan karbon atomu ve serbest amin grupları aracılığı ile gerçekleşir, bu durum antibakterial aktiviteyi engeller. Ancak amin grubunun korumalı olması halinde aşılama, kitosanın özellikle OH taşıyan karbon atomu aracılığı ile gerçekleşir. Aşılama sonrası koruma bozulduğunda ortaya çıkan serbest amin gruplarının antibakterial aktivitede etkin rol aldığı anlaşıldı. Aminoglikozid antibitotiklerinden biri olan gentamisin ile doğal aminopolisakkaritlerden biri olan kitosan ve kitosan türevi, kitosan-*aşı*-poli (N-vinil imidazol), kombinasyonlarının antibakteriyel etkileri incelendi. Gentamisinin amin korumalı metodla sentezlenen kitosan-*aşı*-poli (N-vinil imidazol) ile karışımının, gentamisinin yalnız başına *S.*

epidermidis ve *E. coli*' ye karşı gösterdiği antibakteriyal aktiviteden daha etkin antibakteriyal etkisi olduğu bulundu. *S. epidermidise karşın g*entamisin yalnız başına 8.2±0.2 mm inhibasyon zonu oluştururken, gentamisinin amin korumalı metodla sentezlenen kitosan-*aşı*-poli (N-vinil imidazol) ile karışımının inhibaston zonunu 25.8±0.7 mm ye artırdığı ölçüldü. Böyle kombinasyonların iyileştirilmiş antibakteriyal aktivite ve düşük dozda gentamisin kullanımını sağayacak potansiyel gentamisin türevleri olarak antibiyotik kullanımında aday olabilecekleri düşünüldü.

Kitosan ve kitosan içerikli materyallerin kanla etkileşim özellikleri detaylı olarak incelenmeye çalışıldı. Bu amaçla kitosan-tripolifosfat (TPP) jel boncuklar ve askobil kitosan hazırlandı. Öncelikle, Fe^{3+} baskılanmış ve baskılanmamış kitosan-TPP jel boncuklar oluşturuldu. Baskılama sonucunda yapıda bozunmaya yol açmayan etilen glikol diglisidil eter, EGDE, çapraz baglayıcısı kullanılarak in-situ çapraz bağlama ile yeni bir Fe^{3+} baskılama yöntemi geliştirildi. Fe^{3+} baskılanmış in-situ çapraz bağlanmış jel boncuklar hem daha kararlı hem de Fe^{3+} adsorpsiyonunda daha etkin olduğu anlaşıldı. Sentezlenen jel boncukların potansiyel biyomedikal demir adsorbanı olarak kullanılabilirliği ile ilgili özellikleri insan kanından serum demiri giderme kapasitesini değerlendirmek ile test edildi.Yapılan ön çalışmalar Fe^{3+} baskılanmış kitosan-TPP jel boncukların baskılanmamış kitosan-TPP jel boncuklara göre insan kanında serum demirini azaltmada daha etkili olduğu bulundu. Serum demiri düzeyindeki azalma hemoglobin düzeyindeki azalma ile paralel olduğu tespit edildi. Fe^{3+} baskılanmış kitosan-TPP jel boncukların baskılanmamış kitosan-TPP jel boncukların insan kanı ile etkileşiminin kalsiyum düzeyinde de değişmeye neden olduğu saptandı. Fe^{3+} baskılanmış kitosan-TPP jel boncuklar kalsiyum düzeyini azaltmada baskılanmamış kitosan-TPP jel boncuklara kıyasla daha az etkili olmuştur. Kanla etkileştirilen askorbil kitosanların,

toplam kolesterol, HDL kolesterol, LDL kolesterol, trigliserit ve toplam kalsiyum düzeyine etkisi incelendi. Tam kan sayımı analizi ile ve protrombin zamanına etkisi ile kanla uyumluluğu incelendi. Lipid seviyesini düşürme etkisi artan askorbil grubu ile daha fazla olduğu bulundu. Farklı birkaç fonksiyonel grubun varlığından kaynaklanan non spesifik adsorpsiyon özelliği ile kitosanın şelasyon kapasitesi askorbil kitosan örneklerinde de korundu. Bu davranış, lipid düzeyleri ile birlikte toplam demir seviyelerindede eşzamanlı olarak azalma ile örneklendirilmiştir. Ayrıca askorbil kitosanların kanla uyumluluğunun kitosana kıyasla daha az olduğu ve antikoagülan aktivitenin kitosana kıyasla askorbil kitosan ile arttığı görüldü. Askorbik asite kıyasla askorbil kitosanların kanla uyumluluğunun iyileştirildiği bulundu.

Özetle, farklı biyomedikal alanlarda kitosan-esaslı malzemelerin uygunluğu incelendi. Sonuçlarımızda biyoteknoloji ve biyomedikal uygulamalarda bu özel polimerin önemi vurgulandı.

Anahtar Kelimeler: Kitosan, N-vinil imidazol, antibakteriyal aktivite, tripolifosfat, Fe^{3+} adsorpsiyonu, baskılama, kitosan-kan etkileşimi, kolesterol düşürme aktivitesi, vitamin C, biyomedikal uygulamalar.

This thesis is dedicated to the memory of my dear father, Ismail N. YALINCA

&

to my dear friend, Albert K. HANSEN.

ACKNOWLEDGMENTS

I wish to thank everyone who made my PhD education possible.

I would like to thank my supervisors Prof. Dr. Elvan Yılmaz and Assoc. Prof. Dr. Bahar Taneri for their valuable guidance, critical discussions, and continued advice throughout this study. Without their support, this thesis would not have been completed.

I am very grateful to Prof.Dr. Nahide Gökçora, Prof.Dr.Osman Yılmaz and all my teachers for their kind support.

I am thankful to Eastern Mediterranean University for funding the project (BAP 0808), as it gave me the opportunity to spend my summer in Canada in a well-developed laboratory. I would like to thank Prof. Dr. Hasan Uludağ at University of Alberta for giving me the opportunity for laboratory experience. Interaction and working with his group members have been a great learning experience for me.

I am especially thankful to my dear friend, Fatma Topal Bullici for allowing me to use her Bio Lab and for guiding me with biochemical techniques throughout this study, for her collaborating, assisting, and providing me a pleasant atmosphere to work in.

I would like to thank Assoc. Prof. Dr. Dizem Arifler and Assoc. Prof. Dr. Şükrü Tüzmen for their constructive comments and suggestions for the improvement of this thesis.

I would like to thank senior physics teacher Mr. Altay İstillozlu, Dr. Kıvanç Yüney, Evrim Dalyan Eberdes and Mr. Hasan Arslan for their valuable help to complete my thesis.

Special thanks to my dear friends, Dr. Guilin Wang, Dr. Vanessa Incani, Dr. Betsabeh Khoramian, Hars Deep Sign, Çilem Aydıntan, Tayyibeh Tahamtan, Anıl Kılıç, Maryam Pakseresht, Mr. Sezgin Aydin, Amir Hossein Seyyedi and Dr. Hasan Oylum for their support.

I am very much indebted to Prof. Dr. Mehmet Altınay for his support and encouragement in my education. I am grateful to Mr. Albert K. Hansen who has guided me throughout my education life. Without his support, this thesis would not have been completed.

I would like to acknowledge all the blood donors for their blood used in our study. I would like to convey my appreciation to my family for their patience and love throughout the duration of the thesis.

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LIST OF ABBREVIATIONS

BP	Baird Parker
C-13 NMR	Carbon-13 Nuclear Magnetic Resonance (or ^{13}C NMR)
cm	Centimeter
CAN	Cerium (IV) Ammonium Nitrate
COS	Columbia Agar
CBC	Complete Blood Count
L1	Deferiprone (Ferriprox®)
˚C	Degrees Celcius
DD	Degre of Deacetylation
DNA	Deoxyribonucleicacid
DFO	Desferrioxamine, (Desferal®)
DSC	Differential Scanning Calorimetry
EMB	Eosin Methylene Blue
E. coli	Escherichia coli
EGDE	Ethylenediglycidylether
FT-IR	Fourier Transform Infrared Spectroscopy
HGB	Haemoglobin
HCT	Hematocrit
HDL	High Density Lipoprotein
H_2O_2	Hydrogen Peroxide
HEMA	Hydroxyethylenemetaacrylate

LDL	Low Density Lipoprotein
MSA	Mannitol Salt Agar
MCV	Mean Corpuscular Volume
mRNA	Messenger Ribonucleicacid
MAAP	Methacrylamidoantipyrine
mM	Milli Molar Concentration
M	Molar Concentration
$((M_1w))^-$	Molecular weight
NAG	N-Acetyl Glucosamine
NAM	N-Acetyl Muramic Acid
nm	Nanometer
DMF	N,N-dimethylformamide
GA	N- Glucosamine
PLT	Platelets
PAMAM	Polyamidoamine
PE	Polyethylene
PEI	Polyethylenimine
PHMB	Polyhexamethylene Biguanide
PLL	Polylysine
PMMA	Polymethylmethacrylate
PNVI	Poly (N-vinyl imidazole)
PP	Polypropylene
PS	Polystyrene
PTFE	Polytetraflouoethylene

PU	Polyurethane
PVC	Polyvinylchloride
KBr	Potassium Bromide
PT	Prothrombin Time
RBC	Red Blood Cell
RNA	Ribonucleicacid
rRNA	Ribosomal Ribonucleicacid
rpm	Round Per Minute
SEM	Scanning Electron Microscopy
S. epidermidis	Staphylococcus epidermidis
Θ	Theta
TNBS	2, 4, 6-Tri Nitro-Benzene Sulfonicacid
TPP	Tripolyphosphate
v/v	Volume To Volume Ratio
w/w	Weight To Weight Ratio
WBC	White Blood Cell
WHO	World Health Organization
XRD	X-ray Diffraction

Chapter 1

INTRODUCTION

Advantageous characteristics of chitosan and its derivatives have been reported in many studies. However, it should be noted that the interactions between living cells and chitosan and/or chitosan-based materials are still under study. Hence, it is of utmost importance to elucidate the biological responses and the biochemical changes induced by the chitosan-based biomaterial along with the prime effect under investigation. The main aim of this work is to investigate the biochemical properties and physiological activity of chitosan and chitosan-based materials in two main biomedical domains. Antibacterial actions against gram-positive bacteria and gram-negative bacteria were studied. Also, blood contact properties of chitosan and of chitosan-based materials, namely iron adsorption, lipid lowering activities, together with their influence on blood components were explored *in-vitro*. A broad investigation of chitosan and some of its derivatives as efficient biomaterials was carried out.

The thesis is arranged in 5 chapters. Chapter 1 presents a brief introduction. The remaining chapters of the thesis are organized as follows: Chapter 2 includes literature review to the concept of polymeric biomaterials and their current applications. In addition to this an overview is given for the composition, the basic chemical and physical characteristics of chitosan and its biological properties with applications of chitosan-based materials in biomedical and pharmaceutical field with special emphasis

on the literature related to the specific applications subject of this thesis. Chapter 3 describes details of the experimental set up used in this thesis. The formulations, the chemical characterizations and biochemical analysis are demonstrated in detail. The antibacterial activity of chitosan-*graft*-PNVI in combination with gentamicin is focused in Chapter 4 section 1. Chapter 4 section 2 addresses the issues involved with the blood interactions of chitosan and its derivatives such as Chitosan-TPP bioadsorbents and ascorbyl chitosans. Finally, Chapter 5 summarizes the results obtained in this work, and includes both conclusions and future perspectives.

Chapter 2

LITERATURE SURVEY

2.1 Polymers as Biomaterials

Today, natural polymers as well as synthetic polymers are used as constituents for biomaterials [1-6]. Their exceptional chemistry, physical and biological functionalities, easy to use properties in bioengineering allow for special functions in a wide variety of biomedical fields. Utilizing the tools of chemistry, polymers with different material requirements are designed and are used as biomaterials in implants (cardiovascular, ophthalmic, dental, orthopaedic), drug delivery, gene delivery, and scaffolds for tissue engineering [6-7].

2.1.1 Non-degradable Polymers as Biomaterials

Due to their relative biological inertness to biodegradation, in comparison to carbohydrates and biological polymers, synthetic polymers are used in biological applications when biomaterial is required to stay within the body without any deformation [2]. The coating of a pacemaker or a replacement lens in the eye are some of the examples. Non-degradable polymers can be used as hoses for heart-lung machines. Poly (methyl metacrylate) (PMMA), polyethylene (PE), polyurethanes (PU), polypropene (PP), polytetraflouoethylene (PTFE) and silicone belong to the class of

non-degradable polymers. Today, PMMA and silicone are basic materials of ocular lenses. The main materials for vascular grafts are PU and Teflon [2, 8-9].

2.1.2 Degradable Polymers as Biomaterials

Degradable polymers are mostly preferred as biomaterials when a second operation for removal needs to be avoided. The general pivotal characteristics of biodegradable biomaterials can be explained as below:

- The degradation products should be safe, and capable of being metabolized; no harmful degraded product should be formed and should remain in body.
- The material should have suitable properties for its application. The alteration in its properties upon degradation should be compatible with the curing [2, 10].

Polyesters like polyglycolides, polylactides, poly (lactide-co-glycolides), polydioxannone, polycaprolactone, poly (trimethylene carbonate) belong to a class of biodegradable polymers which are widely used as biomaterials. Hydrolytically degradable polymers used as biomaterials include polyurethanes, polyester amide, poly ortho esters, polyanhydrides, and polyphosphazenes polyphosphoesters. In the group of enzymatically degradable polymers used as biomaterials, proteins and polyamino acids like collagen, albumin, fibrin, elastin as well as peptides are found. Human and non-human origin polysaccharides are also enzymatically degradable polymers as biomaterials. Human origin polysaccharides, hyaluronic acid and chondroitin sulfate, have been investigated especially in tissue repair and wound healing. Chitosan and alginic acid are extensively investigated polysaccharides of non-human origin used as wound dressings, drug delivery vehicles, cell delivery vehicles [2, 10-11].

2.2 Physical and Chemical Requirements of Polymers as Biomaterials

Specific applications require the biomaterial to possess specific properties in order to be suitable for use. One application differs from another, rising different needs. Those needs could be completely contradictory. For instance, when repairing or replacing part of the tissue, the polymeric material used for building the scaffold should be able to decompose at the same time as the body's own cells propagate its own extracellular matrix and will gradually be replaced by the biodegradable tissue. Biomaterials are designed in such a way that complications associated with the host response are eliminated or decreased. Biocompatibility, sterilizability, sufficient mechanical strength, adequate physical properties, and manufacturability are qualities needed for polymeric materials like other biomaterials as shown in Table 1.

Table 1. Requirements for polymers as biomaterials adopted from [4].

Property	Advantages
Biocompatibility	Nontoxic, nonpyrogenic, noncarcinogenic, noninflammatory, blood compatible, nonallergic response.
Sterilizability	Gamma radiation, gas, dry heat and steam autoclaving.
Functionality	Elasticity, strength, durability.
Manufacturability	Machinable, moldable, extrudable.

1. Biocompatibility: Biocompatibility is a primary requirement of biomaterial for a wide range of biomedical applications [3-7, 12]. The material should not interfere with the system or cause undesired reactions, but instead it should contribute to the well being of a patient and progress of an implant. It is, however, foreseen that inflammation may occur in the beginning which is not always bad, but in some cases an inflammation is needed for the healing process. It should be mentioned, that a protractedly period of

inflammation could be the sign of dying cells or a rejection of an implant suggesting the necessity of an intervention. Design of biomaterials has the goal of reducing or, ideally, eliminating the complications associated with the host response. A material's biocompatibility is very much influenced by the molecular weight, its solubility, the composition and the particular form of the implant. Also, its hydrophobicity and its hydrophilicity, the water absorption capacity, the degradation and the surface characteristics affect material's biocompatibility [1-4].

2. Sterilizability: The material must be able to sustain a proper sterilization such as gamma irradiation, gas and steam autoclaving. When using high-powered gamma radiation, certain polymers will emit the gas formaldehyde due to the depolymerization. For the same reason polymers with these properties should be sterilized by ethyleneoxide gas [4].

3. Functionality: The functionality of an implant or other biomedical devices are subject to their capability for being designed and molded into the wanted shape, in order to optimize the human well-being and overall system performance [1, 6].

4. Manufacturability: It is not difficult to find biocompatible materials, but it is difficult to find usable biocompatible materials suitable for production. For that reason, production of medical devices accelerates slowly [4].

2.3 Challenges of Polymers as Biomaterials

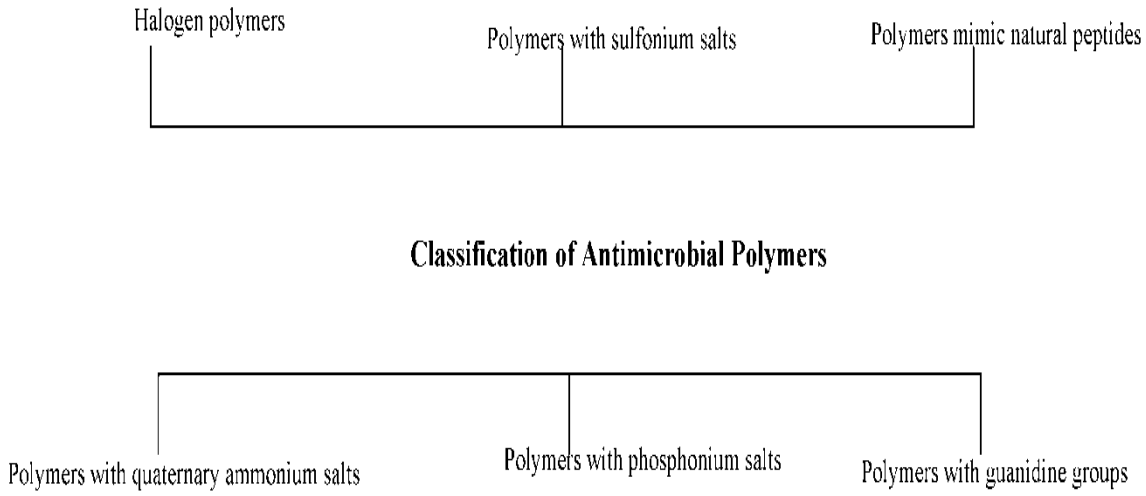
In all biomedical applications, it is critical to minimize any unacceptable and undesired biological interactions with the biomaterial. Still, there are in front of us enormous crucial challenges for the development of biomaterials to be used in the future [13]. One of the challenges is the creation of the futuristic materials that will admit to

noninvasive diagnostic methods as for instance delivery of complexes of protein-chemical drugs. Another provocative major challenge is the design of non viral gene delivery systems which are not only safe but also capable of delivering the genes into applicable cells. Undesired adhesions (tissues adhering together creating blockage) and restenosis of an artery that has previously been physically opened on patients suffering from cardiovascular disease, are common problems caused by biomaterial in biomedical applications. Another challenge is to create a platform for constructing complex tissue structures. Lastly, development of new techniques and procedures for studying the biomaterials for their surface properties and connections with proteins and deoxyribonucleicacid (DNA) is highly needed [1-2, 5-6, 10-12].

2.4 Selected Current Applications of Polymers as Biomaterials

2.4.1 Antibacterial Agents

Antimicrobial agents assist to diminish, fight or stop infections caused by pathogens. The interest in using polymers that possess antimicrobial properties has grown due to their intrinsic character. They have been widely and successfully used in many cases. The antimicrobial polymers are classified in Scheme 1 according to their chemical structure based on [14-16].



Scheme 1. Classification of antimicrobial polymers.

The first polymer studied as an antibacterial agent by Gilbert *et al.* was the polyhexamethylenebiguanide chloride (PHMB). PHMB connection with the cell envelope of *E. coli* was intended to be: (1) a very swift approach of the cell surface , which have a negative charge, toward PHMB, with a firm and specific adsorption to phosphorus-containing compounds; (2) Due to the damage on the outer membrane, the PHMB engages with the inner membrane; (3) When PHMB is bound to phospholipids, the permeability of the inner membrane will increase resulting in loss of K+ and bacteriostasis; (4) Bactericiodal effect occurs when all functions of the membrane are lost and the intracellular fluids are precipitated [15].

Antimicrobial polymers and disinfectants are mostly particularly positively charged; while the greatest part of the bacterial cell walls are negatively charged due to the cell fluid which mostly consists of phosphatidylethanolamine. Quaternary aromatic or heterocyclic charged polymers that bear nitrogen atoms, polysiloxanes, hyperbranched or dendritic polymers, oxazolines, polymers with quaternary nitrogen atoms within the main chains are reported in the subclass of quaternary nitrogen

including polymers having the antimicrobial action. Moreover, guanidine including cationic polymers, polymers including free halogens, polymers including phosphonium and sulfo derivatives, phenol and benzoic acid derived polymers and organometallic polymers have potential being used as antimicrobial agents [14-15].

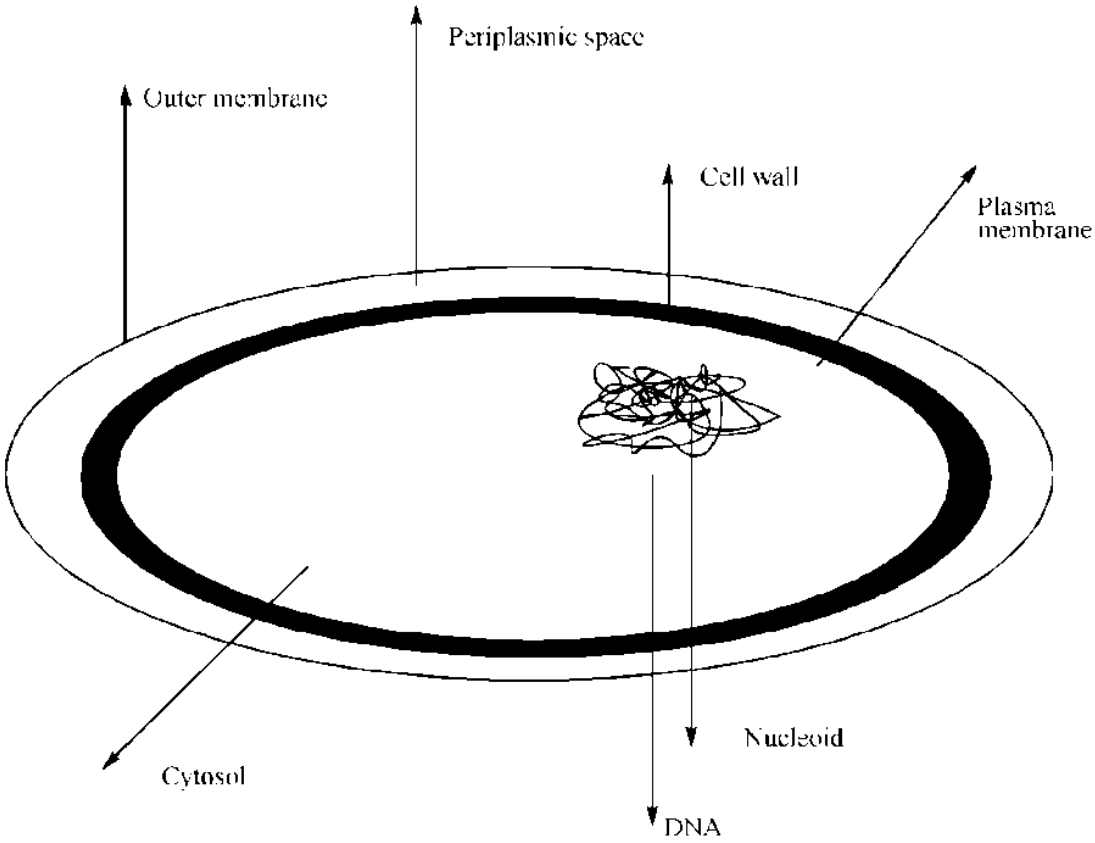
Polymers bearing a positive charge on the quaternary ammonium–phosphonium have been shown to have antimicrobial properties in solution and on surfaces. They hypothesized that bacteria are killed because the outer cell wall and cytoplasmic membrane are damaged and subsequent cell lysis occurs. When cyclic halogen containing polyamines are used for targeted administration, the oxidative halogen targeted receptor proteins (thiol and amino groups) are affected and when in contact with a cell, the cell will be inhibited. Hydrophilicity, hydrophobicity or the molecular weight are all influencing characteristics of the polymer determining the antimicrobial action [14-16].

The majority of polymers that kill or inhibit the growth of microbes, are composed of cationic hydrophilic/hyrophobic molecules targeting a site on the plasma membrane [14-15]. There are positively charged hydrophilic polar polymers and non-polar polymers with hydrophobic functional groups or random copolymers which are made up of both hydrophobic and hydrophilic functional group. These structures enhanced surface-activity characteristics that provide higher interaction (adsorption or absorption) and improved binding affinity for negatively charged bacteria cell in order to cause effective damage of structural composition and integrity of cell membrane, followed by cell membrane disruption, leakage of cytoplasmic contents, and cell lysis [14-16].

2.4.1.1 The Bacterial Cellular Components, Biological Processes

Bacteria are small (0.1 to 10 μm) single-celled prokaryotic microorganisms. They can be found in soil, water, organic matter, or inside and outside the bodies of plants, animals and humans except in the blood and spinal fluid [17]. The general structure of a bacterial cell is illustrated in Scheme 2. The cell wall surrounding the bacterium acts to protect the cell mechanically and chemically from its environment. The cell wall is made of a unique interwoven polymer called peptidoglycan, which makes the cell wall rigid. Just inside the cell wall there is the cytoplasmic membrane, surrounding the cytoplasm. The cell does not include mitochondria, lysosomes, endoplasmic reticulum, or other organelles. Bacterial cells are prokaryotic, which means that they do not contain nucleus, but only ribosomes and a single, double-stranded DNA chromosome. Some bacteria have a circular DNA called plasmid. Although they do not have nucleus, they contain all the chemical elements of nucleic acid and protein synthesis. The nutritional requirements vary greatly and most bacteria are free-living, if an appropriate energy source is available [17-20].

Bacteria can be classified as gram-positive and gram-negative by the differences in their outer cell layers. In the structure of gram-positive bacteria (in Scheme 3) the cell wall surrounding their plasma membrane is thick (25 nm) and includes teichoic acids, whereas gram-negative bacteria (in Scheme 4) have a thin (3 nm) cell wall and a second outer phospholipid membrane that function as an additional permeability barrier [17-18].



Scheme 2. Bacterial cell structure based on [17, 19].

The bacterial cell wall protects the bacteria and prevents it from collapsing when undergoing osmotic lysis. It is made up of a rigid layer of peptidoglycan, a polymer consisting of sugar residues and amino acids. N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) are linked by glycosidic bond to form the peptidoglycan. The peptidoglycan forms a highly crosslinked, hydrophilic network, but is quite open in structure and allows for transport of both charged and uncharged molecules up to 50 kDa. The crosslinking of amino acids is the main strength for the peptidoglycan structure. The cell membrane is semi permeable and composed of a bilayer of phospholipids with hydrophilic, polar head groups and hydrophobic fatty acid chains, giving it amphiphilic and self-associating properties. In general, small, uncharged molecules, *e.g.* ethanol, can pass directly over the membrane, whereas large,

charged molecules are actively transported by membrane-associated proteins. Other proteins, such as receptors and enzymes, are present as well. In contrast with the plasma membrane, the outer membrane's permeability allows the passage of relatively large molecules (molecular weight > 1000 Da) due to porin proteins, which form pores in the lipid bilayer. Between the outer membrane and the cell wall is the periplasm, a space occupied by proteins secreted from the cell [18-21].

Growth of bacterial cell division by binary fission requires three complex metabolic processes: metabolism, which from the nutrient substances present in the environment produces the cell material; regulation and coordination of the hundreds of independent biochemical processes of metabolism result in an orderly and efficient synthesis of cell components and structures in the right proportions leading to an exact replica and cell division, which results in the formation of two independent living units from one [19].

Metabolism of prokaryotic cells is highly complex. The bacterial cell synthesizes itself and generates enough energy for active transport, motility (in some species), and other activities by as many as 2000 chemical reactions. These reactions can be classified according to their function in the metabolic processes of fueling, biosynthesis, polymerization, and assembly. The development of a bacteria population can be explained in four different growth phases: the lag phase, the log phase, the stationary phase, and the death phase. During the lag phase, the cells are actually quite active in adjusting the levels of vital cellular constituents necessary for growth in the new medium. Eventually net growth can be detected, and after a brief period of accelerating growth, the culture enters a phase of constant, maximal growth rate, called the exponential or logarithmic phase of growth. During this phase the generation time is

constant. During this phase, cell number, and total cell mass, and amount of any given component of the cells increase at the same exponential rate. Such growth is called balanced growth, or steady state growth. Constant growth rate requires that there should be no change in the supply of nutrients or the concentration of toxic by-products from the metabolism (such as organic acids). This constancy can exist for only a short time (hours) in an ordinary culture vessel. Then growth becomes progressively limited (decelerating phase) and eventually stops (stationary phase). Cells in the stationary phase are different from those in the exponential phase. There are some important parameters such as temperature, available oxygen, nutrients and inorganic compounds which influence bacterial growth [1-19].

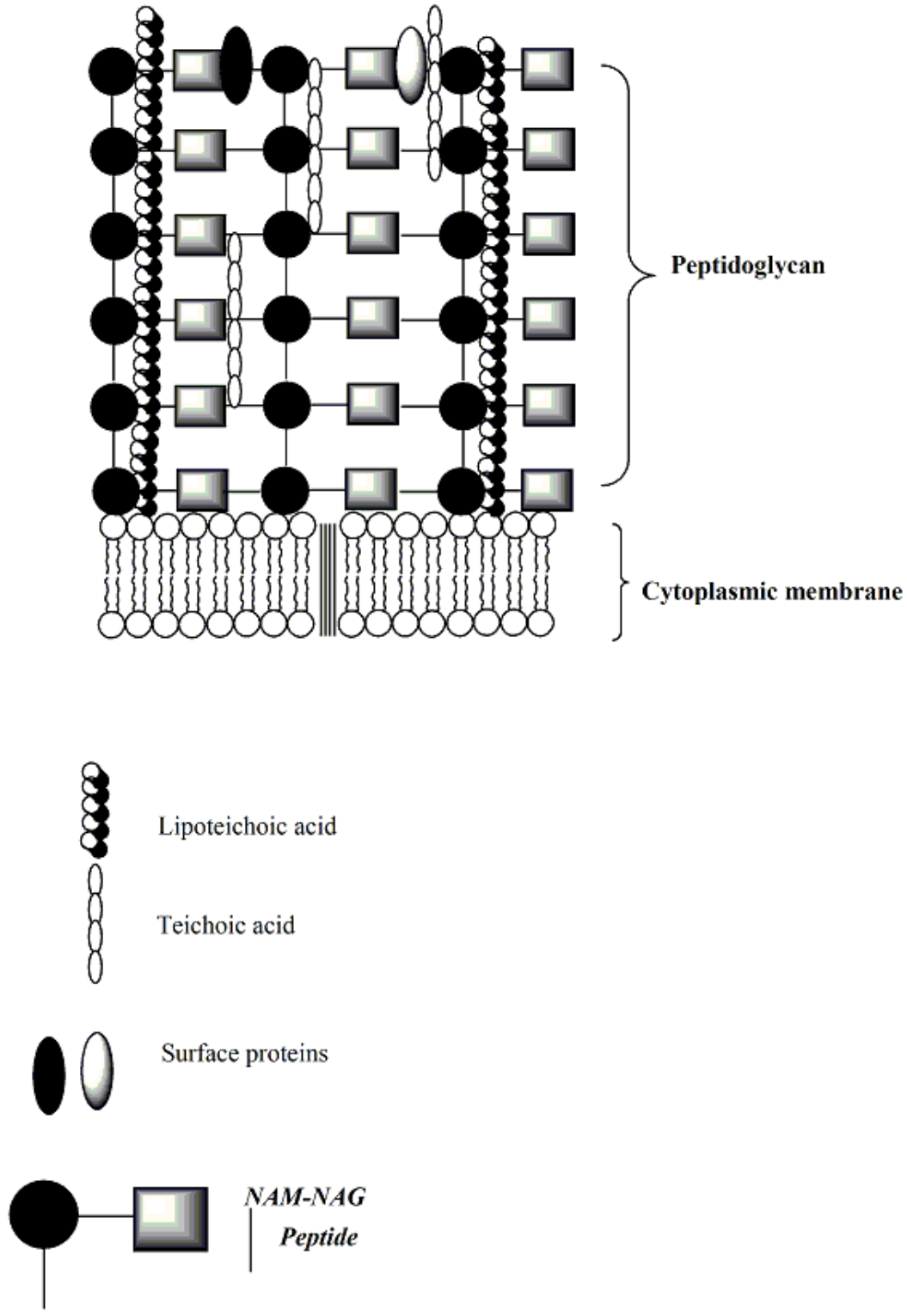
2.4.1.2 The Modes of Antibacterial Agents

The antimicrobial agents can be grouped in three main classes according to the following: (1) the target microorganisms, (2) applications, (3) mechanisms of action [18]. Mechanism of action of an antimicrobial agent could be as follow:

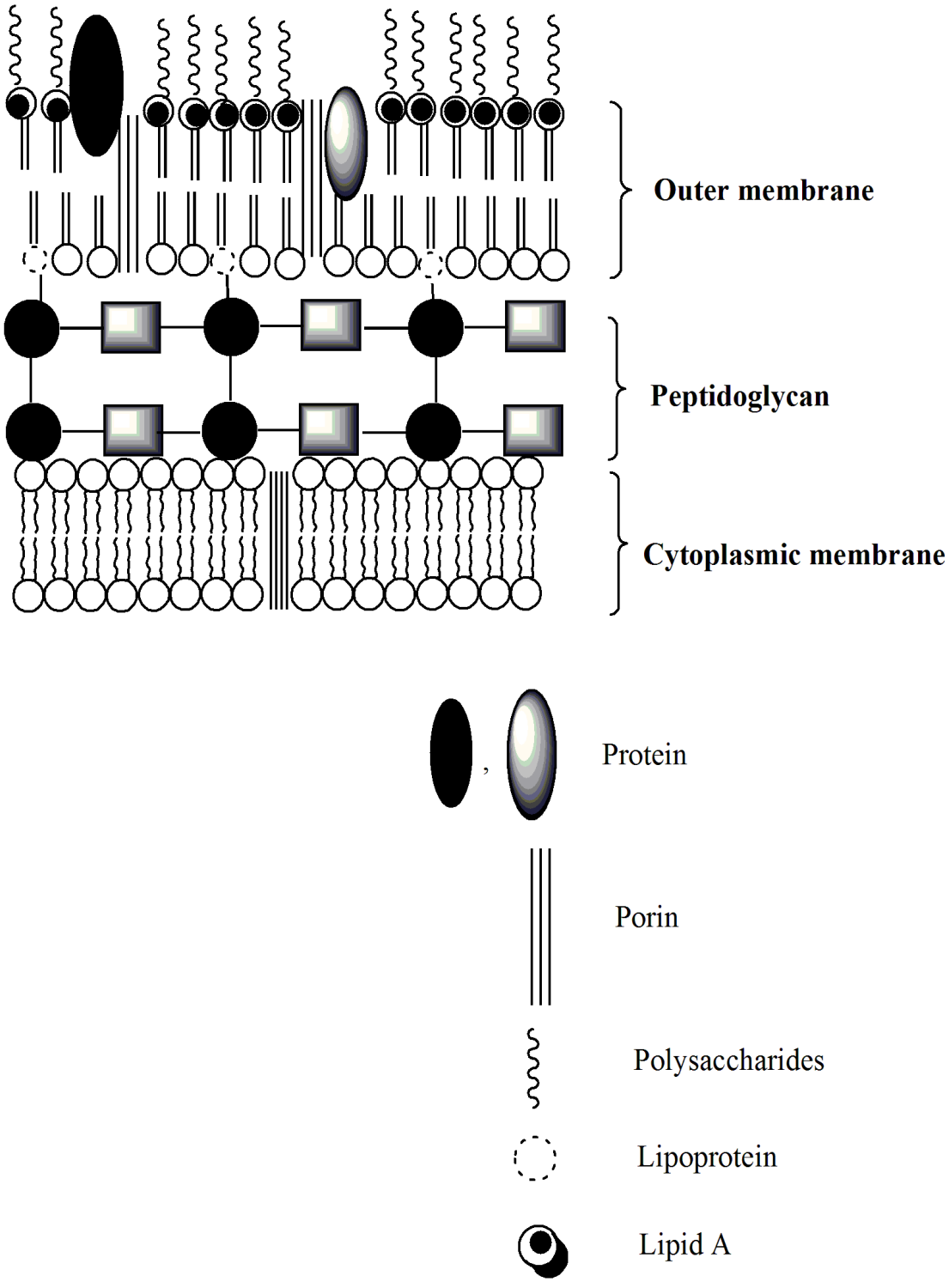
1. Inhibit cell wall synthesis.
2. Interference with membrane integrity.
3. Inhibit nucleic acid synthesis.
4. Inhibit protein synthesis.
5. Inhibition of synthesis of essential small molecules.

For example, phosphonomycin, cycloserine, bacitracin, glycopeptides, lipoglycopeptides, penicillins, cephalosporins, carbapenems, monobactams inhibits the cell wall synthesis. Polymyxins, Lipopeptides and peptide antibiotics interfere with cell membrane integrity. Quinolones, nitroimidazoles and rifamycins are used for inhibiting

nucleic acid synthesis. Aminoglycosides are used as inhibitors of 30S ribosomal subunits. Chloramphenicol, lincomycin and clindamycin, macrolides, ketolides, streptogramins, fusidic acid, oxazolidinones inhibit amide bond formation on bacterial ribosomes by binding to the 50S subunit. Sulfonamides serve as inhibitors of dihydropteroate synthetase which is essential small molecules [18].



Scheme 3. Gram-positive cell wall structure based on [17-21].



Scheme 4. Gram-negative cell wall structure based on [17-21].

2.4.1.3 Antibiotic Resistance

Antibiotics represent one of the greatest advances in modern therapeutic medication. Antibiotics usually act on specific targets within the cell, such as a specific enzyme. Because of their functional selectivity they are very effective but also very sensitive to any changes in the bacterial metabolism. Antibiotic resistance is not a new natural biological phenomenon, but only recently people become aware of the problems [22-24].

Throughout the 1950s and 1960s new types of antimicrobials were developed for in the 1970s and 1980s to be modified in order to be in front of the resistance. Bacterial resistance proved to be a challenge difficult to overcome. Years of use and also misuse of antibiotics have led to mutations in bacterial genes encoding the target protein, which can prevent the antibiotic drugs from inactivating the target protein. Mutations in the baterial DNA may also be the reason for a steady increase in the research and production of antibiotic`s target enzymes, therefore antibiotic may not inactivate all of them. Alternatively, bacteria can produce antibiotic inactivating enzyme or the bacteria may change its cell membrane or cell wall. Therefore, such a change of the protein structure of the bacterial cell wall may cause that the antibiotic effect on the cell wall is negligible. Hence, the bacterial mutations survive better in an antibiotic environment and continue to be a reason for infection and disease. The hospitals are struggling to fight of infections there earlier were easy to cure. After mutations and bacterial resistance, the mutant use in order to remain resistant, different biochemical types of resistance mechanisms such as inactivating antibiotic, target modification and altered permeability [22-24]. The World Health Organization (WHO) Global Strategy defines the appropriate use of antimicrobials being the most cost-effective use if these maximize the clinical

curative healing and at the same time minimizing adversed drug reaction and antimicrobial resistance [24].

Therapeutic difficulties are now caused by strains of certain bacteria such as enterococci and tuberculosis bacteria. They have the ability to acquire resistance to the most useful and possibly to all agents currently in use. Therefore, antimicrobial resistance has become an increasingly vital problem, which has serious implications for prevention and treatment of infectious bacterial diseases. In order to fight evolving patterns of resistance, new drugs are continually being introduced to market. Combining antibacterial agents either to improve the efficacy or hamper the rapid emergence of resistance, an alternative approach to single-antibiotic therapy could be used. Treatment with combinatorial antibiotic combination aims to improve the antimicrobial activity of antibiotics and to reduce dosing regimens to a level, which has the capacity to reduce the rate of attainment of resistance in pathogens. Many patients take multiple antimicrobial drugs simultaneously. This can have diverse effects on bacterial survival. Combinations of antimicrobial drugs not only prevent or minimize the emergence of resistant strains, but also decrease the toxicity of individual drugs. Moreover, combination of antibiotics used to take advantage of the synergistic effect. However, some antibiotic interactions have caused death or severe side reactions such as loss of therapeutic effect of one of combined the drug, toxicity, incompatibility. Side effects are increasing when coadministration of drugs causing similar problems take place [25-26].

In serious conditions prior confirmatory diagnosis and reliable data not yet is available, combinations of antimicrobial therapy are frequently used to provide the best supportive coverage for serious infections. However, the evolution of antibacterial resistance is evolving fast so today in many cases, the outcome of the therapy is not

always respose with a satisfactory response, and even combined antibiotics have been used [26].

2.4.1.4 Aminoglycoside Antibiotic Resistance

Mechanisms of aminoglycoside resistance can be caused by: reduced uptake or decreased cell permeability, alterations at the ribosomal binding sites, or production of aminoglycoside modifying enzymes [18, 24, 27-29]. (Table 2)

Table 2. Mechanisms of aminoglycoside antibiotics resistance based on [18, 24, 27-29].

Mechanisms of Aminoglycoside Antibiotics Resistance
Altered uptake,
Modification of ribosomal proteins and ribosomal ribonucleicacid (rRNA) by mutations,
Modification of antibiotics by enzymes,
rRNA modification by enzymes,
Modification of the antibiotic target.

2.4.1.5 Mechanism of Antibacterial Activity of Chitosan

Chitosan is known to have antibacterial potential due to its cationic polyelectrolyte nature, which provides the ability to bind effectively with negatively charged molecules. Although the exact mechanism of antibacterial action of chitosan is not very well known, it is established that the polycationic nature plays an important role. Hence, the charge density and the number of amine groups determine the antibacterial functionality of chitosan. The degree of electrostatic interaction between polycationic chitosan and the cell surface depends upon the anionic charge density on the bacteria cell surface. The binding of chitosan onto the cell surface may also occur via hydrogen bonding. Antibacterial activity of chitosan closely correlates with the

properties of the bacterial cell such as smoothness, roughness, hydrophobicity or hydrophilicity of cell surface. Antibacterial modes begin with interactions (polymer-cell surface interaction either H-bonding or ionic interactions) at the cell surface and compromise the cell wall. Molecular linkage between chitosan and cell surface leads to disturbance of the functions of the cell membrane. The second proposed mechanism is the binding of chitosan with bacterial nucleic acid. This leads to either the inhibition of protein synthesis or interference of its metabolic processes, contributing to bacterial death. The last proposed mechanism is the metal chelation capability of chitosan which leads to complexation of vital trace elements and blockage of crucial nutrients for bacteria growth [30-32]. Moreover, there are many intrinsic and extrinsic parameters including molecular weight, degree of deacetylation, charge density, water solubility, concentration, temperature, pH and type of microorganisms affects the degree of antibacterial activity of chitosan.

2.4.1.6 Mechanism of Antibacterial Activity of Aminoglycosides

Aminoglycoside group of antimicrobics all contain a six-membered aminocyclitol ring attached to amino sugars. The exact ring structure, number and character of the amino sugar residues differ from agent to agent [18]. Most aminoglycosides are bacteriocidal rather than bacteriostatic against a wide range of bacteria, but only those organisms that are able to transport them into the cell by a mechanism that involves oxidative phosphorylation. Against strict anaerobes or facultative organisms fermentatively metabolizing (e.g., streptococci) they have little or no activity. It appears highly probable that aminoglycoside activity against facultative organisms is similarly reduced *in-vivo* when the oxidation–reduction potential is low.

The aminoglycosides inhibit protein synthesis by binding to the bacterial ribosomes directly or with the help of other proteins. This binding destabilizes the ribosomes, blocks initiation complexes, and thus prevents elongation of polypeptide chains. The agents may also cause distortion of the site of attachment of messenger ribonucleic acid (mRNA), mistranslation of codons, and failure to produce the correct amino acid sequence in proteins. The newer and more active aminoglycosides bind to various sites on both 30S and 50S subunit proteins, and have therefore compared to the first aminoglycoside (streptomycin) which bound to only 30S, a broader spectrum and less susceptibility to resistance due to binding site mutation [18, 25, 29].

Gentamicin and tobramycin have an extended spectrum, which includes staphylococci; enterobacteriaceae; and of particular importance, Pseudomonas *aeruginosa (P. aeruginosa).* Other aminoglycosides as Streptomycin and amikacin are now mostly used in combination with other agents in the therapy of tuberculosis and other mycobacterial diseases. One of the most toxic aminoglycosides is Neomycin. As it is poorly absorbed, it is used in topical preparations and as an oral preparation before certain types of intestinal surgery [18, 20]. All of the aminoglycosides are toxic to the vestibular and auditory branches of the eighth cranial nerve to varying degrees; this damage can lead to complete and irreversible loss of hearing and balance. These agents may also be toxic to the kidneys. For instance, gentamicin includes possible harm to ears and kidneys. It is also known that adverse effects, side effects and toxicity of gentamicin changes from person to person. It is usually dosed by body weight [18, 20, 25, 29].

2.4.2 Fe^{3+} Chelators and Fat Binders

There is limited study about the fat binding and Fe^{3+} chelating properties of polymeric biomaterials. Reports are available on the preparation of Fe^{3+} selective

imprinted polymer adsorbents based on poly (hydroxyethylenemetaacrylate), poly (HEMA), prepared by the classical imprinting technique and tested for analytical purposes [33-34]. The Fe^{3+} imprinted poly(MAAP-EGDMA) beads by complexation of Fe^{3+} ions with methacrylamidoantipyrine (MAAP) was synthesized for the selective removal of Fe^{3+} ions from water sample investigated by Karabork *et al*. [35]. These ion imprinted beads were shown as selective bioadsorbent for Fe^{3+} ions with competitive Cu^{2+}/Fe^{3+}, Zn^{2+}/Fe^{3+}, Co^{2+}/Fe^{3+}, Al^{3+}/Fe^{3+} adsorption studies. They also found these ion imprinted beads are reusable without considerable loss of adsorption capacity [35].

A report by Gore *et al.* [36] investigated role of crosslinkers on molecularly imprinted polymers for both selectivity and adsorption capacity of cholesterol from aqueous solution. Membranes based on cholesterol imprinted methylmethacrylate-co-acrylic acid copolymer for selective removal of cholesterol were prepared by Ciardelli *et al.* [37].

2.4.3 Contact of Biomaterials with Blood

The basic components of blood are different kinds of blood cells and fluids. The blood cells are suspended in a liquid matrix (plasma), which makes the blood liquid [17, 38]. Biomaterial´s compatibility is defined as its ability to perform with a suitable response related to a specific application. Biocompatibility is one of the fundamental characteristics which show the compatibility of material within the hosts, since surface properties of material determine its biocompatibility [39-41]. For instance, the balance of hydrophobicity/hydrophilicity, charge density and protein adsorption capacity are fundamental factors for biocompatibility.

Blood compatibility is often referred to as hemocompatibility and is one aspect of biocompatibility [5, 7, 10, 12, 17, 42]. The blood compatibility is expressed by the

surface the reactivity of biomaterial with blood ingredients as red blood cells (RBCs), white blood cells (WBCs), platelets (PLT) and blood proteins. An indication of how different artificial materials interact with blood constituents is indicated in the changes of plasma coagulation properties on incubation with these materials [41]. Devices in contact with blood as components of blood bags, catheters, cardiovascular implants, large vascular grafts, stents, artificial heart, oxygenators, micro and nanoparticles for drug delivery all contains polymeric materials [13]. When biomaterial gets in contact with blood, there is a fast adsorption of plasma proteins onto its surface. The role of surface of biomaterial and its blood properties (local conditions of flow and blood composition) are significant factors when blood-material interaction is determined. The chemical composition, crystallinity, morphology and surface tension of biomaterials affect protein adsorption and blood clotting pathways in blood contact applications. In vitro, enzyme linked immunosorbent assay (ELISA), cell culture assays and blood compatibility tests are used to provide information about biological interactions between surface of polymers and blood in terms of possible incompatibility. Activated partial thromboplastin time, partial thromboplastin time, platelet number, and leukocyte numbers are biological coagulation parameters to evaluate blood compatibility of biomaterial. Adverse host responses, such as activation of the cascade systems (coagulation, fibrinolytic, etc) and activation of platelets and leukocytes are seen in some cases. These responses cause formation of fibrin or degradation (phagocytosis in combination with liberation of enzymes and free radicals), therefore such systems are not useful for blood contact application [38-41].

Blood coagulation occurs when soluble plasma proteins are activated. The activation is a response to vascular injury and formation of a fibrin clot takes place

arresting blood flow. In the process, thrombin production leads to activation of platelet and its aggregation which leads to a final clot (thrombus). Surface-mediated reactions (intrinsic pathway), or exposure to factors derived from damaged tissue (extrinsic pathway) both can trigger the coagulation. Both process leads to formation of insoluble fibrin gel. Defects in the clotting of blood are related to coagulation disorders. Any deficiencies of the protein factors involved in coagulation can result in haemorrhages after minor injuries [17, 38]. Blood clotting is the one of the main reason leading to failure for application in blood contacting biomaterials. The lack of the biocompatibility of biomaterial is the main problem in its blood contact applications. The resistance of biomaterial towards adsorption of blood components makes it compatible for blood contacting application. The changes in blood components due to their interaction with surface of biomaterial may cause challenges including possibility of clot formation, haemolysis and inflammation. Today, silicone, polyolefin, polyvinylchloride, polytetrafluorethylene and polyesters are indicated as exhibiting thrombogenic and anticoagulant effect, whereas antithrombogenic properties are claimed for polysiloxanes and polyurethanes.

The surface blood compatibility of the polysulfones was investigated by Ishihara K. *et al* [43]. They blended polysulfones with phospholipids. It was found that, this modification improved blood compatibility and decreased protein adsorption compared with that of unmodified polysulfones membrane [43]. Chen J. *et al.* [44] studied the improvement of biocompatibility of titanium as blood contact biomaterial. For this aim, they coated titanium with collagen/heparin. They investigated the adhesion and growth of endothelial progenitor cells on its surface. It was found that collagen/ heparin coating useful for enhancing hemocompatibility and biocompatibility of

Titanium as blood contact biomaterial [44]. In the report of Faxälv L. *et al* [45], blood compatibility of various hydrogels coating on plastic materials (PS) from monomers was evaluated for use in blood contact applications. The obtained hydrogels showed improved resistance to haematological challenges comparison to the PS substrate material without surface treatment.

2.4.4 Other Biomedical Applications

Polymeric biomaterials are used as sutures, blood vessels and other soft tissues, due to their good resilience and easy production characteristics. Although many polymers are easily synthesized and could be used as biomaterials only 10 to 20 polymers are mainly used in medical device fabrications from disposable to long-term implants. Polymeric biomaterials have some drawbacks such as poor strength and easy deformation with time. Some of the more frequently used polymeric materials in biomedical application: PE, PP, PMMA, PU, polystyrene (PS) and its co-polymers, polyesters, polyamides (Nylons), fluoropolymers, rubbers, polyacetal, polysulfone (PSU), and polycarbonate, biodegradable polymers. PVC is used as flexible containers as for blood bag systems and solution bag, packaging for syringes and other medical devices. Biomedical application of PU is used in breast prostheses, bone adhesives, skin dressing, suture material, vascular grafts, blood bags and blood oxygenation tubing. PE is used in orthopaedic implants. PMMA is to be used in implantable ocular lens, bone cement, membrane for blood dialyzer and blood pump and reservoirs. Tissue culture flasks are made up of PS [1-4].

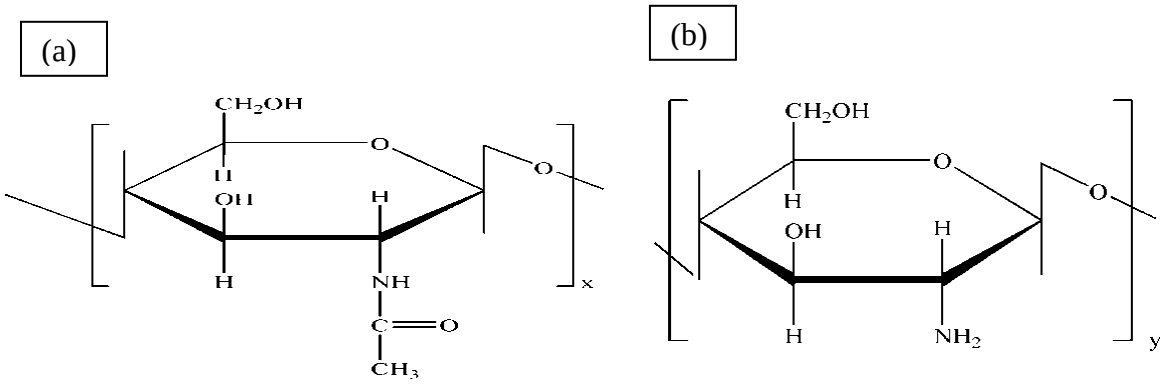
Poly-L-(lysine) (PLL) and its derivatives, polyamidoamine (PAMAM) starburst dendrimers, branched polyethylene imines (PEI), Diethylaminoethyl (DEAE) dextrans and its derivatives, poly(dimethylaminoethyl methacrylates), and polysaccharide-

oligoamine conjugates are among the most studied polymeric carriers to be used for gene delivery [46-53]. Chitosan-based materials were investigated as non-viral gene carriers under supervision of Prof.Dr. Elvan Yilmaz and Assoc.Prof.Dr. Bahar Taneri with collaboration of Prof.Dr. Hasan Uludag. We achieved strong DNA binding with synthesized chitosan derivatives without cytotoxicity. However, poor transfection efficiency was the challenge of the research. Further studies are needed to modify chitosan for enhancing transfection efficiency with minimum cytotoxicity.

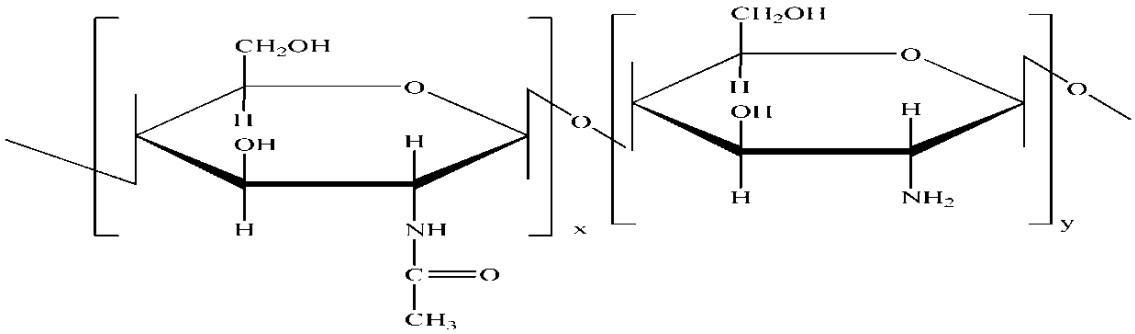
2.5 Chitosan: Composition, Structure and Properties

Chitosan was first discovered by C. Rouget in the 19th century [54]. Chitosan is the collective name for the deacetylated chitins. The properties of chitosan change according to its degree of deacetylation. The ideal chemical structure of chitin is a linear carbohydrate where all units are composed of N-acetyl-glucosamine units (NAG). N-glucosamine (GA) is the ideal chemical structure of chitosan. The ideal fully acetylated form of glucopyranose is chitin and the fully deacteylated form of glucopyranose is chitosan which are shown in Scheme 5 (a) and (b), respectively [54-57].

In reality, these biopolymers do not exist as 100% acetylated chitin or as 100% deacetylated chitosan. They are present as copolymers, as shown in Scheme 6. If the number of acetamido groups (degree of acetylation) is more than 50%, then the biopolymer is defined as chitin. The biopolymer is termed as chitosan when the amino group is predominant [54-55]. Purity, degrees of deacetylation, viscosity, molecular weight solubility, crystallinty and the content of moisture, ash and protein of chitin and of chitosan may change widely, because during the manufacturing process different factors might alter the characteristics of the end-product [54-58].



Scheme 5. Ideal chemical structures of (a) chitin and (b) chitosan [55]. (x: 100% acetylated, y: 100% deacetylated)



Scheme 6. Chemical structures of chitin and chitosan representing the copolymer character of the biopolymer [55]. (chitin copolymer if x>y; chitosan copolymer if y>x)

Chitosan-based materials have attracted a lot of attention due to their versatility, relatively low prices and absence of biological hazards. The physicochemical and biological characteristics are listed in Table 3. The chemical and biological characteristics of chitosan are influenced markedly by the molecular weight, viscosity, degree of deacetylation, crystallinity index, charge density and pKa. The molecular properties of chitosan are strongly influenced by the biological origin of chitin. As chitin is extracted from seashells, the current main commercial sources of chitosan are seashell

materials originating mostly from shrimp and crab shells [54-62]. Chitosan is a semi crystalline polymer. The polymorphism of chitosan highly depends on its degree of deacetylation. The presence of primary amine groups at C-2 position and both primary and secondary hydroxyl groups at the C-3 and C-6 positions confer significant functionality allowing chemical modifications for specific applications. Chitosan is not soluble in water, in alkaline or in organic solvents. Since chitosan has polycationic nature at pH less than 6, establishes ionic bonding with a variety of anionic molecules including peptides, proteins, nucleic acids, genes, fatty acids, anionic polysaccharides, and others dependent upon its degree of deacetylation and molecular weight. Chitosan becomes soluble in an aqueous acidic solution due to the protonation of the free amine groups on the C-2 position of the N-glucosamine repeating units. The amino groups of chitosan confer a highly positive charge density, have pKa values around 6.5 and therefore chitosan behaves as a polycation at acidic and neutral pH. Its solubilization depends highly on the degree of deacetylation and molecular weight. Being a polyelectrolyte, it is able to form gels via electrostatic interactions. The characteristics of polyelectrolyte depend on polymer concentration, pH, temperature, charge density and so on. It is necessary to point out that the degree of deacetylation determines the content of free amine groups in its structure. Chitosan is insoluble in acidic aqueous solution when its degree of deacetylation is below 60%. The presence of alcohol, amine, amide and ether functional groups of chitosan structure cause hydrogen bonds. Interchain association by hydrogen bonding leads to decrease polymer solubility. Formation of hydrogen bonds by means of intermolecular and intramolecular forces play a significant role in chitosan solubility

The remarkable biological properties of chitosan are its biodegradability, biocompatibility and bioresorbability. Bioresobability means ability to be completely eliminated from host through biological pathways. Ability of being decomposed by biological actions refers to biodegradability. The other important biological property of chitosan is its biocompatibility. Additionally, antioxidant, antitumor, haemostatic, analgesic, hypocholesterolemic properties of chitosan has also been reported [42, 58, 61-66].

Table 3. Physicochemical and biological characteristics of chitosan [54, 58, 61-66].

Physicochemical Characteristics	Biological Characteristics
Naturally occurring polysaccharide.	Biodegradable and biocompatible.
Linear polyamine.	Antimicrobial activity.
Reactive amino groups.	Antitumor activity.
Reactive hydroxyl groups.	Antioxidant.
Polyelectrolyte character.	Antigenotoxic.
High to low viscosity.	Ant carcinogenic.
Low to high molecular weight ranges.	Stimulates wound healing.
Cationic polyamine at pH less than 6.5.	Regenerative effect on tissues.
Forms gels with polyanions.	Promote weight loss.
Possesses high affinity to bind metal ions.	Decrease cholesterol.
Chelates with many transitional metals.	Adhesion to cells.
Absorbs fatty acid and lipids.	Haemostatic.

2.6 Applications of Chitosan

The relative low cost of chitosan and its versatility have lead to a substantial progress during the past 45 years. In the mid-eighties waste water treatments, processing of food, and metal ion chelation were the major targets for the applications of chitosan. Today´s applications have turned more into the direction of high valued products, e.g. cosmetics, drug carriers, protein carriers, gene carriers, feed additives, semi-permeable membranes and pharmaceutics [54].

The first studies on chitosan´s chelating capability go back about 35 years [54]. One of the most basic applications of chitosan is for the chelation capacity with many

toxic metal ions including Cu^{2+}, Cd^{3+}, Ag^{+}, Zn^{2+}, Pb^{2+}, Fe^{3+}, Mn^{2+} from waste water [54, 58, 67-69]. Since the presence of free amine groups on chitosan, it has ability to bind to different metallic ions via adsorption, ion exchange, inter/intra molecular electrostatic interactions or chelation phenomena. Chitosan possesses high density of amino groups in its backbone that can interact with molecules having a negative charge [54-60]. A report by Guibal *et al.* [68] investigated that the adsorption of metals, dyes, and organic molecules onto chitosan. Crini *et al.* [69] reviewed removal of anionic dyes via chitosan derivatives.

Various types of membranes and filters made of chitosan have been developed and are now in use all over in the world for purifying water, potable as well as waste water. Muzzarelli *et al.* [70] developed chitosan in the paper industry [54]. Since chitosan is naturally occurring and is a biodegradable polymer, it does not cause pollution. Therefore, it has numerous potential applications in agriculture [54]. In crop care, it is used for seed coating for better germination. Also leaves are coated with chitosan to protect against fungal infections. To protect the environment, fertilizers coated with polymer are widely used together with the coated seeds. When the release into the soil is controlled the nature and properties of soil will be improved [54, 58, 62]. Because of its high binding affinity towards soil, chitosan has been and is still very much used in the food industry. It is also used as food preservative against microbial attack or deterioration, formation of edible films, stabilizing agent in ice cream and juice, act as dietary fiber and as functional food ingredients. For example, it is used to extend the shelf life of foods, due to its antimicrobial property. Moreover, in the case of beverages, it is used to remove dyes, and solids [54, 58, 62].

2.7 Biomedical & Pharmaceutical Applications of Chitosan Derived Materials

Chitosan possesses unique characteristics such as hydrophilicity, biocompatibility, biodegradability, antibacterial properties, antifungal properties and remarkable complexation ability with biological molecules; it is a suitable candidate for biological applications [42, 59-66].

Applications involve biomedical and pharmaceutical applications such as: an excipient in various forms (tablets, hydrogels, sponges, flakes, powders, films, fibers etc.) for a diversity of delivery methods (inhalation, oral, nasal, parenteral, transdermal); membranes for dialyzer for haemodialysis and surgical dressing products, artificial skins, soft contacts, immobilization of cells and enzymes by encapsulation. Other reports on the usage of chitosan and its derivatives in cosmetics consist of nail varnish, moisturisers, fixatives for a variety of purposes and hair care products as conditioners due to absence of allergenic property, cationic nature and gel forming ability of chitosan [54].

Controlled release system provides an adequate way for administering the release of a lower concentration of drugs in the body with the benefit of fewer side effects. Controlled release dosage forms enhance the safety and reliability of drug delivery. Drug delivery and drug administering systems are topics of high interest, and owed to the intense research, we understand better the basics in the chemistry of chitin and chitosan. Their chemical reactions, biotic decomposition, their impact on different tissues, mucoadhesive polymers, chemical bonding of chitosan with inorganic

compounds, are some of main issues of interest. The main reason for the big interest in chitosan is that as a biocompatible polymer [42, 54-66].

Chitosan has also been found to have hypolipidemic effect when it is administered with diet. Uptake of lipids, fatty acids and cholesterol by chitosan can be associated with its cationic nature and amphiphilicity. A variety of hypolipidemic formulations including chitosan were prepared for oral administration. Chitosan has become a useful dietary ingredient because of its hypolipidemic activity [42, 54-55, 58, 62-66, 71-79].

Chitosan exhibits haemostatic effect and is used in forms of chitosan-based sponges and bandages for surgical treatment and wound protection [54, 62]. Anticoagulant membranes for ultra filtration of blood were prepared by immobilization of bioactive molecules such as heparin, hirudin, or antithrombin on chitosan [80-89].

Today the trend is to use natural materials as implantable devices. There are two main reasons: (1) natural materials have been showed to support a faster healing and due to its natural origin, it is anticipated to have a greater compatibility with humans (2) for the purpose of tissue engineering where cells are planted onto the biomaterial implants. Chitin and chitosan have been used in orthopaedic as well as periodontal applications [59, 90]. Chitosan is a very good additive in biomaterials and contribute to versatility in design. For instance, it is possible to use chitosan in creating soft contacts, or to make artificial skins.

An enormous attention in last few years has been directed towards the antibacterial properties of chitosan and its compounds, because of problems related to the synthetic chemical agents and their bacterial resistance. Chitosan exhibited a broad-spectrum antimicrobial activity against gram-positive as well as gram-negative bacteria

and fungus [54]. In section 4.1, we detail the antibacterial properties of chitosan and its derivatives.

Under various physiological pHs, chitosan able to bind cells via electrostatic interactions between negatively charged cell membrane and polycationic chitosan. Chitosan and its derivatives are used as supports for enzymes and cell immobilization mainly by entrapment and membrane confinement. Refinement of cell transplantation techniques for hormone delivery is in under development [42, 54, 62].

Chitosan is among the main type of non viral gene carriers currently investigated, as recently reviewed by Jayakumar *et al.* [91]. Due to the cationic nature of chitosan, electrostatic interaction between negatively charged DNA or RNA and chitosan forms polyplexes in which nucleic acid becomes more protected against enzymatic degradation. Mac Laughlin *et al*. [92] reported that molecular weight, degree of deacetylation, the N/P ratio where the N is the amount of amino groups of chitosan and P the amount of DNA phosphate groups are vital parameters to obtain chitosan-based polyplexes. Factors that affect the transfection efficiency of chitosan/DNA complexes are the degree of deacetylation of chitosan, molecular weight, charge ratio as well as the factors related to environment such as pH, serum and the cell type [93-100]. Unfortunately, it is unknown how these modifications affect the formations of polyelectrolyte between chitosan and nucleic acid. Hydrophilic (PEG, PEI, PVP), hydrophobic (deoxycholic acid N-dodecyl group addition, alkylation), pH sensitive (uraconic acid containing an imidazole group), temperature sensitive modifications (N-isopropylacrylamide) have been carried out on chitosan for a variety of reasons. Ligands such as galactose, mannose, folate and transferrin have been introduced on the chitosan structure to design cell-specific delivery systems [93, 100].

2.8 Antibacterial Activity of Chitosan and its Derivatives

Various salts of chitosan, quaternized chitosan-based materials, carboxyalkylated chitosan-based materials, chitosan-based materials with sulfonyl groups, carbohydrate-branched chitosan-based materials, chitosan-amino acid conjugates, chitosan-iodine complexes, miscellaneous chitosan-based materials, chitosan-PVA blends and water soluble chitosan-based materials were designed as antibacterial agents against broad spectrum of bacteria [101].

Chitosan-*graft*-PNVI films synthesized by redox reaction in acidic solution without N-protection was tested against *Pseudomonas aeruginosa* (*P. aeruginosa), E. coli, Bacillus subtilis (B. subtilis) and Staphylococcus aureus (S. aureus)* using the inhibition zone method by Caner *et al.* [102]. Grafted products exhibited an antibacterial activity higher than chitosan film itself [102]. It was found that the antibacterial activity increased with increasing percent grafting and film thickness against all of investigated gram-negative and gram-positive bacteria. The antibacterial activity of the grafted product was further improved by protecting the free amine groups of chitosan during poly (N-vinyl imidazole) grafting by this thesis author. In a study by Sabaa *et al.* [103], carboxymethyl chitosan was synthesized and modified by graft copolymerization of N-vinyl imidazole onto its backbone [103]. They investigated the antibacterial activity and antifungal activity of chitosan, carboxymethylchitosan and their grafted copolymers against *S. aureus, E. coli*, plant pathogenic fungus Fusarium *oxysporum (F. oxysporum)* and the human pathogenic fungus Aspergillus *fumigatus* (*A. fumigatus)* respectively. It was found out that the antibacterial activities of these derivatives against *E. coli* are

stronger than against *S. aureus.* They also demonstrated that PNVI grafting onto chitosan and carboxymethylchitosan inhibited investigated fungal growth [103].

2.9 Blood Interactions of Chitosan Derivatives

Factors influencing blood-material interactions are many, for instance thrombosis, consumption or destruction of blood components, activation of inflammatory and immunological pathways [41]. In the report of Janvikul *et al*. [85] the comparison of chitin, chitosan, and their derivatives including N-acetylated chitosan, N-sulfated chitosan, N, O-carboxymethylchitosan and N-(2-hydroxy) propyl-3-trimethylammoniumchitosanchloride on *in-vitro* human blood coagulation and platelet activation were explored. Water soluble N, O-carboxymethylchitosan, N-acetylated chitosan and chitin promotes coagulation, causing faster fibrin formation. The other samples; Chitosan, N-sulfatedchitosan and N-(2-hydroxy) propyl-3-trimethylammonium chitosanchloride even delay fibrinolysis and not significant effect on whole blood coagulation time. N, O-carboxymethylchitosan was the highest activity on platelet adhesion. It was found that chitin had a better blood coagulation activity than chitosan. Liu *et al*. [104] investigated solubility, anticoagulant and antibacterial activity of hydroxyethyl chitosan. They found the chitosan derivative showed a good anticoagulant and antibacterial activity against *E.coli* and *Enterococcus* compared to unmodified chitosan. Malette *et al*. [87] found that chitosan in acetic acid solution caused blood to coagulate, even in the presence of a blood thinning agent. A study by Klokkevold *et al*. [88] also reported that chitosan in acetic acid solution stopped bleeding in rabbits. In contrast, Tokura *et al.* [89] produce antithrombogenic medical devices as artificial blood vessels or fibers by immobilization sulfated chitosan oligomers on the chitosan. The

exact molecular mechanism of hemostatic activity of chitosan is not yet fully understood.

2.9.1 Chitosan- TPP Gels for Fe^{3+} Chelation

Iron is a trace element present in human body as a part of haemoglobin, myoglobin, transferrin, ferritin, and haemosiderin. It is also found as a component of some enzymes. Iron released from haemoglobin becomes bound to the protein transferrin and is thus transported in the body. Excess iron is stored in ferritin and haemosiderin. Whenever an imbalance occurs in iron metabolism, non-transferrin-bound iron is found in the blood plasma. This phenomenon is observed in the case of an anomaly called atransferrinemia, which causes all iron acquired remain as non-transferrin-bound iron. Another case is when plasma transferrin becomes saturated due to excessive iron-overload. This is a serious problem encountered by thalassaemia patients who need frequent blood transfusions. Non-transferrin-bound iron is soluble complexes of the ferric ion. Although the identity of these complexes is not very well known, there is evidence that two of these complexes are ferric-citrate and ternary ferric-citrate-acetate [105]. Non-transferrin bound iron is harmful because it may lead to oxidative damage in the cells. Free radicals are produced in this process which can be the reason to a fatal harm to the body tissues. The Fenton Reaction as shown below, is the most renowned process during which free radicals are produced [106].

$$O_2^-. + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$2O_2^-. + 2H^+ \rightarrow H_2O_2 + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow .OH + OH^- + Fe^{3+}$$

$$3O_2^-. + 2H^+ \rightarrow .OH + OH^- + 2O_2$$

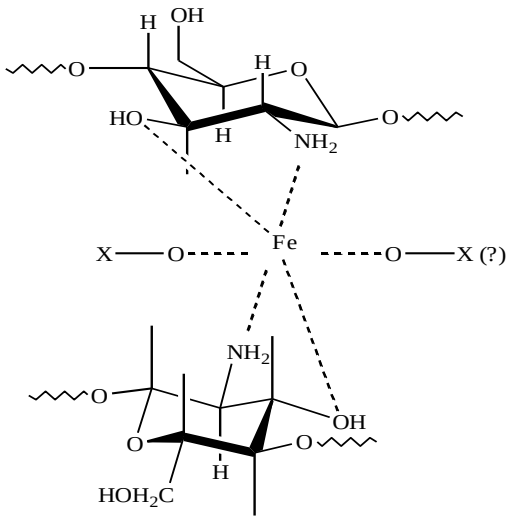
The hydroxyl radical produced is highly reactive; it attacks lipids, proteins and DNA; hence, the excess iron may very well be toxic to the human organism.

Desferrioxamine (DFO, Desferal®) and deferiprone (L1, Ferriprox®) are the commercially available drugs used as iron chelators for the treatment of iron overload encountered in thalassaemia patients. DFO is the best iron chelator known. It is a hexadentate iron chelator which effectively removes excess iron from the blood. It also acts as a free radical trap minimizing the harmful effects of the oxidative damage. One important disadvantage of DFO is that it has to be administered via the parenteral route since it is prone to enzymatic degradation in the gastrointestinal track. So, treatment with DFO is painful and expensive. Furthermore, DFO is not iron selective, and upon prolonged use it may cause growth retardation and loss of hearing and vision [106]. L1 has the advantage of being administered orally. However, it is only a bidentate ligand, hence not as effective as DFO, and it has many side effects, such as to bring down the number of leucocytes, causing gastrointestinal problems and developing zinc deficiency [106-107].

No known chelator for treating iron overload possesses all the properties as listed below, for an ideal iron chelation therapy: (i) specificity and affinity for iron (ii) low molecular weight for gastrointestinal absorption (iii) enough lipophilicity for gastrointestinal absorption via the oral route, (iv) sufficient hydrophilicity to limit liver

absorption (v) slow rate of metabolism (vi) no iron redistribution. Furthermore, it should be relatively non toxic and has a low cost [106-109]. Iron-chelating agents are gaining increasing importance as they have been found to be useful not only in the treatment of toxic iron but also in the therapy for non-iron overloaded diseases [107].

Reports on chitosan-iron systems are limited in literature. Early studies on interactions of chitosan with iron and other transition metals are investigated in Chitin by Muzarelli [54]. Iron-chitosan complex formation has been proposed as either a penta or hexa coordinated Fe^{3+}. It has been studied through Mossbauer spectroscopy that two moles of amino group and four moles of oxygen atoms from two different chitosan backbone chelate the Fe^{3+} (Scheme 7) [110].



Scheme 7. Fe^{3+} chelation by chitosan adopted from [110].

A previous study carried out by Burke *et al*. [111], has revealed that chitosan flakes could successfully adsorb free or citrate-sorbitol complexed Fe^{3+} from solution, and reduce ferritin level in the blood serum samples of thalassaemia patients. In this study, an earlier investigation initiated as a master thesis [112] by the author of this

thesis was further expanded and explored in detail. Chitosan TPP gels were investigated, in-vitro, in human blood as bioadsorbents for Fe^{3+}.

2.9.2 Chitosan as Hypolipidemic Agent

The efficient hypolipidemic dietary fibers have some unique properties as follows:

1. The indigestibility in the upper gastrointestinal tract
2. High viscosity
3. Polymeric nature

Chitosan already possesses these above mentioned characteristics. As chitosan is a weak base, in its cationic form it is able to bind lipids or fats. Also, it is edible but indigestible. Some proposed mechanisms as the viscosity effect of chitosan, the electrostatic interaction between positively charged amino groups on chitosan and negatively charged lipids or fat molecules, the entrapment of lipid and free fatty acids and inhibition of pancreatic lipase has been thought of in order to explain hypocholesterolemic and hypolipidemic action of chitosan. These are compromising that. Among these factors, the viscosity parameter may not be of a critical importance for lipid lowering action of chitosan [42, 54, 55, 58, 62-66, 71-79].

Regarding possible mechanisms of chitosan as cholesterol lowering agents, it should be noted that animal studies might not be predictive of outcomes in humans. Due to the presence of chitinase enzymes in the digestive systems of numerous animals, results from these animals might be different than that of humans [42, 71].

In this thesis, it was explored the physiological role of ascorbic acid and ascorbyl chitosans as blood coagulate or blood anticoagulant agents. The effect of chitosanand ascorbyl chitosans on total cholesterol, triglyceride, high density lipoprotein

(HDL) cholesterol, low density lipoprotein (LDL cholesterol) levels were investigated, *in-vitro*. Ascorbic acid is essential for life and health. ascorbic acid has numerous vital physiological roles in biological systems. It serves as an efficient antioxidant and functions as a cofactor in various metabolic processes. The physical and chemical instability of ascorbic acid leads to limitation in the usage of ascorbic acid. Due to its instability, it is oxidized and it is converted to dehydroascorbic acid by exposure to light, air and high temperature [113-114]. A variety of ascorbic acid derivatives compromising metal salts (Na, Ca salts), ethers, esters and the polysaccharide based materials have been synthesized. Since ascorbic acid inhibits oxidative modification of LDL cholesterol, it is useful for a reduction in the risk of coronary artery diseases. Antioxidants may influence coronary artery disease with different mechanisms including improvement in endothelial function, inhibition of platelet aggregation and reduction in the threat of plaque rupture. Ascorbic acid may contribute to atherosclerosis treatment with reducing lipid peroxidation and increasing antioxidant level [52, 113-117]. Kanauchi *et al*. [73] reported that the addition of ascorbic acid to chitosan causes a larger increase in fecal fat excretion without affecting protein digestibility.

Chapter 3

EXPERIMENTAL

3.1 Materials

Materials/test kits and their manufacturers used in chitosan-based material in antibacterial study are recorded in Table 4. All reagents except N-vinyl imidazole (NVI) were used without any further purification. NVI (Merck) was vacuum distilled before use. Materials/test kits and their manufacturers used in chitosan TPP as bioadsorbent study are listed in Table 5. All reagents were used as they received. Buffer solutions used in chitosan TPP as bioadsorbent experiments were prepared using potassium chloride and hydrochloric acid. Fe^{3+} concentration in aqueous solution was analyzed by Shimadzu UV-1201 V visible spectrophotometer. Total iron, albumin and calcium levels in serum were measured by BTS 310 (4.0 ver) spectrophotometer. Materials/ test kits and their manufacturers used in the experiment of blood contact ascorbyl chitosan are listed in Table 6. All reagents were used as such. MT 1C coagulameter (Medical Technologic Industry) was used for determination of prothrombin time. The complete blood cell count (CBC) was measured by using the Medonic CA 530 (20 Parameter system Thor).

Table 4. Materials, test kits and manufacturers for antibacterial study.

Materials/Test kits	Manufacturers
Chitosan flakes ($4.0x10^5$ ($\overline{M_w}$) g/mol, 85% (DD)	Fluka, Germany
Dimethyl formamide	Aldrich, Germany
Phthalic anhydride	Aldrich, Germany
Hydrazine monohydrate	Aldrich, Germany
N-vinyl imidazole	Merck, Germany
Cerium ammonium nitrate	Aldrich, Germany
Acetone	Riedel-de Häen, Germany
Hydrogen peroxide (3.0%)	Aldrich, Germany
Gentamicin (0.3% Genta)	I.E. Ulagay Drug limited, Turkey
Acetic acid	Riedel-de Häen, Germany
2, 4, 6-trinitro-benzenesulfonic acid	Sigma, Germany
Iodine	Sigma Aldrich, Germany
Potassium hydroxide	Sigma, Germany
Ethanol	Riedel-de Häen, Germany
Safranin	Aldrich, Germany
Crystal violet	Aldrich, Germany
Potassium iodide	Aldrich, Germany
ATB suspension	bioMe´rieux, France
Columbia Agar (COS)	bioMe´rieux, France
Mannitol salt agar (MSA)	bioMe´rieux, France
Baird Parker agar (BP)	bioMe´rieux, France

Table 5.Materials, test kits and manufacturers for chitosan-TPP bioadsorbent study.

Materials/Test kits	Manufacturers
Chitosan flakes ($4.0x10^5$ ($\overline{M_w}$) g/mol, 85% (DD))	Fluka, Germany
Sodium hydroxide	Aldrich, Germany
Acetic acid	Aldrich, Germany
Hydrochloric acid	Merck, Germany
Ferric chloride	Aldrich, Germany
Sodium tripolyphosphate pentabasic	Aldrich, Germany
Ethylene diglycidyl ether	Aldrich, Germany
5-sulfosalicylic acid dihydrate	Riedel-de Häen, Germany
Potassium chloride	BDH, UK
Ammonia	Merck, Germany
Ferrimat assay kit	bioMe´rieux, France
Albumin assay kit	MBT, Biomer, Turkey
Calcium assay kit	MBT, Biomer, Turkey

Table 6. Materials, test kits and manufacturers for investigation of ascorbyl chitosan study.

Materials/Test kits	Manufacturers
Chitosan ($4.0x10^5$ g/mol($\overline{M_w}$), 85% (DD)	Sigma, Germany
Ascorbic acid	Supelco, USA
2-Propanol	Aldrich, Germany
RTU cholesterol assay kit	bioMe´rieux, France
HDL cholesterol (C-HDL Ultra) assay kit	bioMe´rieux, France
LDL cholesterol (C-LDL Dir) assay kit	bioMe´rieux, France
Triglycerides Enzymatic PAP 150 (TG PAP 150) assay kit	bioMe´rieux, France
Ferrimat assay kit	bioMe´rieux, France
Calcium assay kit	MBT, Biomer

3.2 Synthesis

3.2.1 Preparation of Chitosan-*graft*-poly (N-Vinyl Imidazole) without N-Protection

1.0 g of chitosan was dispersed in 100 mL of dried dimethyl formamide (DMF) and was stirred overnight followed by 30 minute purging with nitrogen gas. DMF provides a heterogeneous reaction medium in which chitosan is insoluble. The cerium (IV) ammonium nitrate initiator (3.5 g) and different concentrations (0.14M (1.27 mL), 0.10M (0.889 mL) 0.07M (0.635mL) of N-vinyl imidazole were added under nitrogen atmosphere and the reaction was carried out at constant temperature (70 ºC) by stirring at 50 rpm. The resultant solution was poured into 1000 mL of acetone with vigorous stirring for precipitation. Then obtained product was extracted with ethanol in a soxhlet apparatus for 48 hour to remove the homopolymer and dried at room temperature. The obtained products (chitosan-*graft*-poly (N-vinyl imidazole); are referred as Chi-*graft*-PNVI (98), Chi-*graft*-PNVI (85) and Chi-*graft*-PNVI (60), respectively (Table 7).

3.2.2 Preparation of Chitosan-*graft*-poly (N-Vinyl Imidazole) via N-protection

Chitosan was heated with excess phthalic anhydride in dried dimethyl formamide (DMF) to first give phthaloyl chitosan, according to a previously reported procedure [15, 16]. The resultant solution was poured into 1.0 L of acetone with vigorous stirring for precipitation. The product (phthaloyl chitosan) was dried at room temperature. Then 1.0 g of phthaloyl chitosan was dissolved in 100 mL of dried DMF and was stirred overnight followed with 30 min purging with nitrogen gas. The cerium (IV) ammonium nitrate (CAN) initiator (3.5 g) and 0.15M of N-vinyl imidazole were added under nitrogen atmosphere and the reaction was carried out at constant temperature (70 ºC) by stirring at 50 rpm. The resultant solution was precipitated in acetone. The obtained product (pthaloylchitosan-*graft*-poly (N-vinyl imidazole)) was extracted with ethanol in a Soxhlet apparatus for 48 h to remove the homopolymer and dried at room temperature. Then the (pthaloylchitosan-*graft*-poly (N-vinyl imidazole)) (1.0 g) was added to 20 mL of DMF and treated with hydrazine monohydrate at 100 ^{o}C for 2 hours under nitrogen to remove the phthaloyl group. A yellow solution was formed. The final deprotected product precipitated upon cooling. The precipitate was collected, washed thoroughly with water and ethanol and dried to obtain the final product, chitosan-*graft*-poly (N-vinyl imidazole), which is referred as Chi-*graft*-PNVI*(25) throughout the text.

Table 7. Preparation conditions for synthesized chitosan-*graft*-poly (N-vinyl imidazole) samples. (Reaction conditions: 1.0 g chitosan, 3.5 g cerium (IV) ammonium nitrate in 100 mL dried dimethyl formamide).

Sample ID	Concentration of N-vinyl imidazole (M)
Chi-*graft*-PNVI(98)	0.140
Chi-*graft*-PNVI(85)	0.098
Chi-*graft*-PNVI(60)	0.070
Chi-*graft*-PNVI*(25)	0.140

*Chi, Chitosan; PNVI, poly (N-vinyl imidazole); numerical values in paranthesis are the %poly (N-vinyl imidazole) grafting yield determined by gravimetric method. *prepared via amine protection route.*

3.2.3 Preparation of Fe^{3+} Imprinted Beads by *Post Formation* Crosslinking and *In-situ* Crosslinking

Chitosan solution was prepared by dissolving chitosan flakes in 1% (v/v) acetic acid solution. Fe^{3+} ion was used as the template to prepare the imprinted chitosan beads. Two methods were applied. In the first method, chitosan in acetic acid solution was dropped into a coagulation bath consisting of a mixture of sodium tripolyphosphate pentabasic in pH=1.2 buffer and ferric chloride. The beads formed were then collected, washed with distilled water and cleaned from Fe^{3+} ion using 0.06 M ammonia solution until the solution was colourless. The template free beads were washed with distilled water for neutralization. They were then crosslinked by being treated with EGDE. Finally, they were extensively washed with distilled water and dried overnight at 60 °C.

In the second method, the *in-situ* crosslinking method, chitosan dissolved in acetic acid was dropped into a mixture of sodium TPP and ferric chloride solution containing the cross linker, EGDE. The template ion Fe^{3+} was removed and the beads obtained were washed and dried as given above. Average bead diameter was 1mm. The bead preparation conditions have been summarized in Table 8.

Table 8. Preparation conditions for prepared Fe^{3+} imprinted chitosan gel beads (in 1% v/v acetic acid solution using 2% (w/v) chitosan solution in TPP dissolved at pH=1.2 buffer).

Sample ID	**[Fe^{3+}]**	**Method of Crosslinking and EGDE Concentration (v/v)**
N	None	None
N-PC1	None	*post formation, 1%*
N-PC4	None	*post formation, 4%*
N-PC8	None	*post formation, 8%*
N-SC8	None	*in-situ,8%*
I5	5mM	-
I5-PC8	5mM	*post formation, 8%*
I5-SC8	5mM	*in-situ,8%*
I10-SC8	10mM	*in-situ,8%*

*

N: non-imprinted

I5: imprinted with 5mM Fe^{3+} solution

I10: imprinted with 10mM Fe^{3+} solution

P: post-formation crosslinking

S: in-situ crosslinking

C1: 1%EGDE solution

C4: 4% EGDE solution

C8: 8%EGDE solution

3.2.4 Preparation of Chitosan as Blank and Ascorbyl Chitosans

0.5 g of chitosan and ascorbic acid (0.1 mM, 1 mM, 5 mM and 100 mM) were added into 100 mL of isopropyl alcohol (75 %, v/v). The solution was mixed at 60 °C for 6 hours under nitrogen gas to remove any dissolved oxygen from the solution. After 6 hours the polymer was washed with isopropyl alcohol and was dried at 25 °C. The chitosan sample which is used as blank was prepared as 0.5 g of chitosan and 100 mL of isopropyl alcohol (75 %, v/v) was stirred at 60 °C for 6 hours under nitrogen gas. After 6 hours the polymer was washed with isopropyl alcohol and was dried at 25 °C, (Table 9). Preparation conditions of synthesized ascorbyl chitosan samples (0.5 g of dispersed chitosan in 75 % isopropyl alcohol (v/v) solution) have been summarized in Table 9.

Table 9. Preparation conditions for synthesized ascorbyl chitosan samples (0.5 g of dispersed chitosan in 75% isopropyl alcohol (v/v) solution).

Sample ID	Concentration of Ascorbic acid (mM)	Composition by Weight (% Ascorbic acid)
Chi	-	-
ChiVC-0.1	0.1	0.35
ChiVC-1	1.0	3.4
ChiVC-5	5.0	14.9
ChiVC-100	100	78.0

* *Chi, Chitosan; VC, ascorbic acid, numerical values are the molarity of ascorbic acid.*

3.3 Identification and Growth of Bacteria

Bacterial cells were obtained from patient cultures and were propagated. Morphological properties of *S. epidermidis* and *E. coli* were observed by gram staining. Crystal violet as a primary basic dye and red dye safranin as a secondary dye were used

for gram staining. Crystal violet (0.5 g) was dissolved in 100 mL of distilled water. Safranin (0.5 g) was dissolved in 10 mL of 95% ethanol and diluted to 100 mL with distilled water. Then the obtained dye solution was filtered. Iodine crystals (1.0 g) were added into solution in which 2.0 g of potassium iodide were dissolved in 300 mL of distilled water. Ethanol was used as a decolorizing agent. After the decolorizing step the *E. coli* bacteria lost all of their colour. After that *E. coli* cells were made visible by safranin dye. Glass slides read at 40X magnification on a light microscope (Reichert Jung). *S. epidermidis* takes up dark purple colour and *E. coli* appears bright red colour.

Catalase biochemical test was done to check *S. epidermidis* as catalase negative activity. Two colonies was added into ATB suspension medium and incubated at 37 °C for overnight. 5 mL of this bacteria growth culture was used. Then, 3-4 drops 3% H_2O_2 was added into the culture. No bubbling was observed.

Selective medium mannitol salt agar was used to show *S. epidermidis* become white due to lack of ability to ferment mannitol in Figure 1. *S. epidermidis* produced black colonies on Baird Parker agar in Figure 2. Eosin methylene blue agar is used to show *E. coli* colonies become a dark colony or green with a metallic fluorescent sheen in Figure 3.

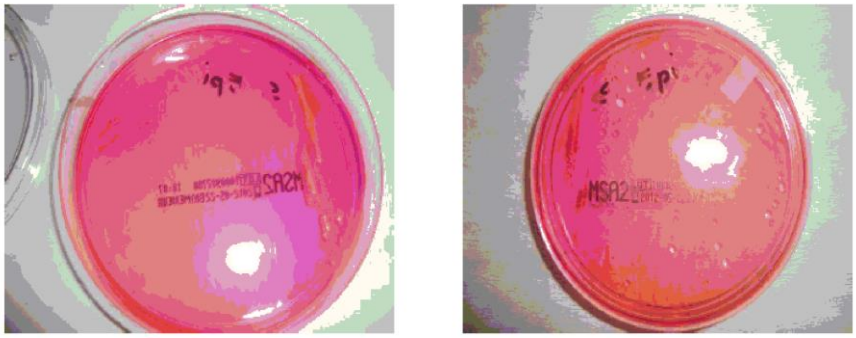


Figure 1.Optical picture of MSA inoculated with *S.epidermidis*.

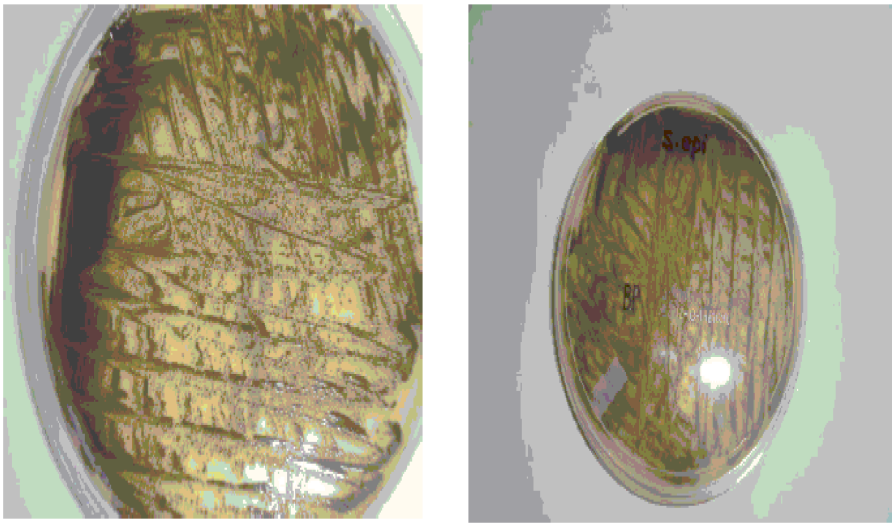


Figure 2. Optical picture of BP agar inoculated with *S.epidermidis*.

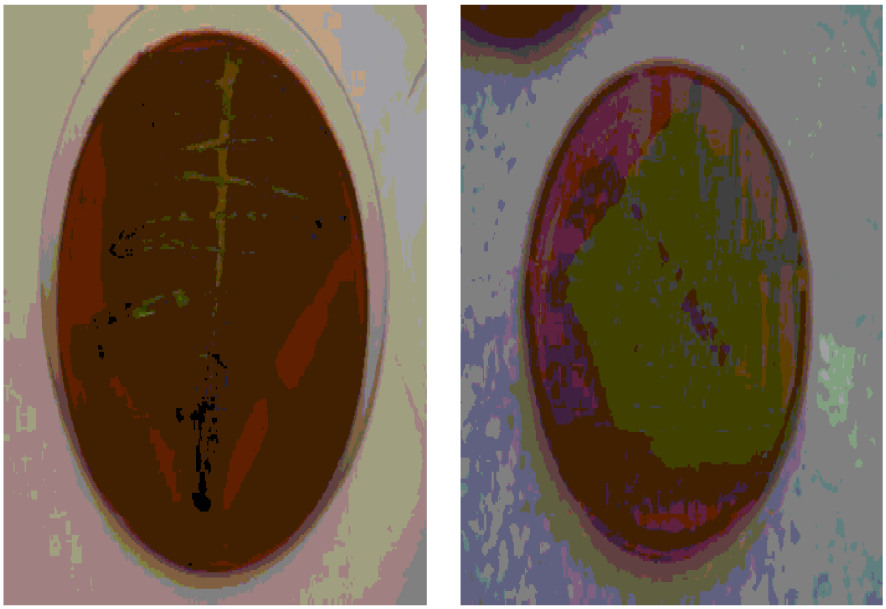


Figure 3. Optical picture of EMB agar inoculated with *E.coli*.

3.4 Preparation of Antibacterial Solutions

0.5 g of chitosan and chitosan-*graft*-poly (N-vinyl imidazole) were dissolved in the 100 mL of 1% acetic acid (v/v) aqueous solution. Then the solution was filtered to remove insoluble impurities. The pH of examined solution and acetic acid used as solvent was adjusted to 5.5 with potassium hydroxide. To prepare serial dilution of examined polymer samples, 90 μL of the sample was mixed with 10 μL of acetic acid for 3 hours. Gentamicin being as a control, 90 μL of gentamicin was diluted into 100 μL with acetic acid. To prepare combinations of examined samples with gentamicin, 90 μL of the examined sample was mixed with 10 μL of gentamicin for 3 hours. 10 μL of gentamicin was diluted into 100 μL with acetic acid as a control. Then all solutions were diluted two-fold, four-fold, eight-fold and sixteen-fold by using 1% acetic acid solution (pH: 5.5).

3.5 Conditions of Incubation

The concentration of *S. epidermidis* and *E. coli* was 8000 cfu/mL by tally counter (cw610). Bacteria were incubated at 37 ºC for 24 hours on a nutrient agar plate before use. Then the added microorganisms (*S. epidermidis* and *E. coli*) were coated on the surface of COS (Columbia Agar + 5% sheep blood COS, bioMerieux), petri dishes. Finally, 10 μL of examined solution was used. The test solutions were applied to the surface of agarose plates containing cultures of bacteria in nutrient. Mean diameter of zone of inhibition and standard deviation of two replicate measurements were calculated.

3.6 Determination of Bacteriostatic or Bacteriocidial Activity

The samples were taken inside inhibition zone created by gentamicin, chitosan, Chi-*graft*-PNVI(60), Chi-*graft*-PNVI(85), Chi-*graft*-PNVI(98), Chi-*graft*-PNVI*(25) and their combinations with gentamicin were applied to the surface of a new agar to check if any bacteria cell growth occurred.

3.7 Swelling and Dissolution of the Chitosan TPP Beads

The swelling behaviour of the prepared beads was measured in pH=1.2 at room temperature at 25 °C. A buffer solution (100 mL) of pH=1.2 was prepared by mixing 25 mL 0.2 M KCl and 42.5 mL 0.2 M HCl. Beads (approximately 0.05 g) were immersed and kept in the buffer solution (pH 1.2) for 6 hours. The rotation speed was 50 rpm and the temperature was 37 °C. The wet mass of swollen gels were determined by blotting with a filter paper to remove the surface water and weighed immediately on an electronic balance. The swelling ratio of the beads was calculated using the equation where, m_d and m_s are the weights of the samples in the dry and swollen states respectively. Each swelling experiment was repeated three times and the average value was taken.

$$\textbf{Swelling ratio (\%)} = \frac{m_s - m_d}{m_d} \times 100$$

The soluble fraction was evaluated by a sample of hydrogel beads (approximately 0.05 g) were immersed and kept in the buffer solution (pH 1.2). The swollen hydrogel samples were taken and dried in oven at 60 °C to constant weight. The soluble fraction was calculated according to the following equation:

$$\textbf{Weight Loss (\%)} = \left(\frac{m_{d1} - m_{d2}}{m_{d1}}\right) \times 100$$

Where m_{d2} is the weight of dried hydrogel after removal of the solvent.

3.8 Adsorption of Fe^{3+} Ion on Chitosan TPP Beads

50 mg bead sample was placed in a 50 ml aqueous Fe^{3+} solution at pH=1.2 and stirred at 60 rpm at 37 °C for 6 hours. 1 mL aliquots were taken at one hour intervals and analyzed for Fe^{3+} concentration by visible spectrophotometer. The amount or Fe^{3+} adsorbed was calculated from the difference between the concentrations of the initial and final solution. All adsorption experiments were done at least in duplicate.

3.9 Determination of Fe^{3+} in Solution by Chitosan TPP Beads

One milliliter Fe^{3+} solution was mixed with 1 mL of 5-sulfosalicylic acid dehydrate, (10% w/v), and completed to 10 mL with pH=1 buffer solution. Twenty-five milliliter 0.2M KCl and 67.0 mL 0.2M HCl is used to prepare 100 mL of the pH=1 buffer solution. The amount of residual Fe^{3+} ion was determined by visible spectrophotometer at 505nm using a Shimadzu UV-1201 V visible spectrophotometer. Then the amount of Fe^{3+} ion adsorbed by chitosan was calculated.

3.10 Ferrimat assay

Blood samples of the volunteers were collected using Vacutest tubes (VACUTEST KIMA Sri ARZERGRANDE, Italy) containing gel and clot activator. The serum was separated by centrifugation for 10 min. Total iron was measured using a commercial Ferrimat assay kit (bioMe´rieux, France). The amount of transferrin bound iron was determined by BTS 310 (4.0 ver) spectrophotometer at 562nm. A calibration was performed for each series of tests.

3.11 Determination of Total Calcium in Blood Serum

10 mg of samples were brought into contact with 100 µL of human serum for 3 hours in vitro. Calcium test kit (MBT, Biomer) were used. Then, 1 mL of reagent, 15µL of standard and 15µL of serum were taken up and incubated for 10 minutes at 37°C. The amount of final total calcium level was determined by BTS 310 (4.0 ver) spectrophotometer at 670 nm.

3.12 Determination of Albumin Analysis in Blood Serum

Blood samples of the volunteers were collected using Vacutest tubes (VACUTEST KIMA Sri ARZERGRANDE, Italy) containing gel and clot activator. The serum was separated by centrifugation for 10 min. Albumin test kit (MBT, Biomer) was used. Then, 1 mLof reagent, 5 µL of standard and 5 µL of serum were taken up and incubated for 10 minutes at 37 °C. The amount of final albumin level was determined by visible spectrophotometer at 630nm.

3.13 Cell Blood Count Measurements

Blood samples of the volunteers were collected using test tubes (Meus sri PIOVE Di SACCO, Italy) containing K_3EDTA. 20 mg of each sample were brought into contact with 400 µL of human blood for 3 hours *in-vitro*. The sampling was carried out by using Medonic CA 530.

3.14 Prothrombin Time Measurements

For prothrombin time measurements, blood samples of the volunteers were collected using 3.5 mL blood vacutest tube (VACUTEST KIMA Sri ARZERGRANDE, Italy) containing Na-citrate (3.2%, 0.109 M) and plasma was separated by centrifugation for 10 min. The test was performed by adding thromboplastin reagent (trini clot pt excel,

rabbit brain). 10 mg from each examined polymer were brought into contact with 200 μL of whole blood for 3 hours *in-vitro*. Prothrombin time analyses of the separated plasma were done by MT 1C coagulameter.

3.15 Total Cholesterol, HDL Cholesterol, LDL Cholesterol and Triglyceride Assay

Blood samples of the volunteers were collected using Vacutest tubes (VACUTEST KIMA Sri ARZERGRANDE, Italy) containing gel and clot activator. The serum was separated by centrifugation for 10 min. 10 mg from each examined polymer were brought into contact with 200 μL of human serum for 3 hours in-vitro. Total cholesterol level was determined at 670nm, HDL cholesterol, LDL cholesterol and triglyceride levels were determined at 505nm by visible spectrophotometry (BTS 310 (4.0 ver)).

3.16 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

FT-IR analysis was carried out at TUBITAK-MAM in Gebze with KBr pellets on a Perkin Elmer Spectrum One FTIR spectrometer.

3.17 Scanning Electron Microscopy (SEM) Analysis

SEM analysis was carried out at TUBITAK-MAM in Gebze by using a JEOL/JSM-633F scanning electron microscope.

3.18 X-ray Diffraction (XRD) Analysis

XRD analysis was carried out at IYTE-MAM in Izmir by using a Phillips X'Pert Pro XRay powder diffractometer (wavelength = 1.54060A˚). The crystallinity indices were determined by the following equation:

$$\mathrm{CrI}\ \% = \frac{(I_{110} - I_{am})}{I_{110}} \times 100$$

3.19 Carbon-13 Nuclear Magnetic Resonance (C-13 NMR) Analysis

C-13 NMR analysis was carried out at METU-Central Lab.

3.20 2, 4, 6-Trinitro-benzenesulfonic Acid (TNBS) Analysis

Primary amines in the chitosan derivatives were determined by TNBS assay. 1 mg/mL of polymer sample was treated with a reaction mixture of 1 ml of borate buffer solution (pH=9.08) and TNBS solution prepared in borate buffer solution by dissolving 22 µL of 5%TNBS in 22 mL of borate buffer. Allowing the reaction to take place for 30 minutes at 37 °C, where the TNBS react with free amine groups and gives a brown colour. The extent of colour intensity is depending upon content of free amino groups in the test sample. Absorbance was measured of the resulting solution after dilution using spectrophotometer (Ultraspec 2000, uv-vis spectrophotometer, Pharmacia Biotech) at 420nm.

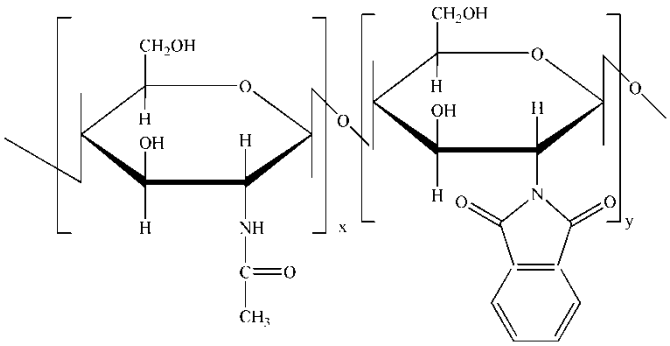
Chapter 4

RESULTS AND DISCUSSIONS

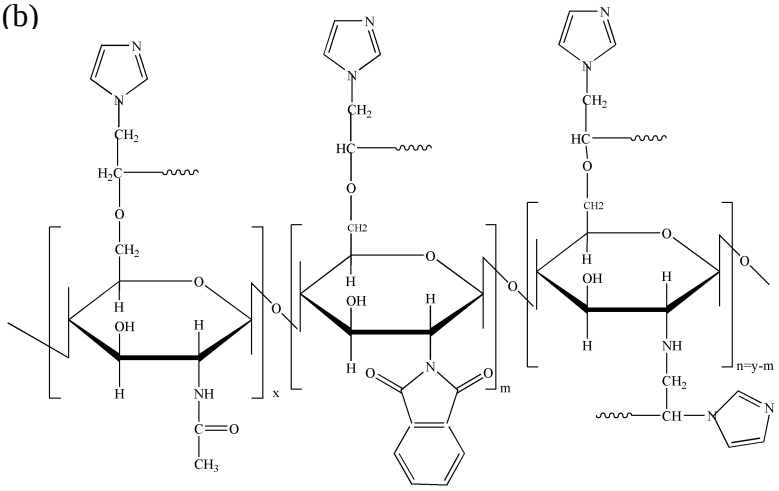
4.1 Antibacterial Activity of Chitosan Derivatives

The antibacterial activities of chitosan and graft copolymer based on chitosan namely chitosan-*graft*-poly (N-vinyl imidazole), (Chi-*graft*-PNVI), were studied against *S. epidermidis* and *E. coli.* The graft copolymer was prepared by two different methods; via an N-protection route and without N-protection to observe the effect of free amine groups on the antibacterial activity against the bacteria studied. It was also investigated whether a combination of each of these products with the antibiotic gentamicin would produce a synergistic effect against *S. epidermidis* and *E. coli* compared to gentamicin alone. The chemical structures of all products are shown in Scheme 8.

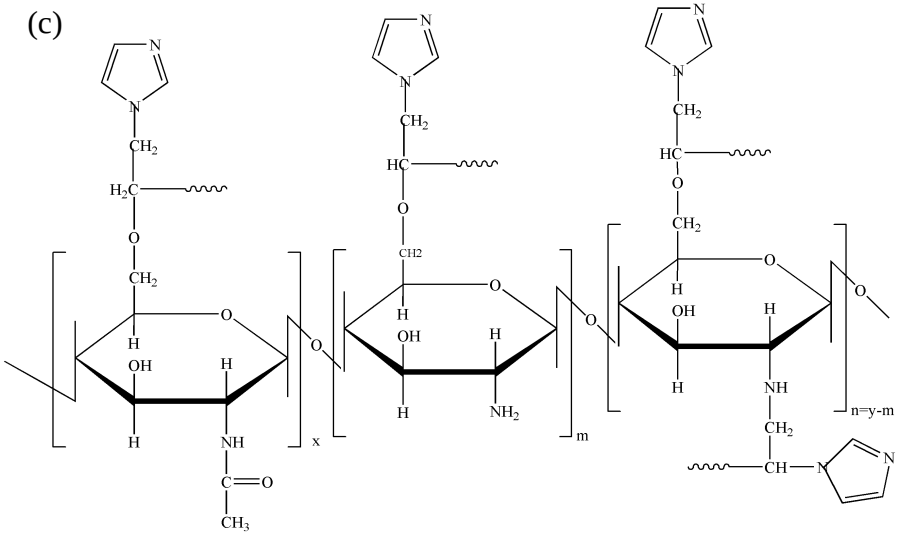
(a)



(b)



(c)



Scheme 8. Chemical structures of (a) Phtaloyl chitosan, (b) PNVI-*graft*-phtaloyl chitosan and (c) NVI-*graft*-phtaloyl chitosan after deprotection.

4.1.1 TNBS Analysis

The amount of amine groups present in the samples prepared was determined by TNBS assay method [33]. The results are presented in Table 10. TNBS assay data confirmed that amine group content of Chi-*graft*-PNVI (98), Chi-*graft*-PNVI (85) and Chi-*graft*-PNVI (60) was 16%, 27% and 44% respectively. The fact that free amine content decreases with increasing grafting yield indicates that amine functionalities serve as grafting sites during redox initiated graft copolymerization. The sample prepared via N-protection route, Chi-*graft*-PNVI*(25), contained the highest fraction of free amine group (66%). This result indicates that amine groups of chitosan were successfully protected during grafting reaction and deprotected after grafting.

Table 10. % Grafting yield by gravimetric method and free -NH_2 groups determined by TNBS analysis.

Sample ID	**%Grafting Yield**	**%Free-NH_2 groups/100 repeating units**
Chi-*graft*-PNVI(98)	98	16
Chi-*graft*-PNVI(85)	85	27
Chi-*graft*-PNVI(60)	60	44
Chi-*graft*-PNVI*(25)	25	66

4.1.2 FT-IR Analysis

The products were examined by FT-IR spectroscopy method and this analysis was carried out at EMU. In the FT-IR spectrum of chitosan given in Figure 4 a), amide I and amide II bands are observed at 1665 cm^{-1} and 1590 cm^{-1}. In the spectrum of *N*-

phthaloyl chitosan (Figure 4 b)), absorptions at 1776 cm^{-1} and 1723 cm^{-1} are characteristic of carbonyl in phthalimide and absorption at 721 cm^{-1} of aromatics. Carbonyl anhydride peaks characteristic of phthaloyl group were observed in the spectrum of the poly (N-vinylimidazole) grafted N- phthaloyl chitosan shown in Figure 4 c). The bands at 1298 cm^{-1} and at about 1199 cm^{-1} can be attributed to -CH_2 bending. 1638 cm^{-1} and 1549 cm^{-1} shows C=C and C=N stretching vibrations of the imidazole ring. In the spectrum of the Chi-*graft*-PNVI*(25) (Figure 4 d)), the absence of the carbonyl stretching in the 1700-1800 cm^{-1} range was taken as evidence of successful deprotection.

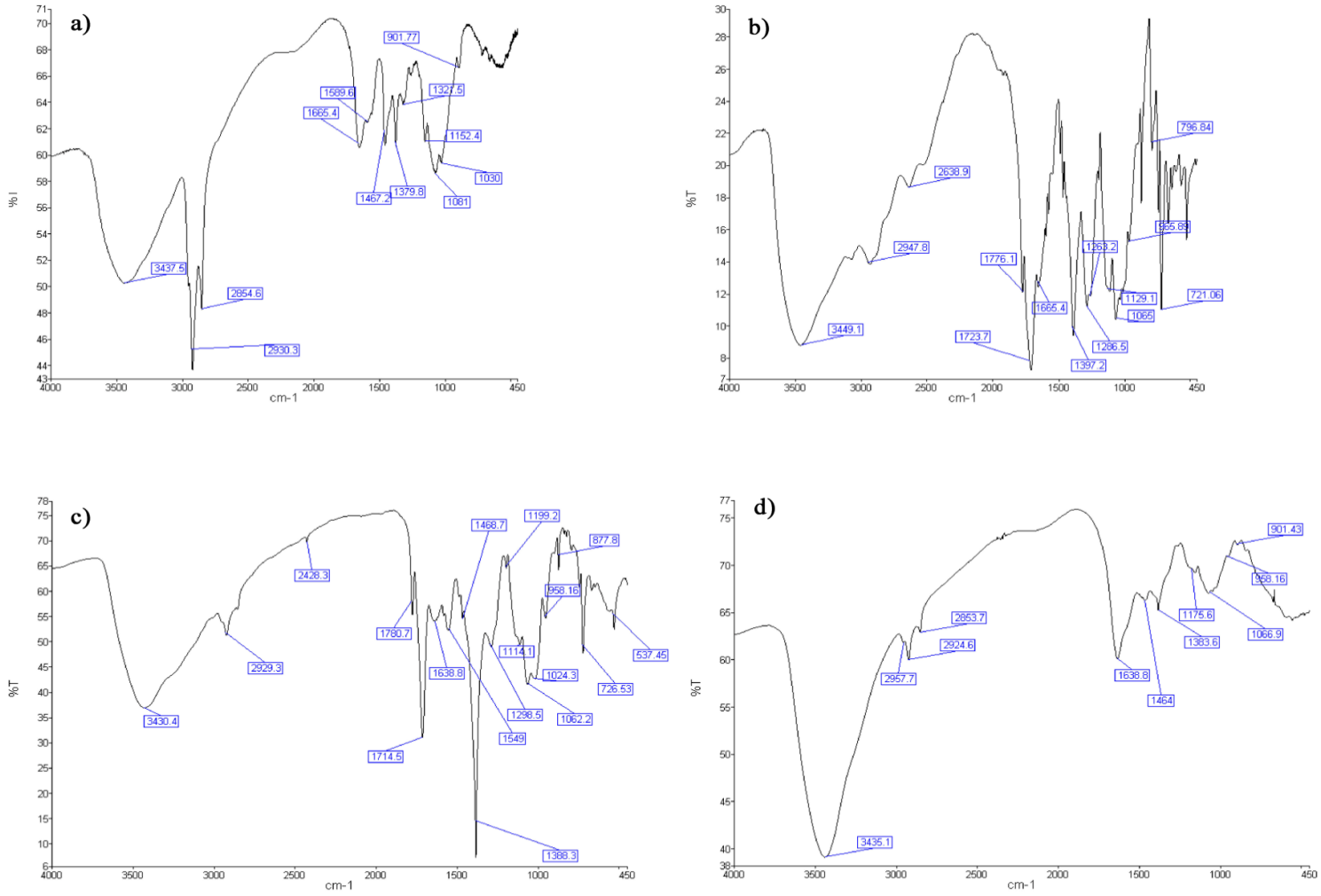


Figure 4. FT-IR spectra of (a) chitosan, (b) phtaloyl chitosan, (c) phtaloyl chitosan-*graft*-PNVI, (d) chitosan-*graft*-PNVI*(25).

4.1.3 Antibacterial Activity

Antibacterial activities of chitosan, Chi-*graft*-PNVI samples and their mixtures with gentamicin have been investigated against *S. epidermidis* and *E. coli*. Antibacterial activity was tested by the inhibition zone measurement method. The antibacterial activity of the samples was examined with respect to their physical, chemical characteristics and concentrations.

Antibacterial activities of Chi-*graft*-PNVI samples are shown in Figure 5 against *S. epidermidis* and *E. coli*. No inhibition was noticed in control media containing acetic acid (pH=5.5) alone which is the solvent used for dissolution and dilution of the samples. The measured inhibition zones of Chitosan, Chi-*graft*-PNVI*(25), Chi-*graft*-PNVI(60) and Chi-*graft*-PNVI(85) is 10.2±0.2 mm, 5.1±0.2 mm, 2.5±0.1 mm and 1.2±0.0 mm, respectively against *S. epidermidis*. Chitosan,and Chi-*graft*-PNVI*(25) showed an inhibition zone of 10.6±0.2 mm, 10.7±0.03 mm, while Chi-*graft*-PNVI(60) and Chi-*graft*-PNVI(85) had an inhibition zone of 6.75±0.1 mm and 4.75±0.03 mm, respectively against *E. coli*. The antibacterial activities of Chi, Chi-graft-PNVI*(25), Chi-graft-PNVI(60), Chi-graft-PNVI(85) against *E.coli* were stronger than against *S.epidermidis*. Among grafted samples, Chi-*graft*-PNVI prepared via amine protection route, Chi-*graft*-PNVI*(25), exhibited the highest inhibition zone against both type of bacteria whereas there was no inhibition zone observed for Chi-*graft*-PNVI (98) against *S. epidermidis* or *E. coli.* Moreover, Chi-*graft*-PNVI*(25) exhibited less antibacterial activity than chitosan itself against *S. epidermidis*, but almost the same antibacterial activity with chitosan itself against *E. coli*. These results indicate decreased antibacterial activity with increasing grafting percentage of PNVI. The observed trend can be explained by the fraction of free amine groups present in the samples. PNVI grafting

onto chitosan proceeds via both OH-bearing carbons and free amine groups which results in a reduced fraction of free amine groups (Table 10) responsible from the antibacterial effect. When the amine group is protected, grafting proceeds mainly from the hydroxyl bearing carbon atoms. Hence, after deprotection there are higher fraction of free amine groups available (Table10) for antibacterial activity leading to improved antibacterial activity. Chi-*graft*-PNVI (98), on the other hand, does not show any measurable antibacterial effect which can be interpreted as there must be a minimum amount of free amine group for inhibitory effect.

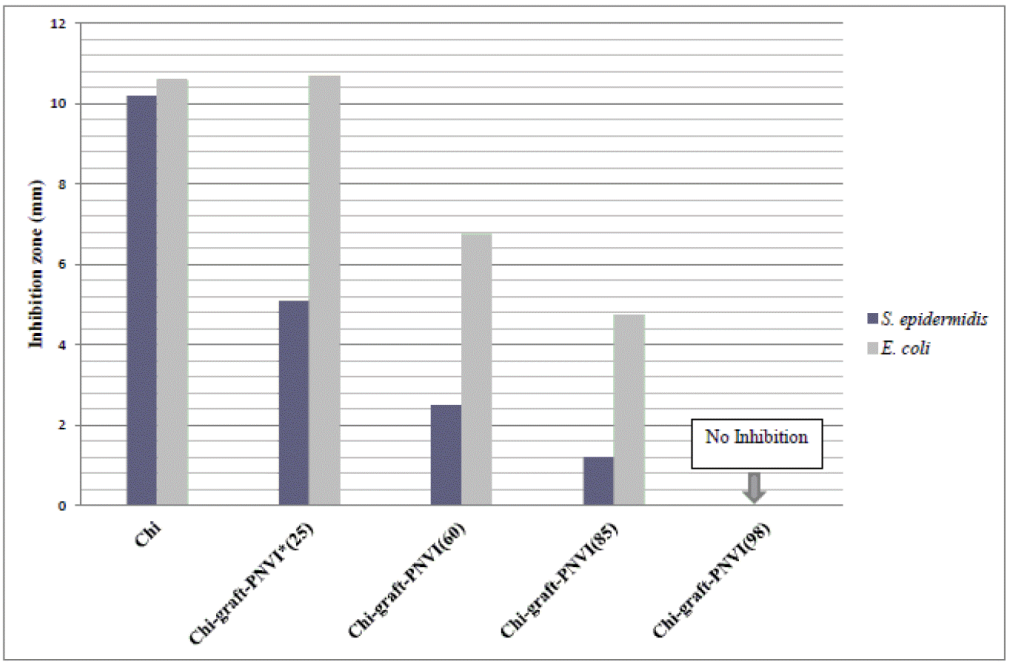


Figure 5. Bar diagram of inhibition zones of chitosan and Chi-*graft*-PNVI samples against *S. epidermidis* and *E. coli.*

The varying antibacterial activity of the products against *S.epidermidis* and *E.coli* can be attributed to the differences in the cell membranes in terms of composition and polarity.

S.epidermidis cell surface might be binding cationic chitosan and PNVI grafted chitosan more strongly than that of E.coli. Hence, diffusion of the polymers into the cell structure is hindered in *S. epidermidis* resulting in smaller inhibition zone measurements. In the outer cell membrane of *E.coli*, lipopolysaccharides and phospholipids are available which make the cell surface more hydrophobic than the cell membrane of *S.epidermidis* which allows higher diffusion ability to the chitosan-based agents because of less interaction. Furthermore, the cell membrane of *E.coli* contains histidine binding proteins which may assist in the transportation of PNVI grafted chitosan chains.

Tables 11 and 12 show the inhibition zone diameter (to nearest), mm obtained with various serial dilutions of the antibacterial agent, gentamicin and those of chitosan and Chi-*graft*-PNVI*(25) (90μL sample+10 μL acetic acid) tested against *S. epidermidis* and *E.coli* respectively. The inhibition zone diameter decreases for all samples upon dilution. The trend observed in Figure 5, is exhibited by the polymer samples at all concentrations against *S.epidermidis.* In other words, chitosan is more effective than the graft copolymer, Chi-*graft*-PNVI*(25), indicating a direct relationship between the free amine content and antibacterial activity.

It is interesting to note that the inhibition zone diameter values measured for the graft copolymer, Chi-*graft*-PNVI*(25) are higher than those of chitosan for diluted solutions. This observation can be taken as further evidence for the smaller degree of interaction of the copolymer with the biopolymers present on the cell wall of *E.coli*. The graft copolymer gains higher mobility upon dilution to be effective over a broader distance whereas chitosan with higher degree of free amine groups has a higher cationic charge density which causes a stronger degree of interaction with the lipopolysaccharides and proteins on the cell wall hindering its diffusion into the cell

structure.The inhibition zone diameter is shown with various serial dilutions antibacterial activity examined samples tested combined with gentamicin against *S. epidermidis* in Table 13 and against E.coli in Table 14.

Table 11. The inhibition zone diameter (to nearest), mm obtained with various serial dilutions antibacterial activity examined samples (90µL sample+10 µL acetic acid) tested against *S.epidermidis*.

Dilution Factor	**Gentamicin**	**Chitosan**	**Chi-*graft*-PNVI*(25)**	**Chi-*graft*-PNVI(98)**
No dilution	13.0±0.7	10.2±0.1	4.95±0.1	No inhibition
1 in 2	12.5±0.0	9.1±0.1	4.85±0.1	No inhibition
1 in 4	12.0±0.1	7.2±0.2	4.10±0.1	No inhibition
1 in 8	10.0±0.28	5.1±0.1	3.25±0.1	No inhibition
1 in 16	8.0±0.0	4.95±0.1	1.13±0.1	No inhibition

Table 12. The inhibition zone diameter (to nearest), mm obtained with various serial dilutions antibacterial activity examined samples (90µL sample+10 µL acetic acid) tested against *E. coli*.

Dilution Factor	**Gentamicin**	**Chitosan**	**Chi-*graft*-PNVI*(25)**	**Chi-*graft*-PNVI (98)**
No dilution	15.0±0.0	10.6±0.1	10.7±0.0	No inhibition
1 in 2	12.5±0.1	9.6±0.2	9.75±0.3	No inhibition
1 in 4	12.0±0.1	7.5±0.0	9.0±0.1	No inhibition
1 in 8	10.0±0.3	6.9±0.1	8.55±0.1	No inhibition
1 in 16	8.0±0.0	5.42±0.1	8.0±0.1	No inhibition

Table 13. The inhibition zone diameter (to nearest), mm obtained with various serial dilutions antibacterial activity examined samples tested combined with gentamicin against *S. epidermidis.*

Dilution Factor	**Gentamicin**	**Chitosan combined with gentamicin**	**Chi-*graft*-PNVI*(25) combined with gentamicin**	**Chi-*graft*-PNVI(98) combined with gentamicin**
No dilution	8.2±0.7	20.2±0.0	25.8±0.7	15.3±0.0
1 in 2	7.8±0	18.4±0.1	24.2±0.2	13.4±0.1
1 in 4	7.1±0.1	15.5±0.1	22.1±0.1	12.3±0.1
1 in 8	5.3±0.0	12.2±0.1	20.95±0.1	10.0±0.1
1 in 16	5.0±0.1	10.5±0.1	19.9±0.1	8.1±0.1

Table 14. The inhibition zone diameter (to nearest), mm obtained with various serial dilutions antibacterial activity examined samples tested combined with gentamicin (90μL sample+10 μL gentamicin) against *E. coli*.

Dilution Factor	**Gentamicin**	**Chitosan combined with gentamicin**	**Chi-*graft*-PNVI*(25) combined with gentamicin**	**Chi-*graft*-PNVI (98) combined with gentamicin**
No dilution	12.2±0.1	20.8±0.1	21.8±0.0	20.2±0.3
1 in 2	10.2±0.1	19.1±0.1	21.3±0.3	18.3±0.3
1 in 4	9.7±0.0	16.6±0.1	19.5±0.0	16.2±0.0
1 in 8	9.5±0.0	16.2±0.0	19.2±0.0	15.8±0.3
1 in 16	8.1±0.1	15.9±0.1	19.1±0.1	15.2±0.0

When chitosan and Chi-*graft*-PNVI samples were combined with gentamicin, it was observed that an increased antibacterial effect was exhibited by all samples against *S. epidermidis* compared to gentamicin alone in Table 13. The inhibition zone of undiluted solution of gentamicin combined with chitosan, Chi-*graft*-PNVI (98) and Chi-*graft*-PNVI*(25) were 20.0±0.0 mm, 15.3±0.0 mm, 25.8±0.7 mm, respectively in Table 13. As it can be observed from these results, Chi-*graft*-PNVI*(25) combined with gentamicin provided an antibacterial activity twice as much as that of gentamicin alone. Gentamicin combined with Chi-*graft*-PNVI (98), gentamicin combined with chitosan exhibited an improved antibacterial activity against *S. epidermidis* in comparison to gentamicin alone. This might be due to different cellular targets of these antibacterial agents. The inhibition zone of undiluted solution of gentamicin combined with chitosan, Chi-*graft*-PNVI (98) and Chi-*graft*-PNVI*(25) were 20.8±0.1 mm, 20.2±0.3 mm, and 21.8±0.0 mm, respectively against *E. coli* in Table 14. Chitosan combined with gentamicin exhibited an actibacterial activity twice as much as that of chitosan itself against *S. epidermidis* and against *E.coli*. Even though Chi-*graft*-PNVI (98) could not produce any measurable antibacterial effect, it might be assisting gentamicin in antibacterial action by opening new access routes into the cell. As it can be observed from these results, Chi-*graft*-PNVI*(25) combined with gentamicin provided an antibacterial activity three times as much as that of gentamicin alone against *E.coli*. The antibacterial activity of Chi-*graft*-PNVI*(25) combined with gentamicin was twice as much as that of Chi-*graft*-PNVI*(25) against *E.coli*. Chi-*graft*-PNVI (98) combined with gentamicin, chitosan combined with gentamicin exhibited an improved antibacterial activity against *S. epidermidis* in comparison to gentamicin alone.

The mobility of gentamicin through the cell membrane is increased when it is in combination with polymeric samples in comparison to gentamicin alone due to the cationic nature of the samples. Repulsion forces develop between the polymeric sample and gentamicin causing increased mobility through the cell membrane.

Inoculated *E. coli* on COS was used as control in Figure 6 (a). SEM images shown in Figures 6 (b) and 6 (c) demonstrate the cell damage caused by gentamicin and Chi-*graft*-PNVI*(25) in combination with gentamicin respectively against *E. coli.* The level of cellular disruption increases with the addition of chitosan-*graft*-PNVI*(25).

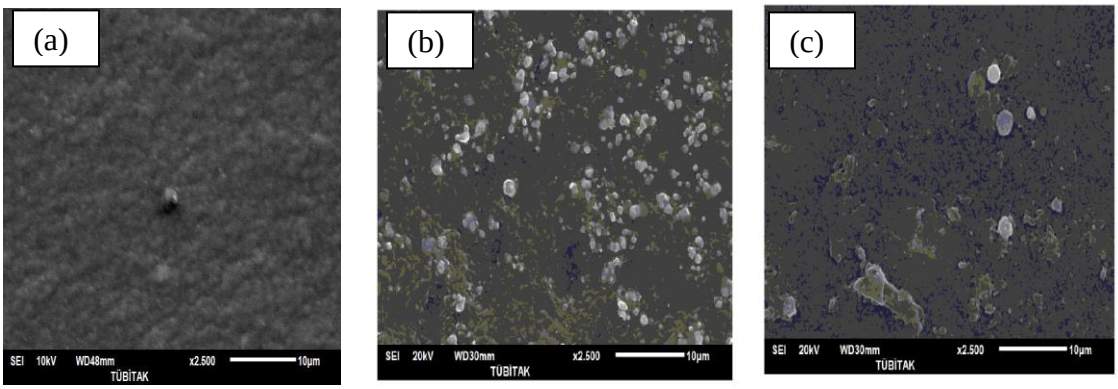


Figure 6. SEM images of (a) control, the cell damage caused by (b) gentamicin and (c) gentamicin combined with chitosan-graft-PNVI*(25) against E. coli.

It was also demonstrated what could be bacteriostatic activity instead of bactericidal activity against both bacteria by our examined samples. The samples were taken inside the inhibition zone formed by gentamicin, chitosan, Chi-*graft*-PNVI (98), Chi-*graft*-PNVI*(25) and their combination were applied to the surface of a new COS agar. After 1 day of incubation, bacteria growth was observed on the agar. Therefore, inhibition of cell development instead of cell death was caused by these tested samples or their combinations. Although the exact mechanism cannot be explained by this simple

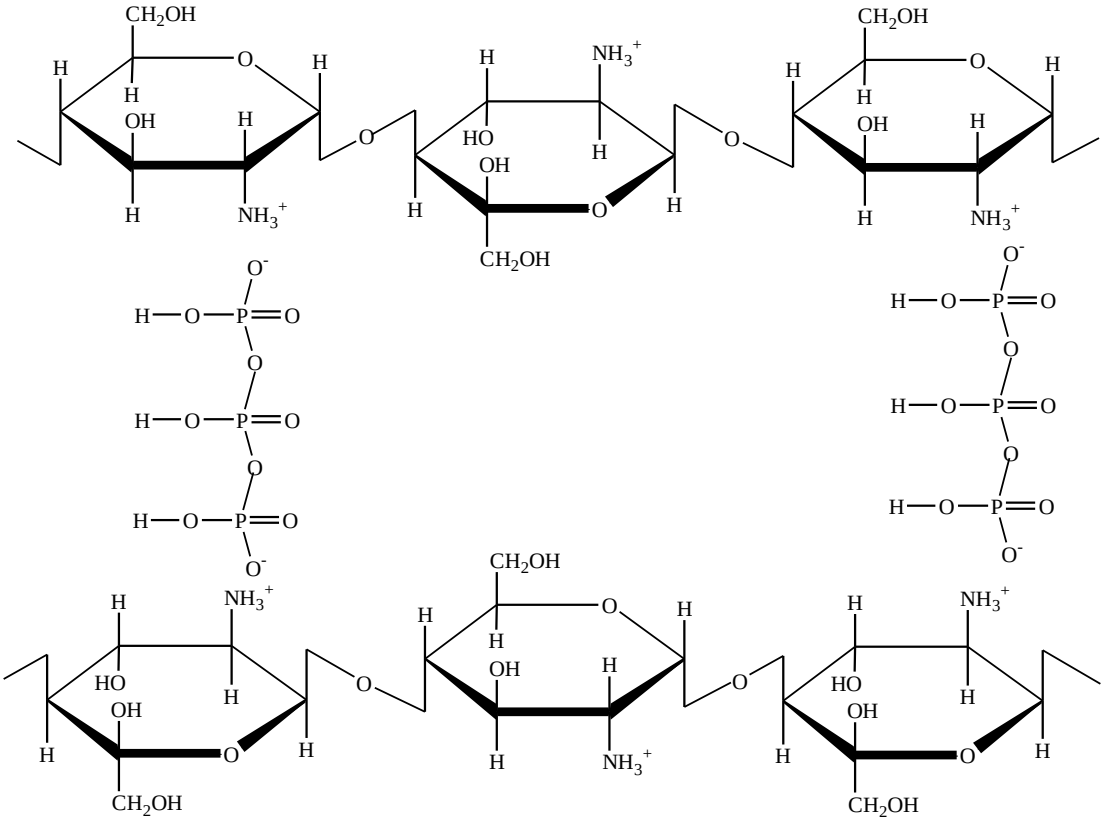
methodology, there are two factors causing bacteriostatic activity. One of the reasons might be that the examined samples interfere with the production of essential proteins needed for the bacteria division in order to multiply fast or properly. Having concentration dependent antibacterial activity leads to bacteriostatic effect rather than bactericidal effect.

4.2 Blood Interactions of Chitosan Derivatives

4.2.1 Chitosan-TPP Gels

Ionically crosslinked chitosan gels are formed by interaction between negatively charged ions, either anions or anionic macromolecules, and protonated amine groups of chitosan [42]. The nature of electrostatic interactions depends on the type, size, charge density of crosslinking agent, charge density of chitosan during the reaction and pH of medium. Physical crosslinking by electrostatic interactions instead of chemical crosslinking is preferable due to avoidance of the possible toxicity. TPP is the most common ionic crosslinker used to prepare chitosan-based systems since ionic gelation via TPP is based on mild operation parameters. TPP is a biosafe multivalent polyanion and fast gelation ability that ionically interact with chitosan [42, 118-120]. The ionic crosslinking reaction between chitosan and TPP is shown in Scheme 9 [118]. Chitosan-TPP based macro, micro and nano particles have been described in the literature [118-122]. Chitosan TPP gels have been prepared by using a technique similar to the ones described by various authors in the literature [118-120]. The basic principle behind the method is the physical gel formation via ionic cross linking between cationic chitosan and polyanions of the TPP in solution. Factors which affect the physical properties of the gels prepared include the pH of the gelling medium and the

concentrations of the gel-forming ions. Furthermore, an easy and reliable method was developed to prepare Fe^{3+} ion imprinted chitosan TPP gels with a higher potential Fe^{3+} affinity. Although a number of articles are available in the literature describing the synthesis and applications of imprinted synthetic organic polymers [123-126], those on imprinting of natural polymers is very limited [127].



Scheme 9. The ionic crosslinking reaction between chitosan and TPP adopted from [118].

This study gives an account of the preparation of nonimprinted and Fe^{3+} imprinted chitosan TPP gel beads including the effect of the preparation conditions and the crosslinking method on the physical properties such as the surface morphology, swelling and dissolution characteristics and on the Fe^{3+} adsorption behaviour. The Fe^{3+} uptake

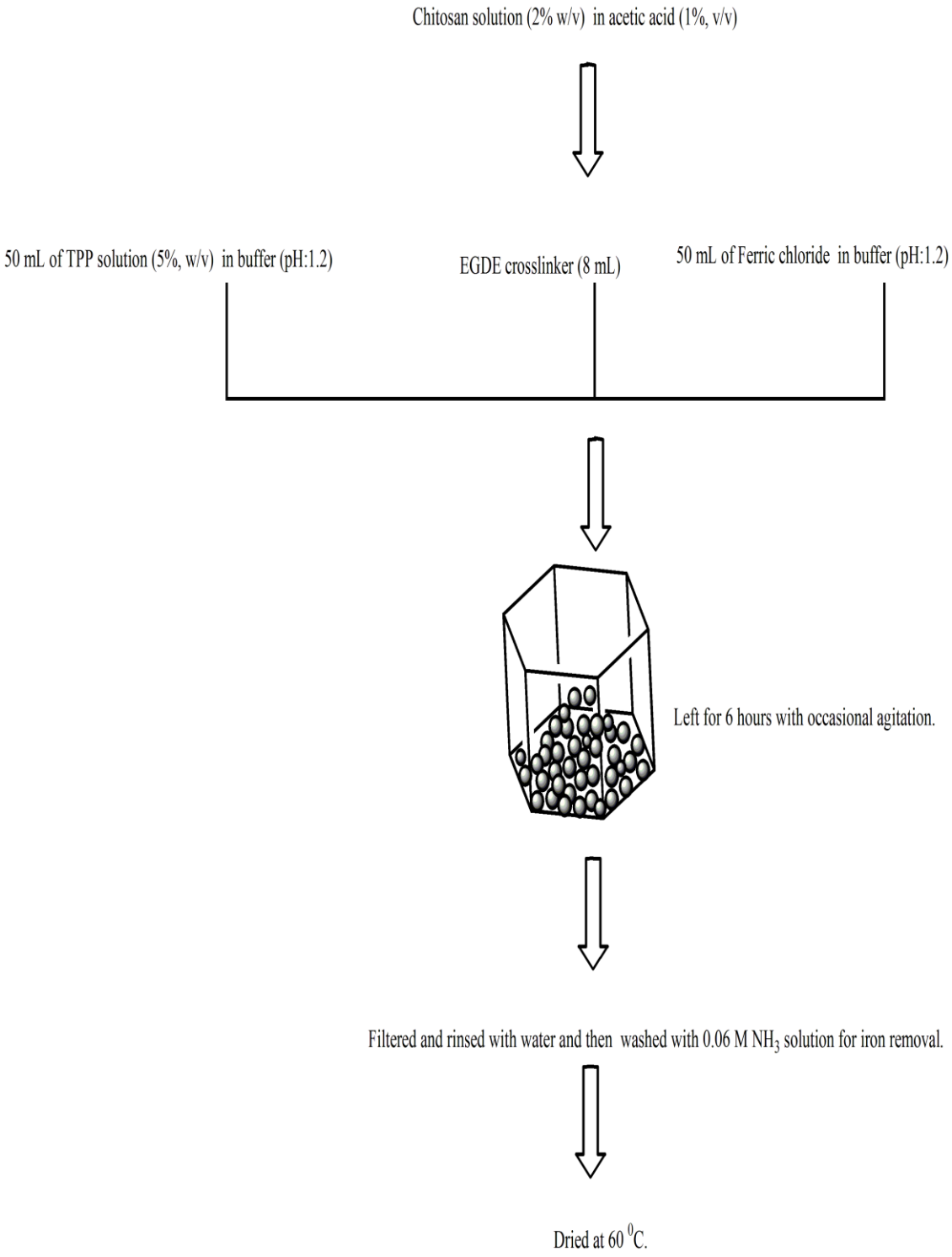
capacities of the beads were evaluated both in aqueous solution and in human blood, *in-vitro*. Accompanying changes in the haemoglobin, albumin and calcium levels have also been reported.

4.2.1.1 Bead Preparation and Bead Morphology

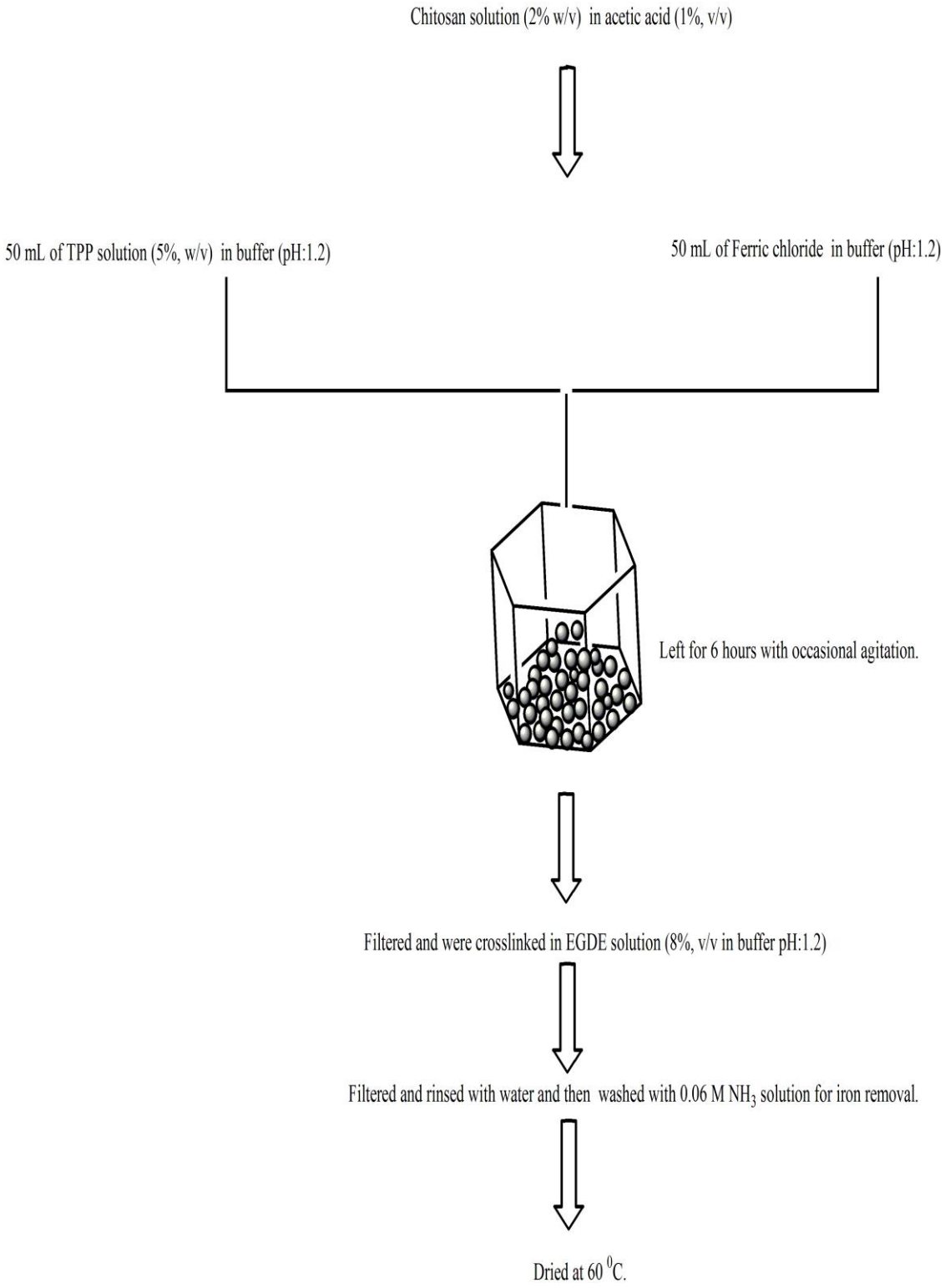
Chitosan TPP beads were prepared under acidic conditions via ionic crosslinking between cationic chitosan macromolecule and TPP ions available in the medium. Gel formation occurs due to the interaction of the polymer molecules with the polyfunctional anion leading to a three dimensional network structure. Spherical, homogeneous beads form instantaneously when the chitosan solution is dropped into the aqueous solution containing the TPP ion. Chemical crosslinking was performed in two different ways; *In-situ* crosslinking was achieved by including EGDE in TPP solution during bead formation (Scheme 10). In the second method, the *post formation* crosslinking, beads were treated with aqueous EGDE solution after they had been formed (Scheme 11).

Preparation of imprinted synthetic polymers basically involves polymerization of the monomer complexed to the template molecule or ion, in the presence of a crosslinker and a suitable porogen. After polymerization is complete, the template molecule and the excess monomer are removed from the system leaving behind an imprinted polymer with specific recognition sites. Imprinting of natural polymers with a given template involves no polymerization stage. The imprinting process needs to be achieved via crosslinking the polymer in the presence of the template followed by removal of the template and the excess crosslinker. Haemoglobin imprinted chitosan-based beads [128], Ni^{2+} ion imprinted chitosan TPP beads [129] and their

characterization has been described in the literature. In both of these studies, surface crosslinking of the chitosan beads was employed rather than carrying out imprinting in the presence of the crosslinker, as described in this article. Reports are available on the preparation of Fe^{3+} selective imprinted polymer adsorbents based on poly (HEMA) prepared by the classical imprinting technique and tested for analytical purposes [33-34]. No report could be found in the literature describing the preparation and characterization of Fe^{3+} imprinted chitosan resins or any other natural polymer. Ion imprinting was carried out by using Fe^{3+} ion as the template. Gel bead formation taking place in the presence of the Fe^{3+} ion allows inclusion of the ion in the network structure. It is known that chemical interaction between Fe^{3+} and chitosan occurs via amine and hydroxyl groups of chitosan and oxygen containing sites available in the medium [110] (Scheme 7). In this case, the phosphate groups available in the medium should also take part in the chelation process (Scheme 12, 13, 14, and 15). Removal of the template ion from the bead leaves behind Fe^{3+} imprinted network structure.

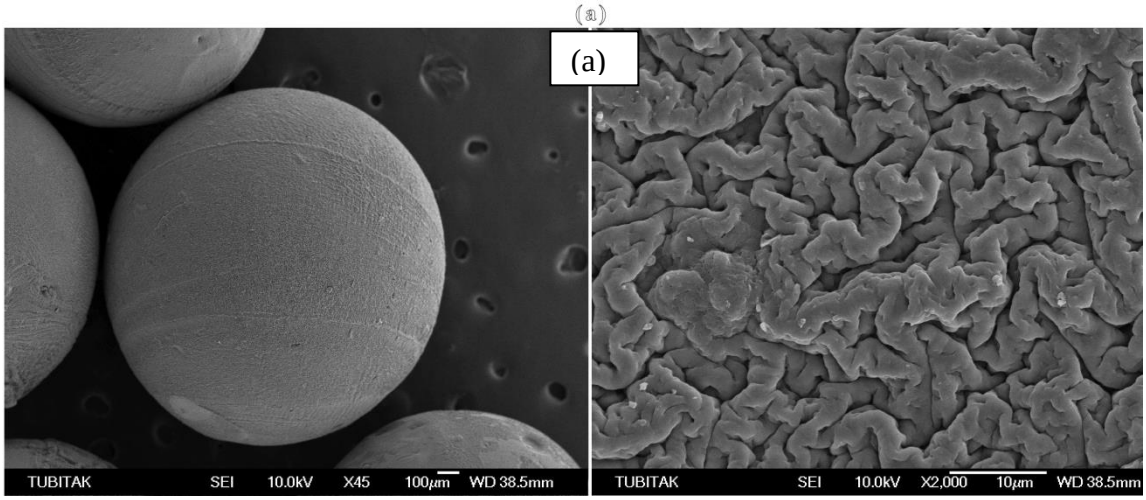


Scheme 10. Imprinted bead fabrication by *in-situ* crosslinking.



Scheme 11. Imprinted bead fabrication by *post formation* crosslinking.

The morphological features of the chitosan TPP beads prepared via *in- situ* crosslinking were studied by scanning electron microscopy (SEM) in the dry state. SEM micrograph of the samples N-SC8, I5-SC8 and I10-SC8 are shown in Figure 7 (a), (b), and (c) respectively. N-SC8, I5-SC8 and I10-SC8 were formed under similar experimental conditions, using a mixture of 5% TPP and 8% EGDE solution. I5-SC8 and I10-SC8 are Fe^{3+} imprinted samples prepared in the presence of 5 mM and 10 mM Fe^{3+} solution. N-C8 is the nonimprinted counterpart of these samples. All beads are spherical with a smooth, nonporous and homogeneous surface as observed at low magnification(x45). The effect of imprinting on the morphology is clearly observable from the micrographs taken at high magnification (x2000). The surface of I10-SC8 is smoother than that of I5-SC8 and the surface of I5-SC8 is smoother than that of N-SC8. This observation indicates that complexation with the template ion Fe^{3+} brings polymer chains closer together during bead formation. Furthermore, the chemical treatment on the beads to remove the template ion does not disturb this compact structure formed.



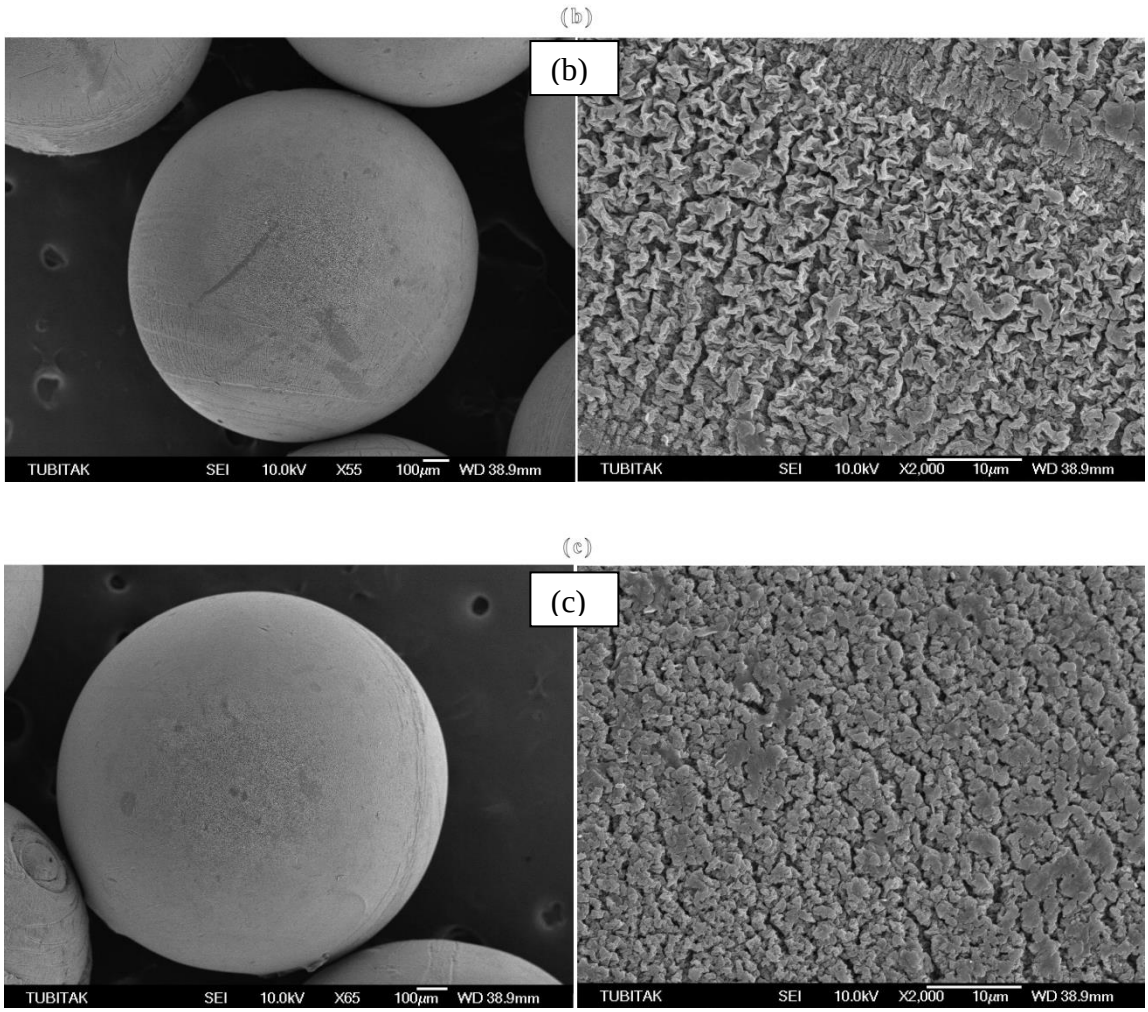


Figure 7. SEM micrographs with x45and x2000 magnification (right) of (a) N-SC8, (b) I5-SC8 and (c) I10-SC8.

4.2.1.2 FT-IR Analysis

FT-IR spectra of N-PC8, I5-PC8, I5-SC8 and N-SC8 are given in Figure 8 (a), (b), (c) and (d). In all chitosan TPP beads, in 3410-3440 cm^{-1} region O-H stretchings and 2870-2890 cm^{-1} region –C-H – stretching vibrations are observed. The C=O stretching at 1735 cm^{-1} is due to the presence of acetate ion of chitosan acetate salt. At 1642-1648 cm^{-1}, amide I band of acetamido group of chitosan is observable. N-H bending of the prototonated amino group of chitosan is available at 1570 cm^{-1}. Since bead formation

was carried out in acidic medium, TPP ion is present in its highly protonated form, the tripolyphosphoric acid, hence P-O stretching vibrations of tripolyphosporic acid are observed at 1247 cm^{-1}. At 1200-900 cm^{-1} C-O stretching vibration of the pyranose ring overlap with P-O and P-O-H stretchings. Sample I5-PC8 does not exhibit any acetate stretching, showing that this group was removed during template ion removal by ammonia. Furthermore, P-O stretching at 1247 cm^{-1} has decreased to a great extent indicating that tripolyphosphoric acid component of the post formation crosslinked and imprinted gel beads was removed during ammonia treatment.

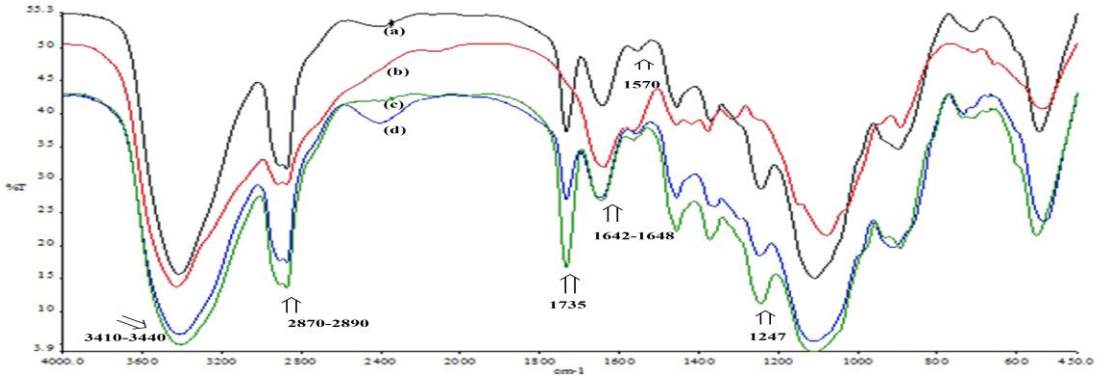


Figure 8. FT-IR spectrum of (a) N-PC8, (b) I5-PC8, (c) I5-SC8, (d) N-SC8.

4.2.1.3 XRD Analysis

The degree of crystallinity of I5-SC8, N-PC8, N-SC8 and chitosan was evaluated by X-ray diffraction method. The diffraction patterns of these samples are shown in Figure 9 (a), (b), (c) and (d) respectively. Chitosan has a strong reflection at 2θ=20°, and 2θ=10° well established in the literature [130]. The crystalline peak at 2θ=20° peak becomes broader and weaker in chitosan gel beads. The second crystalline peak at 2θ=10°, cannot be observed in the samples. The crystallinity indices of N-SC8, I5-SC8 and N-PC8 were determined as, 52.6%, 52.4% and 50% respectively. Crystallinity index of chitosan was determined as 63.6%. XRD results clearly show that

crosslinking and imprinting results in partial loss of crystallinity when compared to raw chitosan.

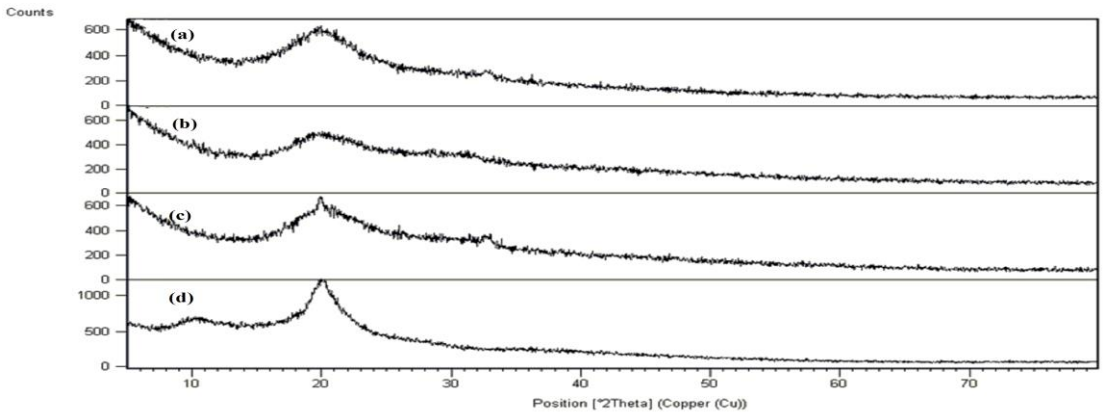


Figure 9. XRD patterns of (a) I5-SC8, (b) N-PC8, (c) N-SC8, (d) chitosan.

4.2.1.4 Differential Scanning Calorimetry (DSC) Analysis

The DSC thermograms of I5-PC8, N-PC8, N-SC8, N-PC1 and I5-SC8 beads are shown in Figure 10 (a), (b), (c), (d) and (e) respectively. Chitosan decomposes during heating with a sharp exothermic peak at 300 °C [131,132]. The I5-SC8 has a decomposition peak at 247 °C, and the N-SC8, N-PC8, NPC1 and I5-PC8 have decomposition peaks at 235 °C, 249 °C, 239 °C, and 273 °C respectively. DSC analysis reveals that all beads except for I5-PC8 have similar thermal decomposition temperatures around 240 °C indicating that they all have a similar ratio of hydrogen bonded amines-to-protonated amines. Loss of hydrogen bonding in chitosan structure is responsible from the exothermic decomposition upon thermal treatment [131]. It can be inferred from the method of bead preparation that I5-PC8 bears a much higher fraction of hydrogen bonded amine groups than the others as explained below. During preparation of imprinted and post crosslinked samples like I5-PC8, amine groups

interact with the template ion, Fe^{3+} during gel formation. After the gel is formed, the ammonia treatment serves a dual purpose; removal of the template ion and neutralization of the gel beads by reformation of free amine groups capable of hydrogen bonding. Hence, I5-PC8 has a much higher thermal decomposition temperature (273 °C) than the others. The endotherms observed in the other samples at around 260 °C and 270 °C can be attributed to the presence of weak ionic interactions between protonated amine groups of chitosan and the TPP ions. The last peak around 280-290 °C should be due to the chemical crosslinks established by the reaction of chitosan with EGDE. Sample N-PC1 cannot exhibit the peak in this region because of low degree of crosslinking. The DSC results are summarized in Table 15.

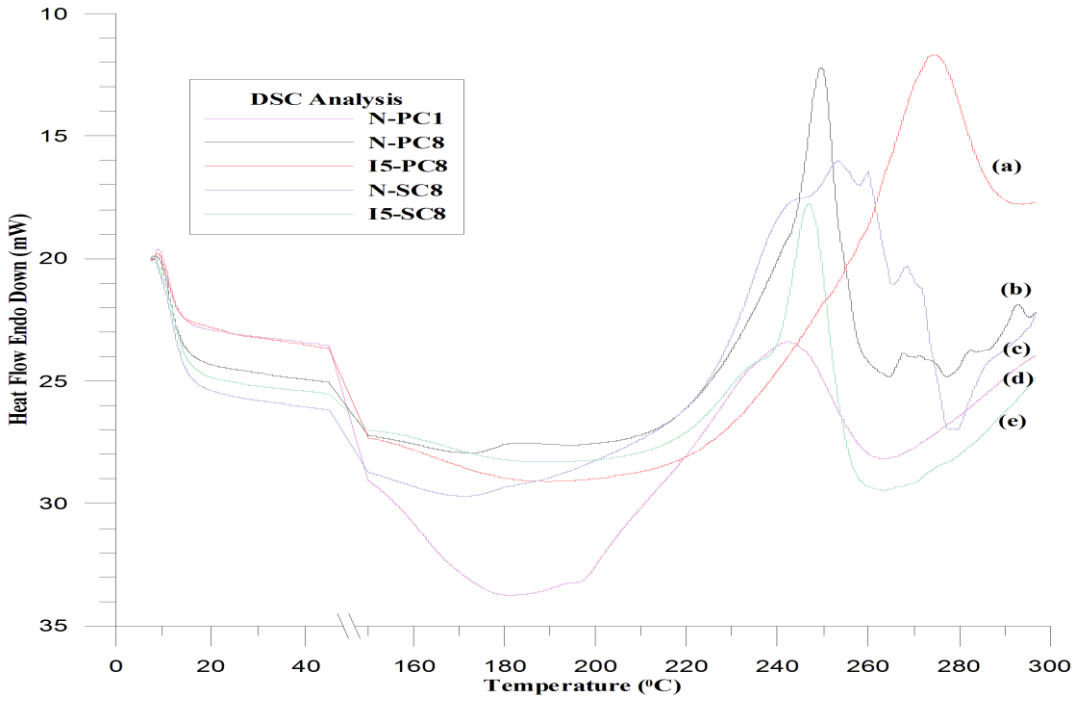


Figure 10. DSC thermogram of (a) I5-PC8, (b) N-PC8, (c) N-SC8, (d) N-PC1 and (e) I5-SC8.

Table 15. DSC analysis of chitosan, N-PC1, N-PC8, I5-PC8, N-SC8 and I5-SC8.

Sample ID	Exotherm (°C)	Endotherm (°C)
Chitosan	300 [131]	-
I5-PC8	273	-
N-PC8	249	260, 275, 290
N-PC1	239	262 (broad)
I5-SC8	235	260 (broad), 270(shoulder), 282 (shoulder)
N-SC8	235	258, 267, 278

4.2.1.5 Physical and Chemical Characteristics of the Chitosan Gel Beads

An account of the overall physical and chemical properties of the gel beads is given below to help to understand the correlation between the physicochemical characteristics and the swelling/dissolution and Fe^{3+} adsorption behaviour in solution. Results of SEM, FT-IR, XRD, and DSC analyses reveal that:

1. All beads have smooth, nonporous surfaces, with layers becoming closer in imprinted and/or crosslinked surfaces. Hence physicochemical processes like swelling and ion adsorption should be taking place mainly on the bead surface. Diffusion of small molecules or ions into inner depth should be limited by the nonporous surface.

2. Gel beads are formed due to weak ionic interactions between protonated chitosan and TPP ions available in the medium under acidic conditions, and chemical crosslinking between the free amines and the etheric end groups of EGDE. Strong ionic interactions are missing as the bead formation took place in acidic medium causing the TPP ion be protonated to a great extent.

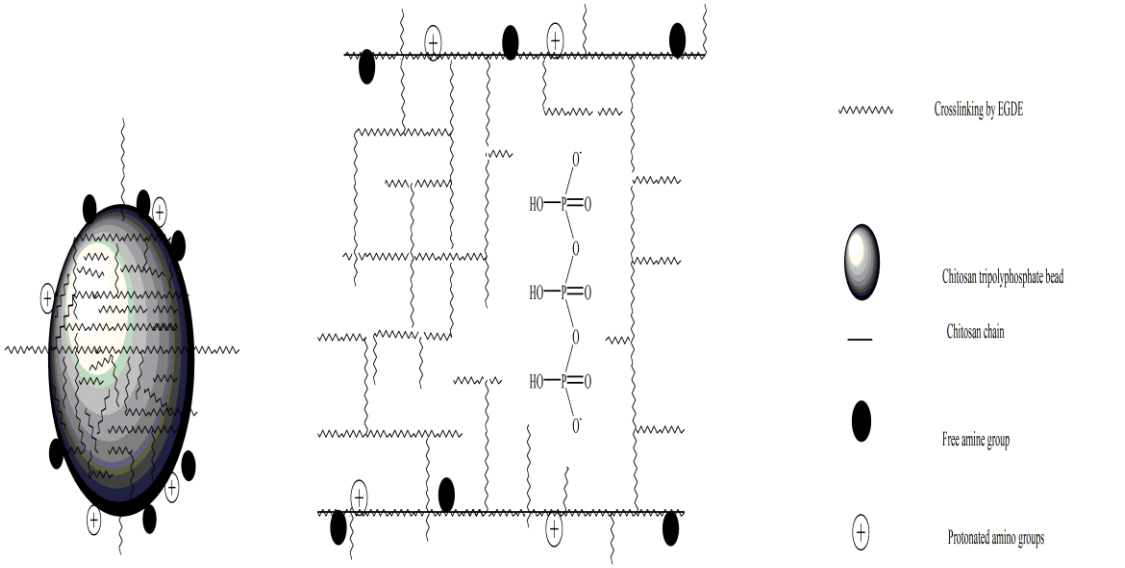
3. During imprinting Fe^{3+} ion interacts with amine and oxygen containing groups available in the medium. Ammonia treatment leaves behind imprinted gel beads with unprotonated amine groups.
4. Crystallinities of all samples are close to each other, hence swelling or Fe^{3+} adsorption should be interpreted independent of sample crystallinity.

4.2.1.6 Swelling and Dissolution Behaviour

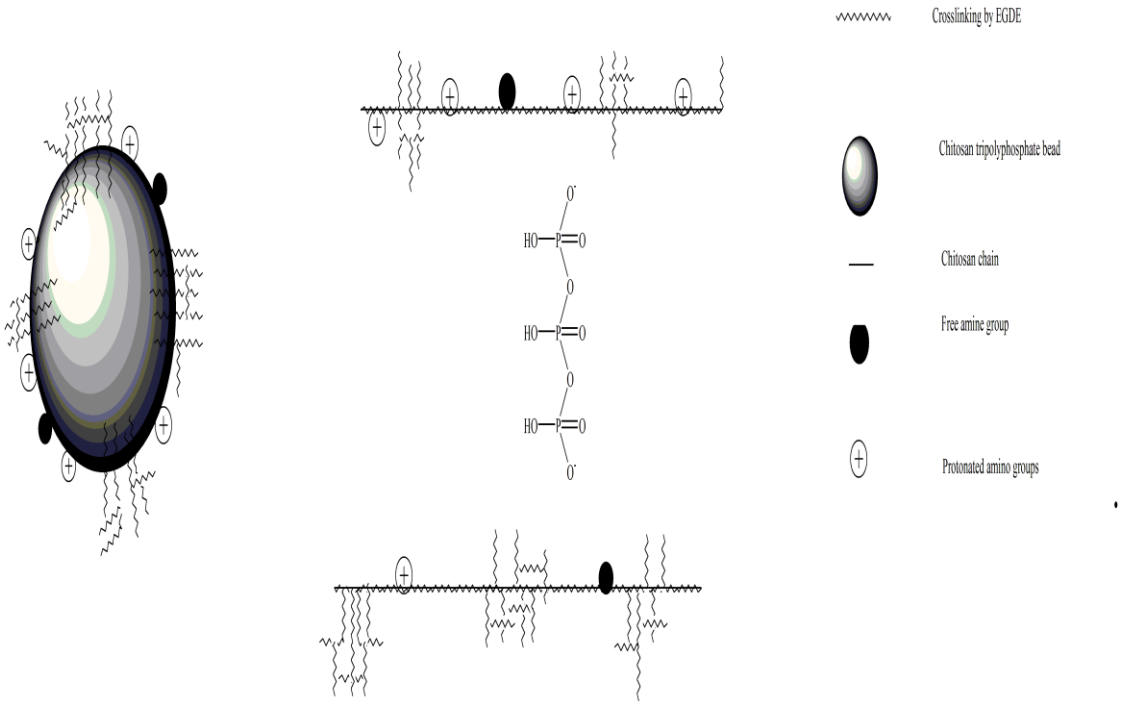
Swelling characteristics of the beads were studied in aqueous solution at pH=1.2, the same pH as the adsorption experiments were carried out. The swelling behaviour of the samples is shown in Figure 11. All beads exhibited similar swelling kinetics. They swelled and reached an equilibrium swelling capacity in time. Noncrosslinked samples as exemplified by samples N and I5 swell to dissolve within 6 hours time. Crosslinking decreases the swelling capacity but protects the samples from dissolving. Therefore, it is necessary to perform chemical crosslinking to be able obtain useful adsorbents. Increasing EGDE concentration used for the reaction, inversely affects the swelling capacity. This effect is illustrated for the nonimprinted samples that were crosslinked via *post formation* crosslinking. For example, N-PC1 swelled to an equilibrium value of 125%, N-PC4 to 62% and N-PC8 to 50%. Not only the degree of crosslinking but also the method of crosslinking affects swelling capacity. Sample N-SC8 prepared via *in-situ* crosslinking using 8% EGDE solution, has an equilibrium swelling capacity of 104% which is higher than that of its counterpart prepared by *post formation* crosslinking, N-PC8. This sample has an equilibrium swelling capacity of 62%. Higher swelling capacity of the *in-situ* crosslinked sample can be attributed to the chemical structure of the bead surface after crosslinking. Repulsion between the

positively charged protonated amine groups is responsible from swelling taking place in acidic medium. During *post formation* crosslinking amine groups on the bead surface are more susceptible to reaction with EGDE molecules. Hence the fraction of free amine groups on the surface of this type of beads is less than that of the *in-situ* crosslinked beads. Therefore, beads crosslinked by *post formation* crosslinking would bear less number of protonated amine groups in acidic medium causing less swelling (Scheme 12 and 13). *In-situ* crosslinked beads, whether imprinted or not, were durable in aqueous medium for weeks without any measurable weight loss, while the *post formation* crosslinked ones disintegrated at the end of 24 hours showing that a weaker network with a lower crosslinking density was formed by *post formation* crosslinking.

Imprinting brings about a huge increase in the swelling capacity. Noncrosslinked but imprinted sample I5 has an equilibrium % swelling value of 1113. *In-situ* crosslinked and imprinted beads I5-SC8 and I10-SC8 have equilibrium % swelling capacity values of 1152% and 1794% respectively. It can be concluded that a high crosslinking density chitosan-TPP-EGDE network is formed during *in-situ* crosslinking and the network could accommodate the template ion successfully via a complex formation. Furthermore, the ammonia treatment applied to remove the template ion did not disturb the chitosan- TPP-EGDE network formed but resulted in neutralization of the protonated amine groups. Hence, these beads bear free amine groups in the dry state. As discussed above, swelling depends primarily on the repulsion between protonated amine groups. As the imprinted beads bear a higher fraction of free amine groups in the dry state, they have a higher potential to swell via protonation of these free amine groups in the acidic swelling medium (Scheme 10 and 11).



Scheme 12. The sketch of nonimprinted beads by *in-situ* crosslinking.



Scheme 13. The sketch of nonimprinted beads by *post formation* crosslinking.

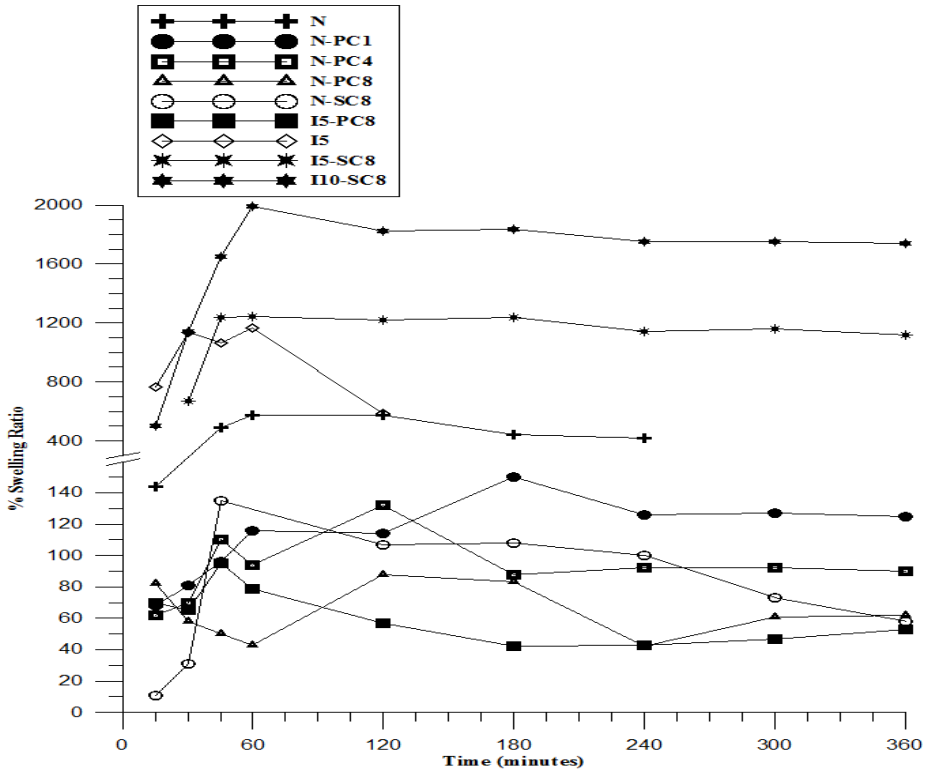
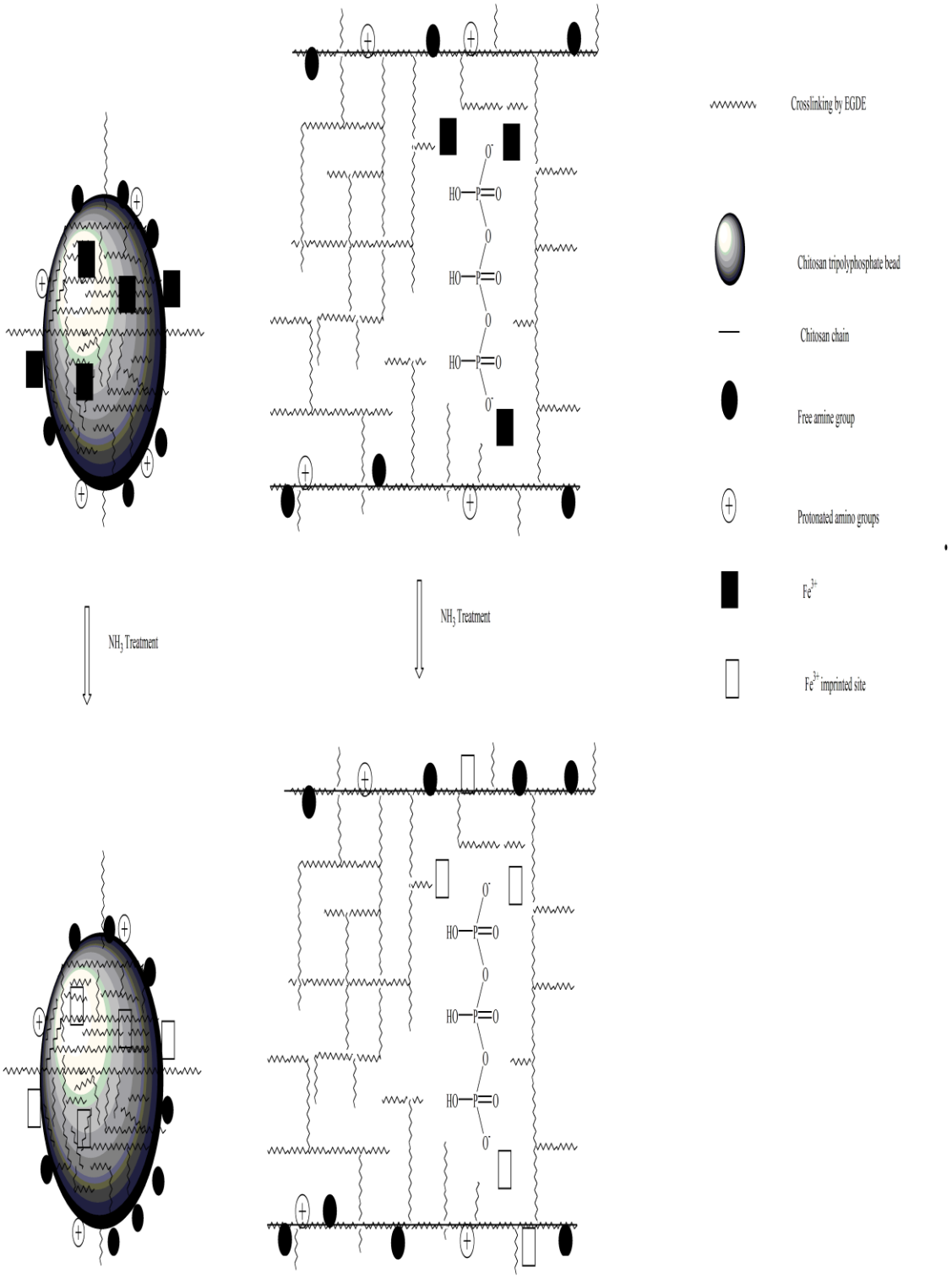
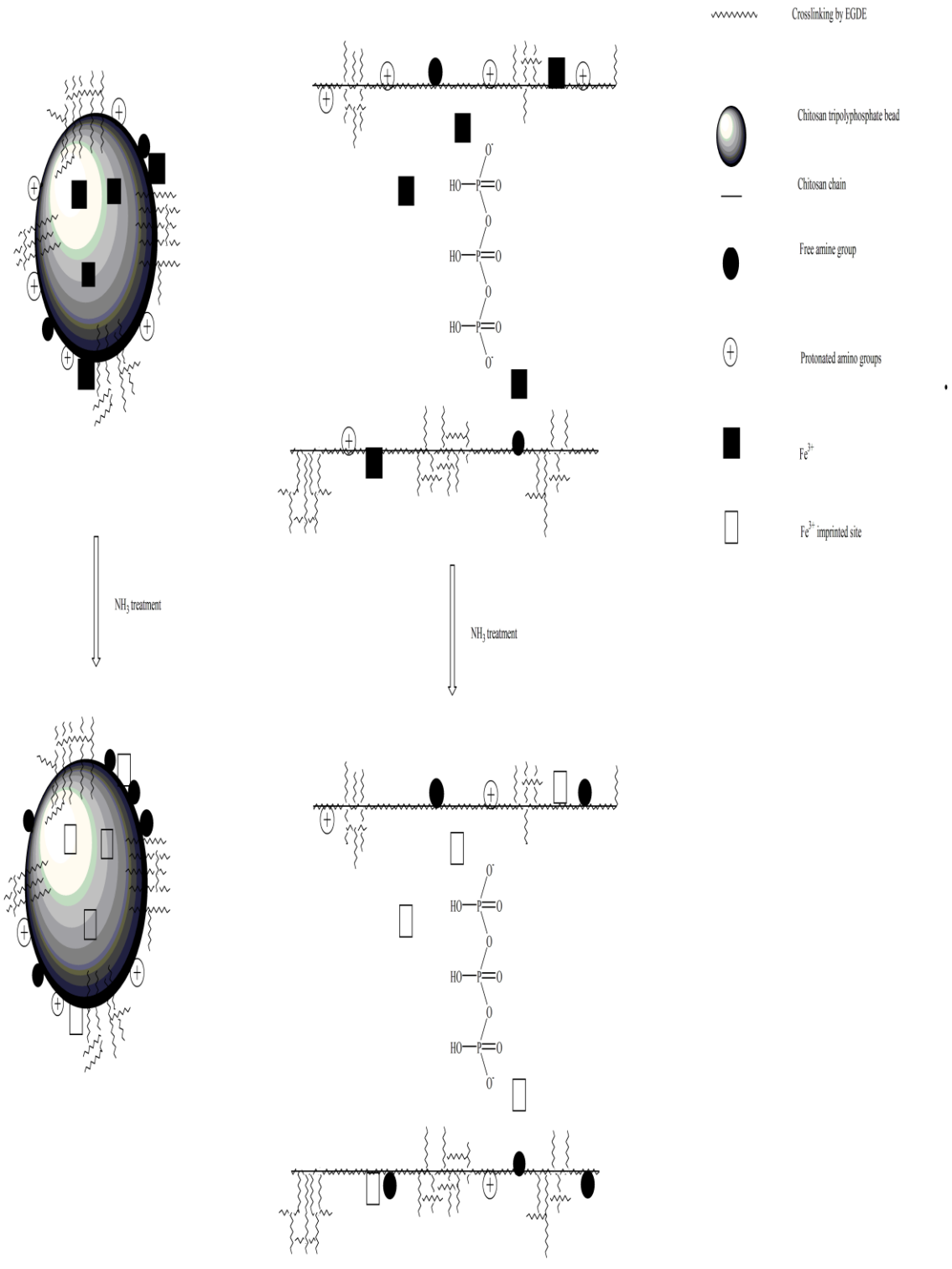


Figure 11. The swelling characteristics of the beads (N-PC1, N-PC4, N-PC8, I5-PC8, N-SC8, I5, I5-SC8, I10-SC8) in aqueous solution at pH=1.2.



Scheme 14. The sketch of imprinted beads by *in-situ* crosslinking.



Scheme 15. The sketch of imprinted beads by *post formation* crosslinking.

4.2.1.7 Fe^{3+} Adsorption Behaviour

SEM analysis given above revealed a smooth, homogeneous and nonporous surface for the chitosan TPP beads (Figure 7). The surface is even smoother, and more compact for the Fe^{3+} imprinted samples. This finding indicates that Fe^{3+} adsorption occurs mainly on the bead surface as lack of pores limits diffusion of ions and molecules into inner depths. The Fe^{3+} ion adsorption behaviour of chitosan TPP beads prepared under various conditions was followed in aqueous solution. The dependence of the Fe^{3+} adsorption capacity on solute concentration and on the structural characteristics of the beads such as crosslinking and imprinting was studied. The adsorption capacity values determined for each sample are listed in Table 16.

Table 16. The swelling and Fe^{3+} adsorption capacities at equilibrium for prepared imprinted chitosan gel beads (in 1% v/v acetic acid solution using 2% (w/v) chitosan solution in TPP dissolved at pH=1.2 buffer).

Sample ID	**Equilibrium swelling (%)**	**Equilibrium Fe^{3+} adsorption capacity (mg) /g chitosan ±Standard deviation**	
		1mM $FeCl_3$	**5mM $FeCl_3$**
N-PC1	125	24.6±0.283	30.7±0.0354
N-PC4	90	20.5±0.0566	21.7±0.212
N-PC8	62	12.9±0.424	19.8±0.184
I5-PC8	50	10.2±0.198	13.1±0.00141
N-SC8	104	17.4±0.0283	39.1±0.0989
I5	1113	9.5±0.0566	31.2±0.0141
I5-SC8	1152	15.9±0.141	39.5±0.282
I10-SC8	1794	17.1±0.0707	53.9±0.0424

It can be followed from Table 16 that all chitosan TPP beads studied have higher equilibrium adsorption capacity values towards Fe^{3+} in 5 mM solution than in 1 mM solution. A higher concentration gradient results in higher adsorption from solution.

Adsorption capacity is limited by the number of available sites for chelation. The equilibrium adsorption capacities of N-PC1, N-PC4 and N-PC8 are 30.7, 21.7 and 19.8 mg/g showing a tendency to decrease with increasing EGDE concentration in the medium since the crosslinking reaction between EGDE and amino groups of chitosan results in a decrease in the fraction of free amino groups on the surface. Fe^{3+} adsorption capacity of the *post formation* crosslinked and Fe^{3+} imprinted and nonimprinted chitosan TPP beads was compared to each other to follow the effect of Fe^{3+} imprinting on the adsorption capacity. Adsorption capacity values of nonimprinted and Fe^{3+} imprinted chitosan TPP beads in 1 mM and 5 mM $FeCl_3$ solution are shown in Table 16. The imprinted sample, I5-PC8 has a lower equilibrium adsorption capacity (13.1 mg/g) than its nonimprinted counterpart, N-PC8. Even though imprinted sites are available on the surface of I5-PC8, amine groups have taken part in the crosslinking reaction on the surface. Hence, they cannot participate in iron chelation. The *in-situ* crosslinked counterpart (I5-SC8), on the other hand, is much more effective (39.5 mg/g) than I5-PC8 or N-PC8. The reason lies behind the fact that crosslinking has occurred homogeneously during bead formation. Specific loss of amine groups on the surface to crosslinking has been avoided. Hence, chelation of Fe^{3+} on the surface may occur more effectively leading to a higher adsorption capacity. The favorable effect of imprinting is more pronounced in the case of I10-SC-8 which has an equilibrium adsorption capacity of 53.9 mg/g.

4.2.1.8 Iron Removal in Human Blood *in-vitro* and a Short Account of Accompanying Changes in the Blood Composition

It is known that about 60% of the iron in human body is found in haemoglobin, 5% in myoglobin. The rest 35% is stored as ferritin or haemosiderin. A very small amount is transported between the body compartments as bound to transferrin. There is a balance between the iron containing species in the blood. In cases of deficiency of iron or iron overload a complex set of biochemical reactions are triggered to overcome the anomaly. Iron removal from blood samples of healthy volunteers was studied by following the serum iron content in the blood serum which is a measure of the transferrin-bound iron. The results are shown in Table 17. Same amounts of *in-situ* crosslinked beads (N-SC8, I5-SC8, I10-SC8) and *post formation* crosslinked beads (N-PC8) was brought into contact with the 500 μL of blood samples for 3 hours. The general behaviour observed was that the imprinting had a significant effect on the iron removal capacity. The *in-situ* crosslinked but nonimprinted beads (N-SC8) removed only 15 μg/dL in blood sample of *sample 1* which corresponds to 20.3% decrease. The *in-situ* crosslinked and Fe^{3+} imprinted beads I5-SC8 and I10-SC8, on the other hand, decreased the serum iron level from74 μg/dL to 17 μg/dL, and to 14.5 μg/dL respectively. These values correspond to 77.0 % and 80.4% decrease in the serum iron level. Sample N-PC8 decreased the serum iron level by 35.1%. A similar trend was been observed in *sample 2* as shown in Table 17. N-SC8, I5-SC8, I10-SC8 and N-PC8 induced a 28.3%, 47.2%. 61.3% and 31.2% decrease in the serum iron level respectively.

One important effect accompanying iron uptake in blood was the change induced on the haemoglobin level. The beads were not only effective in reducing the

serum iron level but they also induced a parallel decrease in the level of the iron containing protein haemoglobin as shown in Table 17. It can be followed that the trend observed for the serum iron removal is valid for haemoglobin level as well. Each sample induced an identical change in the haemoglobin level as it induced for the serum iron level as can be followed in Table18. In *sample 1*, contact with N-SC8 results in a 20.3% decrease in the haemoglobin level as compared to 18.9% decrease in the serum iron level. Similarly I4-SC8, I10-SC8 and N-PC8 induce a 77.0%, 80.4% and 34.5% decrease in the haemoglobin level respectively which is all comparable to the serum iron level changes observed as 77.0%, 80.4% and 35.1% respectively. A similar effect is true for the *sample 2* as well. Hence, it can be deduced from the above given results that when iron present in blood is removed upon contact with the chitosan TPP beads, the iron balance is disturbed. This disturbance is reflected as a decrease in the serum iron level. Further detailed analysis is needed to understand how iron metabolism is affected upon contact with the chitosan TPP beads. Contrary to the decrease in haemoglobin level upon contact with chitosan TPP beads with human blood, a higher reading of free albumin level was obtained. Chitosan is known to have an affinity towards albumin. Hence a decrease in free albumin level would have been the expected behaviour. It can be followed from Table 18 that upon contact with the beads, an interdependent calcium:albumin level disturbance occurs in the most unexpected way. While this is only a slight disturbance in the case of the Fe^{3+} imprinted beads (I10-SC8), it was more pronounced for the nonimprinted ones (N-SC8). In the first blood sample there is a deceptive increase in the albumin level from 58.7 g/L to 73.2 and 60.2 g/L upon contact with N-SC8 and I10-SC8 respectively. Similarly, in blood samples of *sample 2* and *3*, the albumin level apparently increases from 81.2 g/L to 95.1 and 93.8 g/L and from 82.4

g/L to 92.3 and 83.6 g/L upon contact with nonimprinted and imprinted samples respectively. As the total albumin content in blood should remain constant, this change should be due to the detection of free albumin unbound from other biochemical entities to which it was previously bound. This behaviour can be explained by looking at the calcium level changes. Chitosan has an affinity towards calcium and it is known that calcium and albumin levels are interdependent as albumin is the carrier protein for calcium. It can be observed from Table 18 that the nonimprinted sample (N-SC8) adsorbs all calcium in all three blood samples studied. The imprinted sample ((I10-SC8), on the other hand, adsorbs only a fraction of calcium present in the blood sample. When calcium complexed to albumin is transferred to chitosan gel beads, the protein albumin is set free, and hence an increase in the albumin level is recorded. The fact that the imprinted beads do not adsorb all calcium as the nonimprinted ones, is a good indication of their selectivity towards iron bearing molecules in the blood. Although, it is not clear whether it is the iron atom or it is the proteins and other iron carrying molecules that adsorb onto the beads, the fact that iron imprinted beads have higher iron removal capacities and lower calcium affinities than the nonimprinted ones indicates that iron atom present in the iron containing entities should have a role in the adsorption process.

Table 17. Haemoglobin and serum iron level changes in the blood samples of healthy samples after 3 hours of contact *in-vitro* (10 mg of beads with 500 μL blood).

Initial Haemoglobin and iron level	**Final Haemoglobin and iron level, (% *decrease)***			
Sample 1	N-SC8	I5-SC8	I10-SC8	N-PC8
Haemoglobin (14.8 g/dL)	12.0, *(18.9%)*	3.5 *(76.4%)*	2.9, *(80.4%)*	9.7, *(34.5%)*
Iron (74 μg/dL)	59.0, *(20.3%)*	17.0, *(77.0%)*	14.5, *(80.4%)*	48.0, *(35.1%)*
Sample 2				
Haemoglobin (10.6 g/dL)	7.6, *(28.3%)*	5.8, *(45.3%)*	4.2, *(60.4%)*	7.2, *(32.1%)*
Iron (53μg/dL)	38.0, *(28.3%)*	28.0, *(47.2%)*	20.5, *(61.3%)*	36.0, *(32.1%)*

It is also needed to understand if (i) changes other than iron, haemoglobin and albumin levels such as cholesterol level are induced in the blood composition upon contact with the chitosan -based beads and (ii) if the Fe^{3+} imprinted beads would selectively reduce iron in the case of high free and/or stored iron concentrations, i.e. iron overload. More widespread analysis using simulated blood samples and a higher number of real samples is needed to be able to generalize these results and understand the chitosan-based biomaterial-blood interactions.

Table 18. Albumin level changes and total calcium affiinity from blood serums of healthy samples after 3 hours. (blood serum with polymer contact *in-vitro*)

	N-SC8	**I10-SC8**
Sample 1		
Albumin: 58.7 g/L	73.2 g/L	60.2 g/L
Total Calcium: 4.0 mg/dL	0 mg/dL	1.9 mg/dL
Sample 2		
Albumin: 81.2 g/L	95.1 g/L	93.8 g/L
Total Calcium: 7.5 mg/dL	0 mg/dL	6 mg/dL
Sample 3		
Albumin: 82.4 g/L	92.3 g/L	83.6 g/L
Total Calcium: 15.2 mg/dL	0 mg/dL	3.6 mg/dL

4.2.1.9 Prothrombin Time Measurements

The blood samples used were selected from one healthy volunteer and one volunteer using warfarin. Warfarin is probably the best known and most widely used oral anticoagulant. It is used to limit the size of existing blood clots and to prevent formation of new blood clots in high-risk patients. TPP, chitosan and chitosan TPP beads values examined have been shown to delay blood coagulation. The prolonged prothrombin time and extension of the clotting time were observed with blood contact chitosan flake, TPP and chitosan TPP beads (Table 19). TPP had the strongest blood thinning effect. According to obtained data (Table 19), TPP had the highest anticoagulant activity; due to its polyanionic character it caused a huge increase in the prothrombin time. When same amounts of *in-situ* non imprinted crosslinked beads (N-SC8) and *post formation* crosslinked beads (N-PC8) was brought into contact with the

blood samples, N-PC8 and N-SC8 increased prothrombin timefrom 11.8 s to 14.5s and 18.7s, respectively. Since the beads prepared by *post formation* crosslinking are poorer in the amine groups on the bead surface when compared to *in-situ* crosslinked beads, beads crosslinked by *post formation* crosslinking would have weaker anticoagulant activity. We found that N-PC8 had the minimum anticoagulant effect. Additionally, I10-SC8 and chitosan induce anticoagulation from 11.8 s to 15.4s and 15.8s, respectively. N-SC8 increased anticoagulation from 11.8 s to 18.7s whereas, in-situ imprinted crosslinked beads (I10-SC8) induced anticoagulation from 11.8 s to 15.4s.

Table 19. Prothrombin time levels (in seconds) after 3 hours of blood contact chitosan and chitosan TPP beads.

Sample ID	***Volunteer (F)***	***Volunteer (F)***
	Prothrombin time (s)	**Prothrombin time* (s)**
Blank	11.8	25.0
Chitosan	15.8	32.0
TPP	>200	>200
N-PC8	14.5	31.0
N-SC8	18.7	40.1
I10-SC8	15.4	31.8

*volunteer who is using warfarin medicine.

4.2.1.10 CBC Measurements

Since *in-situ* imprinted crosslinked beads (I10-SC8) reduced least RBC count in comparison to that of examined samples. The findings from CBC compositions demonstrated that imprinting is effective for heamocompatibilty (Table 20). The most hemocompatible examined sample was found as I10-SC8. The increase in the mean corpuscular volume (MCV) and hematocrit (HCT) values were indicative of the volume increase of the RBC when chitosan alone was used. A reduction was determined in PLT

values with all examined samples. TPP, N-PC8, N-SC8 and I10-SC8 values examined have been shown to delay blood coagulation. Polyanionic TPP molecules decreased PLT 49.6% but prothrombin value was determined higher than 200s. Therefore, the blood thinning effect of TPP depends not mainly reduction in PLT values. The PLT reduction was obtained with I10-SC8 and N-SC8 as 28.5% and 72.9%, respectively. In addition to this, N-SC8 had higher prolonged prothrombin time than that of I10-SC8. Anticoagulant activity of i*n-situ* non imprinted crosslinked beads (N-SC8) is much more effective than that of *in-situ* imprinted crosslinked beads (I10-SC8). According to Table 18, Ca removal capacity of N-SC8 was higher than I10-SC8. All this corresponds to speed up anticoagulation.

Table 20. The percent changes* in RBC, WBC, PLT, HCT, MCV and HGB levels in the blood samples of healthy volunteer by TPP, N-PC8, N-SC8 and I10-SC8 samples after 3 hours of contact *in vitro*.

	Normal Ranges	**Blank**	**TPP**	**N-PC8**	**N-SC8**	**I10-SC8**
RBC	*3.5-5.50 (10^6 / mm^3)*	4.39	2.62 (-40.3%)	2.03 (-53.8%)	0.69 (-84.3%)	3.26 (-25.7%)
HCT	*35.0-55.0 (%)*	32.9	20.3 (-38.3%)	15.1 (-54.1%)	5.1 (-84.5)	23.9 (-27.4)
WBC	*3.5-10.0 (10^3 / mm^3)*	4.6	3.1 (-32.6)	2.4 (-47.8%)	0.9 (-80.4%)	2.9 (-36.9%)
HGB	*11.5-16.5 (g/dL)*	9.7	6.0 (-38.1%)	4.4 (-54.6%)	1.5 (-84.5%)	7.0 (-27.8)
PLT	*100-400 (10^3 / mm^3)*	274	138 (-49.6%)	185 (-32.5%)	74 (-72.9%)	196 (-28.5%)
MCV	*75.0-100.0 (µm^3)*	75	77.6 (+3.5%)	74.1 (-1.2%)	73.5 (-2%)	73.2 (-2.4%)

4.2.1.11 Antibacterial Activity of Chitosan-TPP Beads

The antibacterial activity of all sythesized chitosan-TPP beads against *E.coli* and *S.epidermidis* was investigated. No inhibition zone was observed by any of chitosan-TPP beads. As the cationic nature of chitosan allows ionic interaction with TPP, due to this ionic interaction may hindered the antibacterial activity of chitosan-TPP beads with cell membrane.

4.2.2 Ascorbyl Chitosans

To date, in the biomedical literature there is very little data on the blood contact properties of chitosan and ascorbic acid. Based on its biosafety and weak basicity characteristics of chitosan, blood-contact ascorbyl chitosans were prepared for investigation of blood-contact properties. Blood contact activities of ascorbyl chitosans were investigated with respect to absorption of lipids and blood components, such as red blood cells, white blood cells, platelets and haemoglobin *in-vitro*.

4.2.2.1 FT-IR Analysis

In the FT-IR spectrum of chitosan shown in Figure 12 characteristic absorption bands of O-H stretching at 3435.32 cm^{-1}, N-C stretching at 2880 cm^{-1} , the amide band at 1649 cm^{-1}, the C-H bending vibrations 1400-1500 cm^{-1} region, the -CH_3 bending at 1380 cm^{-1} and the pyranose C-O-C and C-OH stretching vibrations in the region 1100-900 cm^{-1} are observable. The ascorbic acid spectrum in Figure 13 exhibits all characteristic absorption bands of -C-C, -C=C-, C-H, C-O and O-H linkages in addition to the lactone absorption bands at 1754 cm^{-1} and 1673 cm^{-1}. The ascorbyl chitosan shown in Figure 14 exhibits O-H stretching at 3488 cm^{-1}, the C-H bending vibration at 1400-1300 cm^{-1} region including the -CH_3 bending at 1380 cm^{-1}. The pyranose ring absorptions are also

available in the 1100-900 cm^{-1} region similiar to parent chitosan. The absorption bands at 1720 cm^{-1} and 1627 cm^{-1} have been taken as evidence of ester and amide bond formation between chitosan and ascorbic acid. The -C=C- bending vibrations are available at 738 and 661 cm^{-1}.

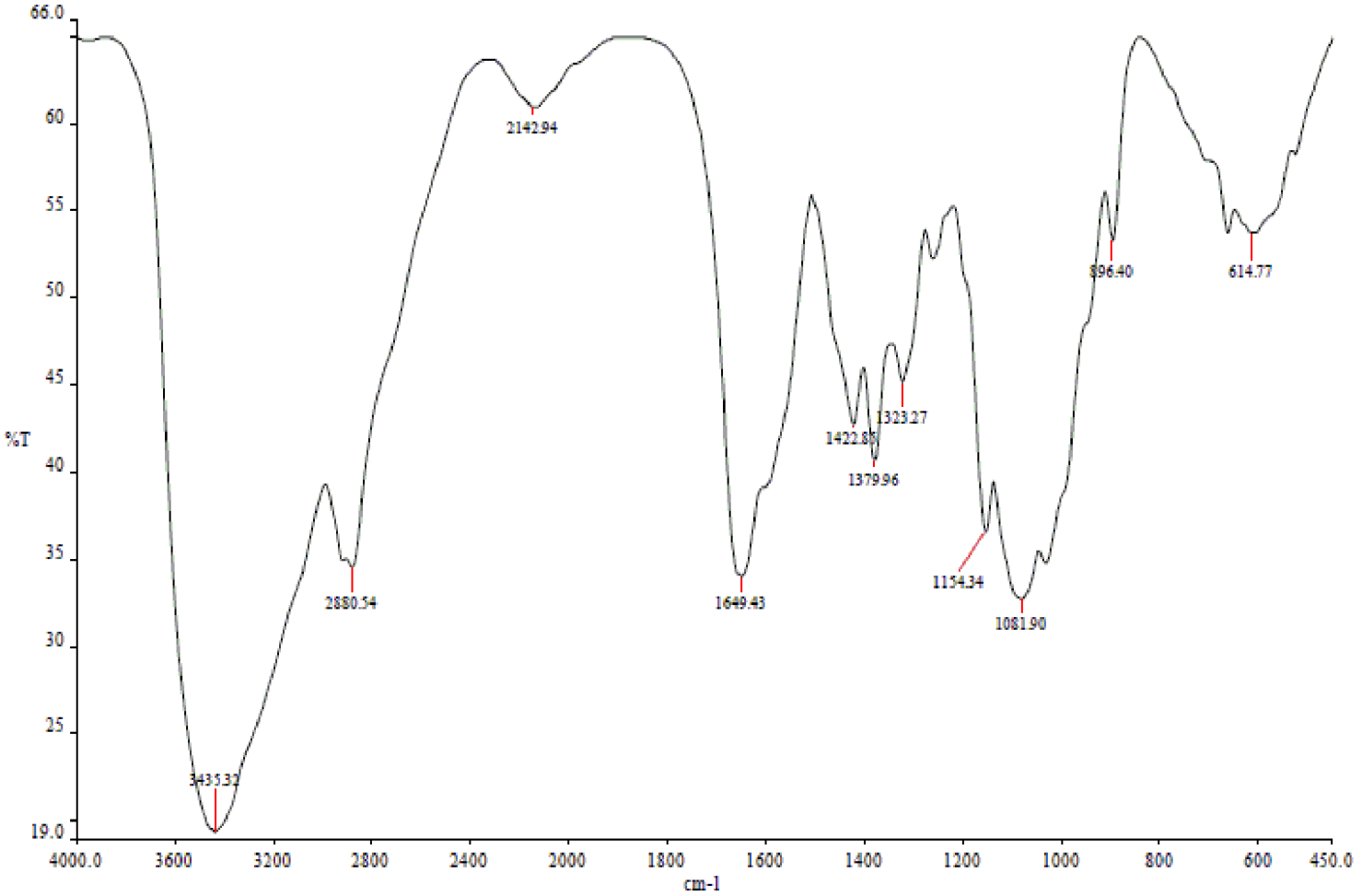


Figure 12. FT-IR spectrum of chitosan.

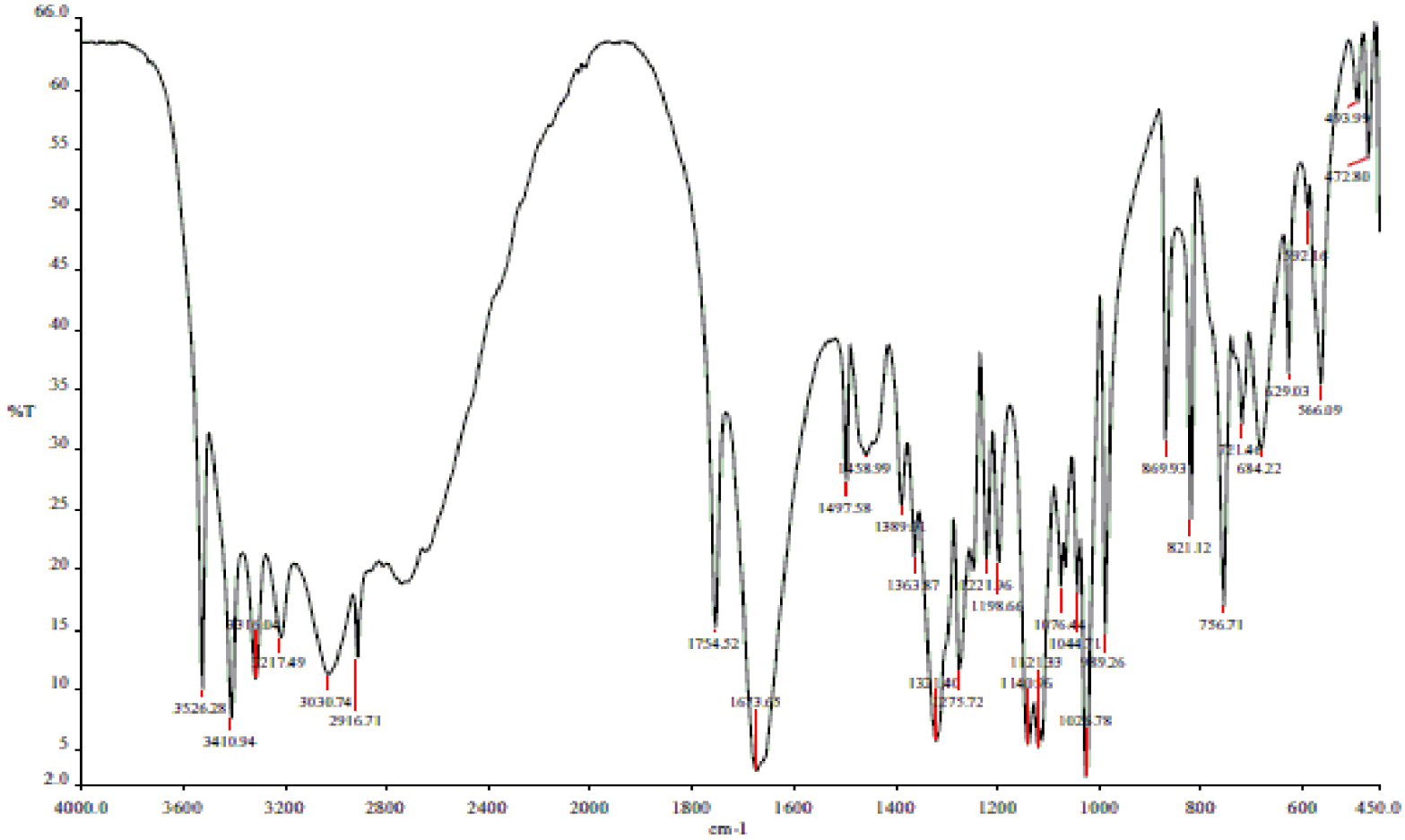


Figure 13. FT-IR spectrum of vitamin C.

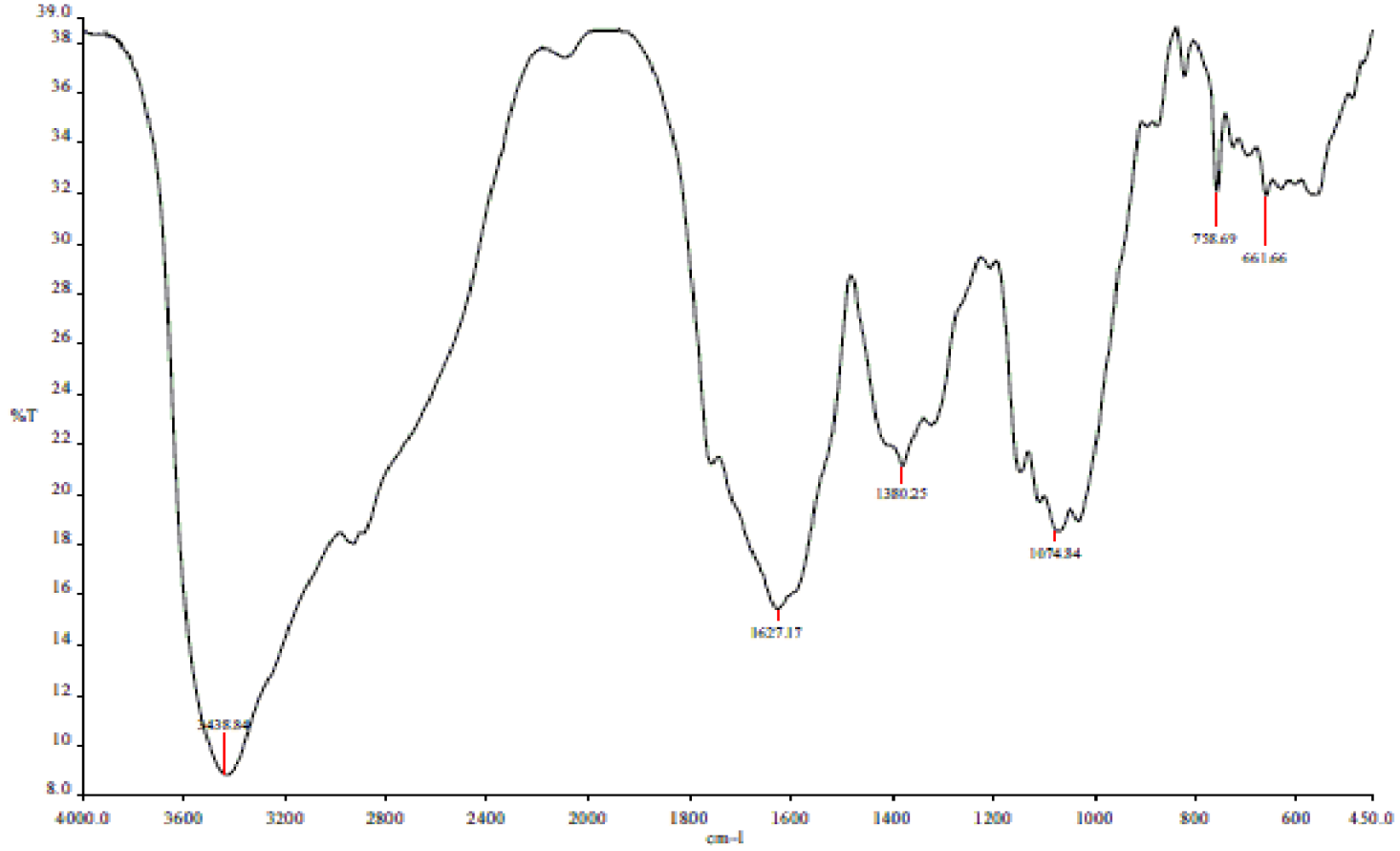


Figure 14. FT-IR spectrum of ascorbyl chitosan (ChiVC-100).

4.2.2.2 C-13 NMR analysis

C-13 NMR spectra of chitosan, ascorbic acid and ascorbyl chitosan are shown in Figures 15, 16 and 17, respectively. In the spectrum of chitosan, the signals at 169.744 ppm and 19.175 belong to the carbonyl carbon and the methyl group of the acetamide groups respectively. The signals at 100.743 ppm, 78.402 ppm, 70.913 ppm, 56.775 ppm, 53.101 ppm are assigned to the ring carbons: C-1, C-4, C-3, C5, C-6 and C-2 respectively. In the spectrum of ascorbic acid, the signal at 170.472 ppm belongs to the carbonyl carbon (C-1). The doublet signals at 151.266 ppm and 147.981 ppm are assigned to C-2. The signal at 114.111 ppm belongs to C-3. The signals at 72.109 ppm and 71.205 ppm are assigned to belong to C-4.The signal at 63.666 ppm belongs to C-5. The triplet signals at 57.587 ppm, 56.004 ppm, 54.644 ppm belong to C-6. In the spectrum of ascorbyl chitosan, the carbonyl carbon of the acetamide group of chitosan is observed at 171.251 ppm. New signals appear at 162.910 ppm and 162.319 ppm which can be attributed to the amide and ester carbonyls of the ascorbyl group. Bis hydroxy functionalities exhibit themselves at 94.467 ppm. The primary and secondary alcohol carbons of the ascorbyl group and chitosan produce overlapping signals in the 50-70 ppm range. The methyl group of chitosan is observed at 18.643 ppm. The proposed structure of the product based on FT-IR and C-13 NMR data is shown in Scheme 16.

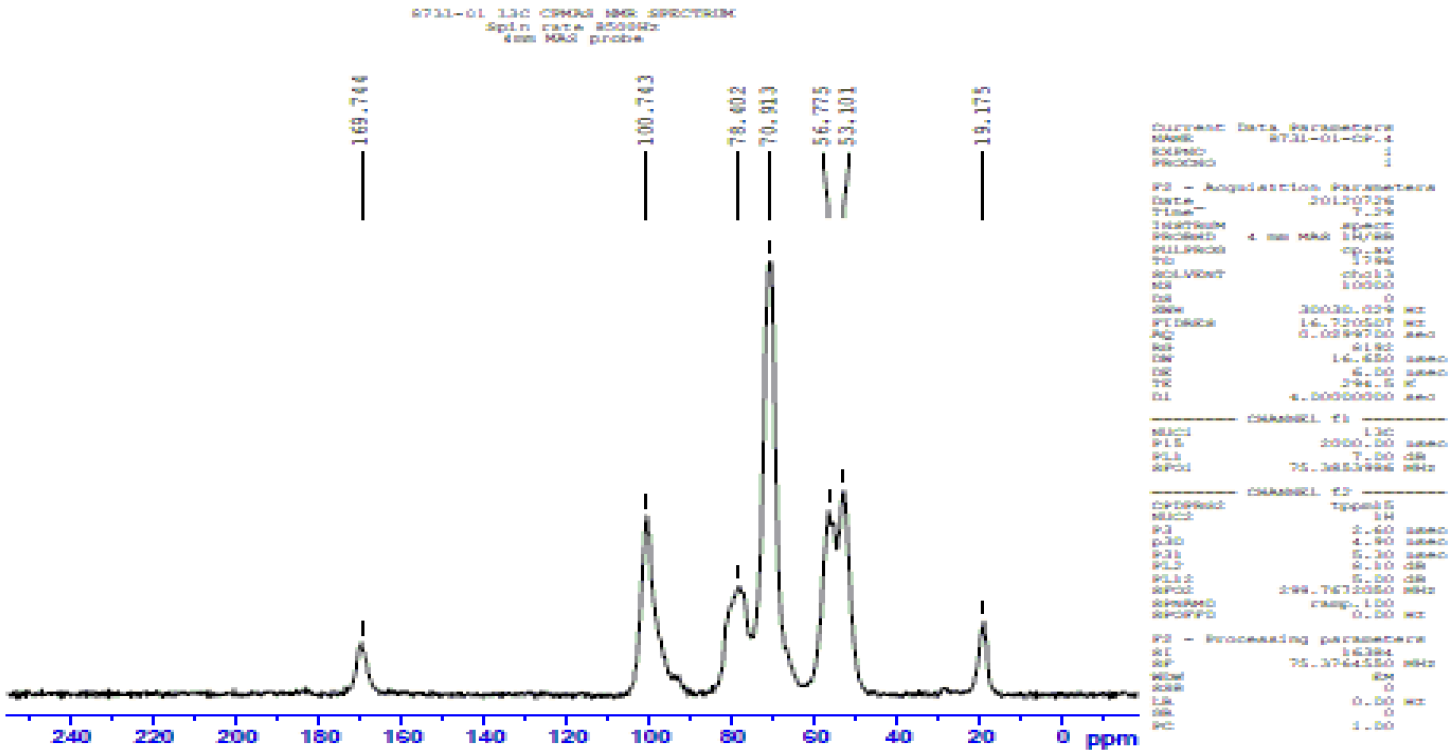


Figure 15. C-13 NMR spectrum of chitosan.

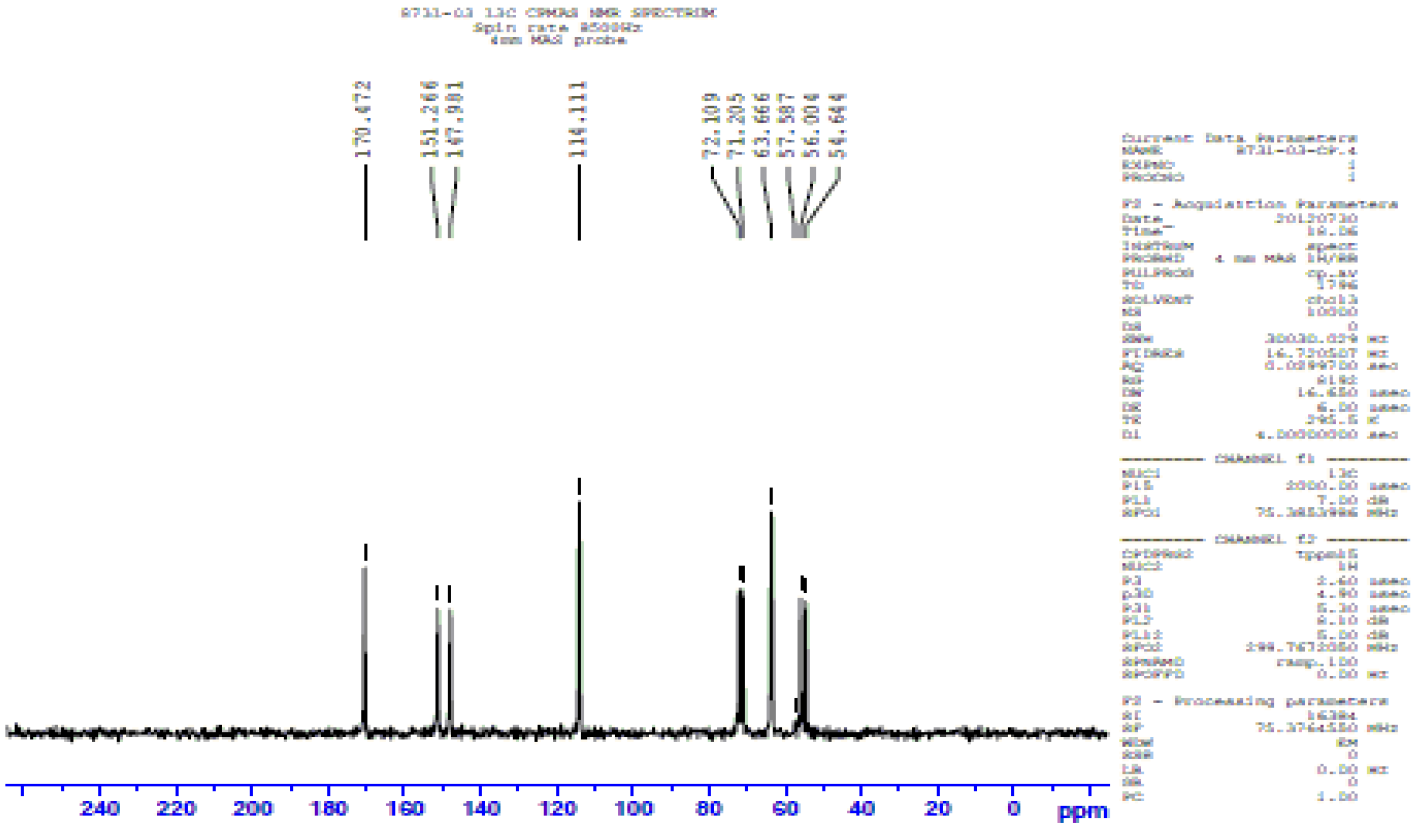


Figure 16. C-13 NMR spectrum of ascorbic acid (VC).

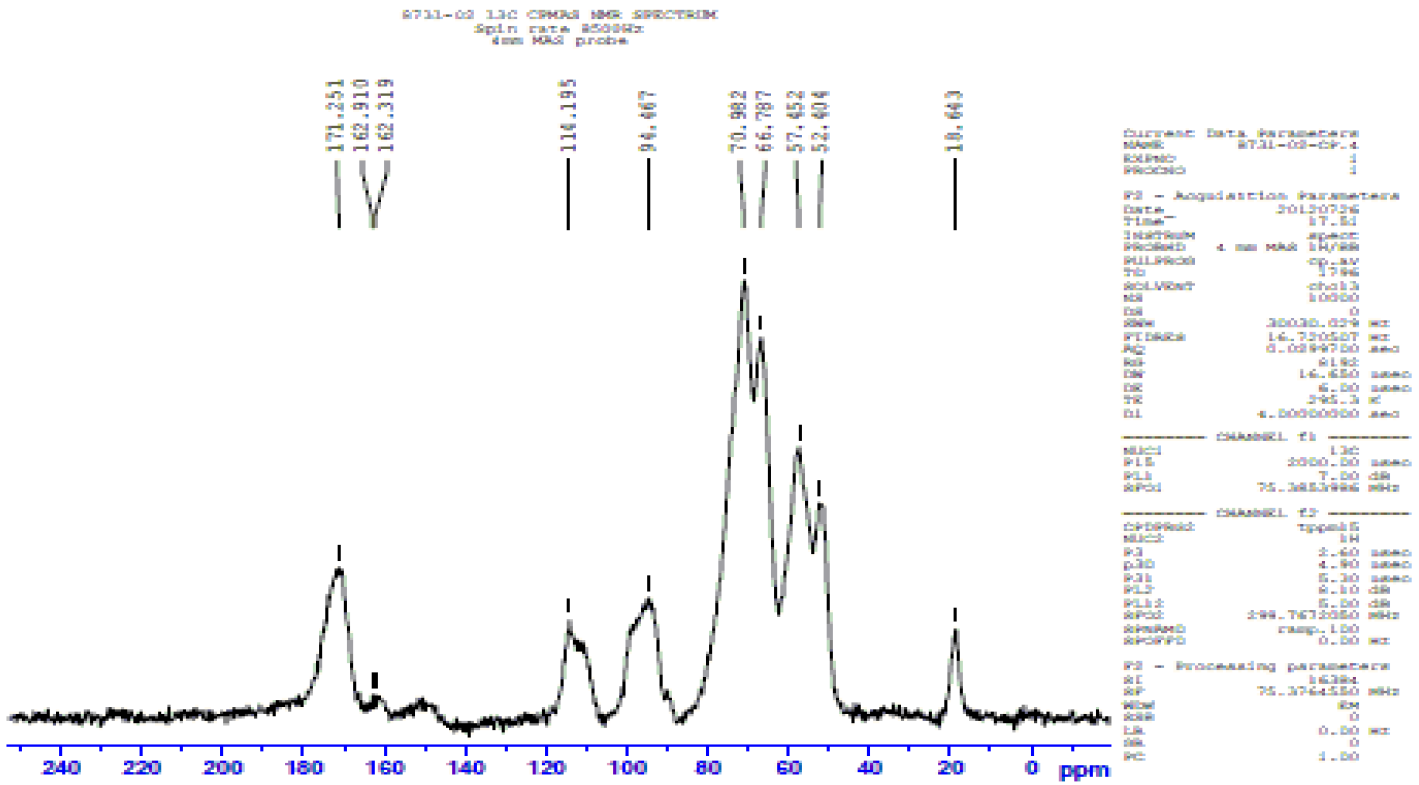
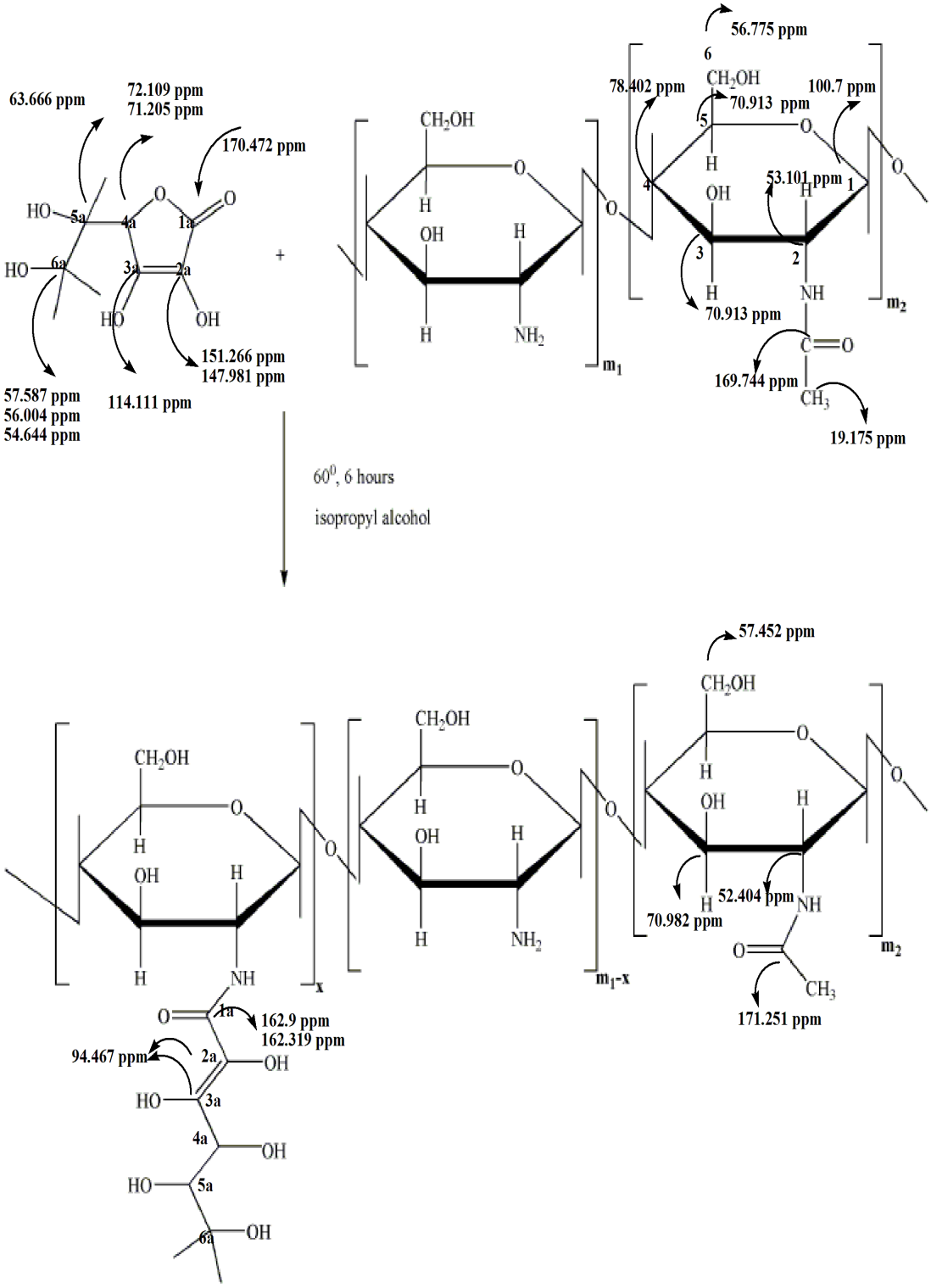


Figure 17. C-13 NMR spectrum of ascorbyl chitosan (ChiVC-100).



Scheme 16. The proposed structure of the ascorbyl chitosan-based on FT-IR and C-13 NMR data.

4.2.2.3 Elemental Analysis

Elemental analysis results of ascorbyl chitosan samples are shown in Table 21. Chitosan was reported to have 39.350 % C, 7.170 % N and 7.150 % H. The ascorbyl chitosan shows decrease in % C, % N and % H values with increasing ascorbyl group content indicating that chitosan repeat units have been modified with ascorbyl group causing an increase in the average molar mass of the repeat unit. The samples were characterized with respect to ascorbyl content by weight and degree of substitution (i.e. degree of ascorbylation), based on elemental analysis results. The theoretical and experimental values are given in Table 22. It should be noted that the accuracy of the experimental results are only reliable with the confidence limits of the method used. Hence, a slightly higher experimental value than the theoretical value has been reported for the first sample. The results show that not all of the ascorbic acid initially present has been substituted on the chitosan backbone.

Table 21. Elemental analysis of chitosan and ascorbyl chitosans.

Sample ID	**C%**	**N%**	**H%**	**O%**
Chi	39.350	7.170	7.150	46.330
ChiVC-0.1	39.320	7.140	7.040	46.530
ChiVC-1	39.490	6.970	7.110	46.430
ChiVC-100	38.570	4.050	5.987	51.393

Table 22. Compositions of ascorbyl chitosans.

Sample ID	**Theoretical ascorbyl content (% weight)**	**Theoretical degree of substitution**	**Experimental ascorbyl content (% weight)**	**Experimental degree of substitution**
ChiVC-0.1	0.35	0.0033	0.42	0.00465
ChiVC-1	3.5	0.033	2.37	0.0269
ChiVC-100	78	3	43.5	0.853

4.2.2.4 XRD Analysis

From the XRD pattern of (a) Chi, (b) ChiVC-1, ChiVC-5 and ChiVC-100 in Figure 18, there is a difference in the intensity of the peaks with increased ascorbylation. It was observed that ascorbylation decreased the crystallinity of chitosan.

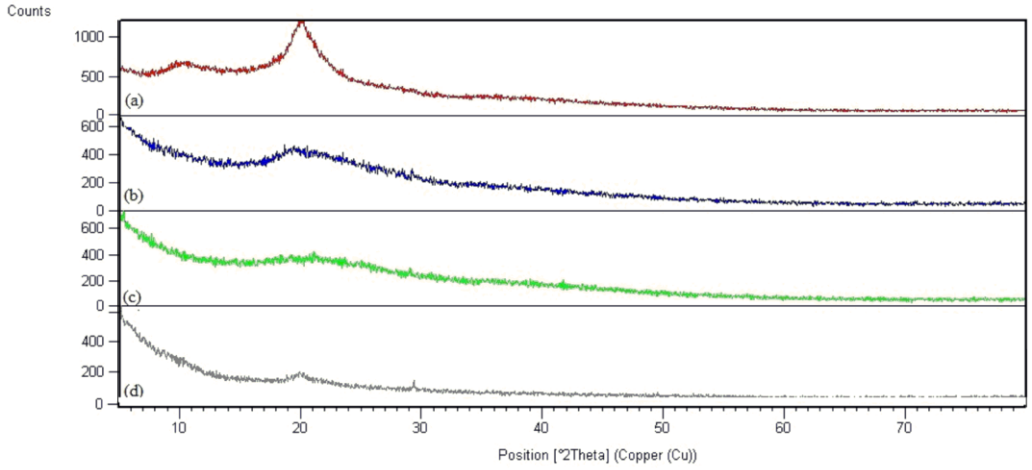


Figure 18. XRD patterns of (a) Chi, (b) ChiVC-1, (c) ChiVC-5 and (d) ChiVC-100.

4.2.2.5 SEM Analysis

The surface morphologies of Chi, blood contact Chi, ChiVC-1, blood contact ChiVC-1, ChiVC-100 and blood contact ChiVC-100 was examined using SEM micrographs with magnification of 500x as shown in Figure 19. All samples have varying degrees of surface roughness. The smoothness of the surface increases upon ascorbylation due to the decrease in the crystallinity. The surface morphologies of the samples after blood contact were examined using SEM micrographs with magnification of 500x as shown in Figure 19. Blood contact samples showed a completely different surface after treatment with blood. Adsorption of blood components on the surfaces are observed in all SEM micrographs of blood contact samples, showing that the samples have poor hemocompatibility.

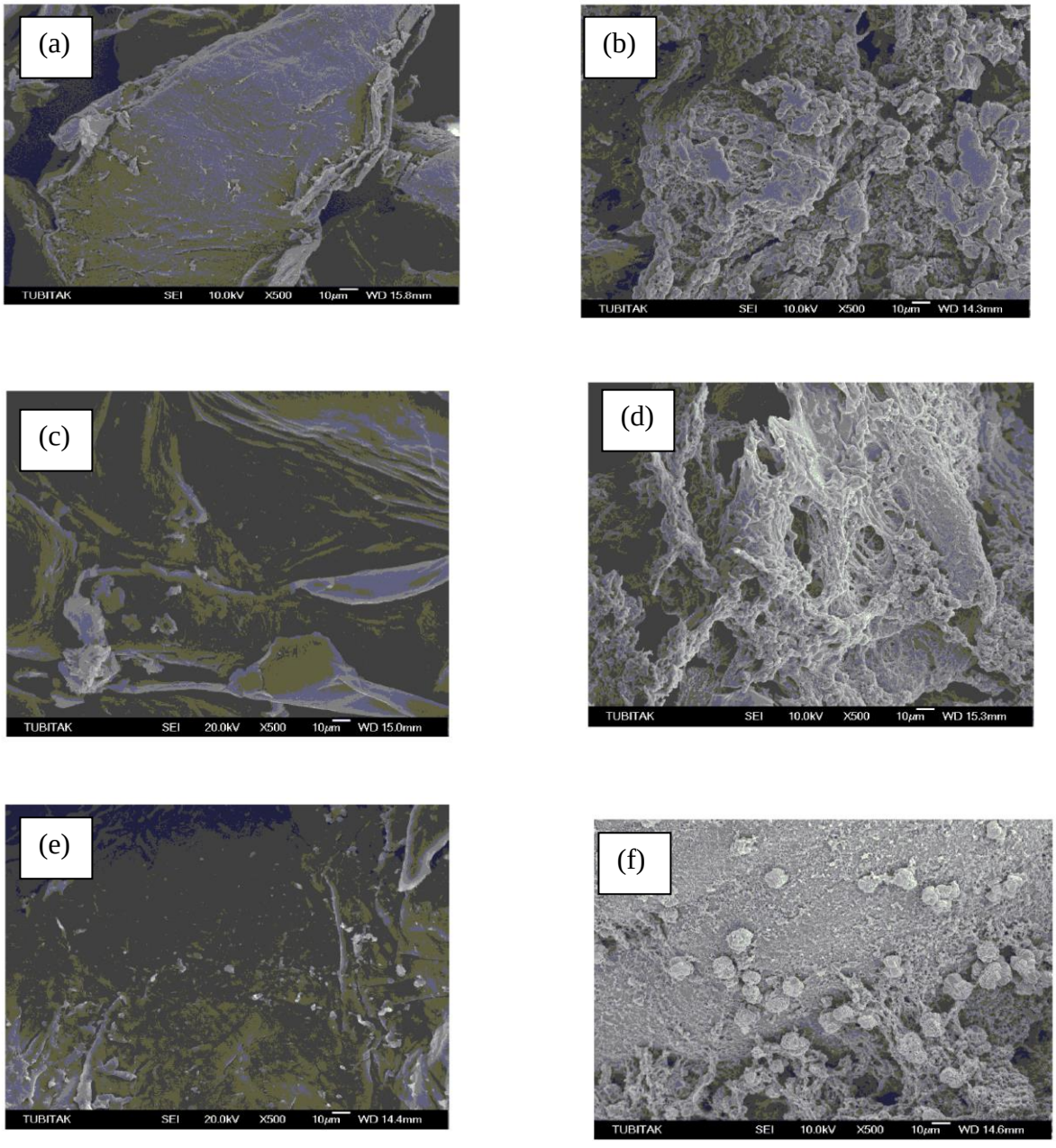


Figure 19. SEM micrograph of (a) Chi, (b) blood contact Chi, (c) ChiVC-1, (d) blood contact ChiVC-1, (e) ChiVC-100, (f) blood contact ChiVC-100.

4.2.2.6 Biochemical Analysis

Chitosan and its derivatives have the potential to interact with blood cellular components, such as blood cells and blood proteins. The lipid lowering activity, the transferrin bound iron chelation capacity, and the influence on blood coagulation were investigated.

4.2.2.6.1 Physiological Role of Chitosan and Its Derivatives in Human Blood

Chitosan and its derivatives have the potential to be used as biomaterials. Hence, the interaction of chitosan and ascorbyl chitosan with blood cellular components, such as blood cells and blood proteins, the transferrin bound iron chelation capacity, calcium ion removal capacity, the lipid lowering activity and the influence on blood coagulation were investigated.

4.2.2.6.2 Total Cholesterol, HDL Cholesterol, LDL Cholesterol and Triglyceride Determination in Human Serum

According to the results given in Table 23, upon contact with chitosan total cholesterol level in the blood sample of *volunteer 1* decreased from 203 mg/dL to 199 mg/dL, which is equivalent to 1.9 % decrease. In the same volunteer's sample, ascorbyl chitosans; ChiVC-0.1 and ChiVC-1, decreased the total cholesterol level from 203 to 196 mg/dL, and to 188 mg/dL, respectively. These values correspond to 3.45 % and 7.39 % decrease in the total cholesterol level. ChiVC-5 reduced the total cholesterol level by 19.7 %. For *volunteer 1*, triglyceride level decreased 2.34 %, 6.97 %, 15.19 % and 43.67 % upon contact with Chi, ChiVC-0.1, ChiVC-1, and ChiVC-5 respectively. It was found that ascorbylation resulted in higher reduction in LDL cholesterol in compare to

chitosan. A similar behaviour was observed in the other examined blood samples (*volunteer 2*, *volunteer 3* and *volunteer 4*) as shown in Table 23.

Chitosan alone had the lowest reduction in total cholesterol levels. The increasing dose of ascorbic acid caused enhanced lowering activity of ascorbyl chitosan on total cholesterol, triglycerides, and HDL cholesterol levels (in Table 23). Smaller ratio of total cholesterol/HDL indicates a less risk for cardiovascular diseases. The total cholesterol/HDL ratio was reduced with increasing ascorbylation.

Table 23. Total cholesterol, triglyceride, HDL choletserol, LDL cholesterol, ferrimat (total iron) and total cholesterol/HDL ratio in the blood samples of healthy volunteers after 3 hours of contact *in vitro*.

Sample ID	**Total Cholesterol (mg/dL)**	**Triglyceride (mg/dL)**	**HDL cholesterol (mg/dL)**	**LDL cholesterol (mg/dL)**	**Ferrimat (µg/dL)**	**Total cholesterol/HDL**
Volunteer 1 (female)						
Blank	203	158	90	81.7	120	2.25
Chi	199	154.3	88	80.4	118	2.47
ChiVC-0.1	196	147	86.7	78.1	114.5	2.50
ChiVC-1	188	134	85.4	75.9	108	2.48
ChiVC-5	163	89	83	62.4	98.8	1.96
Volunteer 2 (female)						
Blank	286	176	70	179.1	69	4.18
Chi	244	159	67.6	145.7	67	3.61
ChiVC-0.1	232	103	64.6	140.9	64	3.59
ChiVC-1	177	85	63	113	60	2.80
ChiVC-5	114.8	47	60.9	53.6	53.2	1.88
Volunteer 3 (male)						
Blank	276	151	66	181.8	58	4.2
Chi	269	147	64	172	57.3	4.2
ChiVC-0.1	266	140	62.3	170	56.2	4.3
ChiVC-1	260	136	60.9	168.8	54	4.3
ChiVC-5	209	90	57	134	49.9	3.66
Volunteer 4 (male)						
Blank	265	159	64	177	47	4.1
Chi	262	157	60.9	175.5	46.7	4.3
ChiVC-0.1	260.4	155.2	60.6	173	46.4	4.3
ChiVC-1	256	147.1	59.2	170.4	44	4.3
ChiVC-5	152	101.9	56.3	140.9	42.3	2.69

4.2.2.6.3 Determination of Serum Iron

Iron removal from blood samples of healthy volunteers was performed by following the serum iron content in the blood serum, which is a measure of the transferrin-bound iron. The behaviour of the ascorbyl chitosan was compared to that of chitosan. The results are shown in Table 23. It was found that chitosan removed only 2 µg/dL in blood sample of *volunteer1*, which corresponds to 1.67 % decrease and did not have a significant iron removal capacity alone. It was observed that the ascorbylation increased the iron removal capacity of chitosan considerably. The ascorbyl chitosan samples; ChiVC-0.1 and ChiVC-1, decreased the serum iron level from 120 to 114.5 µg/dL, and to 108 µg/dL, respectively. These values are within normal range (60-170 µg/dL) and correspond to 4.58 % and 10.0 % decrease in the serum iron levels. ChiVC-5 reduced the serum iron level by 17.7 %. A similar trend was observed in the other examined blood samples (*volunteer 2*, *volunteer 3* and *volunteer 4*) as shown in Table 23. In a previous section (section 4.2), up to 80 % decrease in serum iron was reported by iron imprinted chitosan TPP beads. The iron imprinted chitosan TPP beads have the advantage of being selective towards iron containing molecules. No cholesterol or lipid lowering activity was observed with these samples.

4.2.2.6.4 Total Calcium Analysis in Serum

Removal of calcium ions was observed by all tested samples. It was found that, the highest removal capacity belongs to ascorbic acid, which chelates 65.96 % of total calcium ions. The ascorbyl chitosans chelate more calcium in comparison to chitosan's. The higher removal of total calcium ions reduced coagulation by ascorbyl chitosan samples with increased ascorbyl groups as shown in Table 24. The ascorbyl chitosan

samples; ChiVC-0.1, ChiVC-1, and ChiVC-5 decreased the total calcium level from 9.4 mg/dL to 5.7 mg/dL, 5.0 mg/dL and to 4.9 mg/dL, respectively.

Table 24. Prothrombin time levels (in seconds) after 3 hours of blood contact chitosan and ascorbyl chitosans.

Sample ID	***Volunteer 4***		***Volunteer 9***
	Total Calcium (mg/dL)	**Prothrombin time (s)**	**Prothrombin time* (s)**
Blank	9.4	12	34
Chi	6.3	14.4	38
VC	3.2	>200	>200
ChiVC-0.1	5.7	19.8	45
ChiVC-1	5.0	27.6	46.1
ChiVC-5	4.9	32.4	49
ChiVC-100	3.6	48.8	70.5

4.2.2.6.5 Complete Blood Count Analysis in Whole Blood

A blood contact material is required to have hemocompatibility. That is, it should cause minimium change in the complete blood count compositions. RBC, WBC, PLT, HCT, MCV and HGB are shown in Table 25. Our findings from complete blood count compositions demonstrated that chitosan alone is the most hemocompatible sample in terms of negligible interaction between blood cells and materials mixed with blood. Ascorbyl chitosans had the worse hemocompatibility compared to chitosan alone. This observation was illustrated by the decrease in RBC count (Table 25). The increase in the MCV values was indicative of the volume increase of the RBC when chitosan alone was used. Additionally, the increase in the HCT value with chitosan alone, may also be considered as an increase in the RBC volume during incubation time.

Haemolysis was observed when ascorbic acid came into contact with blood after 15 minutes of incubation at 10 mg/200 µL as illustrated in Table 25 for RBC count when

ascorbic acid alone is added. Ascorbic acid made blood darker compared to blood which was blank. Additionally, the blood which included ascorbic acid became more gelatinous in comparison with control and other blood contact examined materials. Due to these problems associated with ascorbic acid, it was concluded that blood was ascorbic acid incompatible. On the other hand, chitosan and its derivatives did not show haemolysis over 3 hours of incubation at 10 mg/200 µL. This may be explained as ascorbic acid having a stronger electrostatic interaction with blood components compared to ascorbyl chitosans. Negatively charged biological molecules on blood cell membranes may electrostatically interact easier with ascorbic acid mixed with blood than blood mixed with raw chitosan and ascorbyl chitosans due to its more cationic structure. Beside the nonspecific binding of negatively charged biomolecules on blood cell membrane and adsorbed blood components cause the variation in blood characteristics.

A reduction was observed in PLT values dramatically with ascorbic acid alone. This may be the indication of prolonged prothrombin time and extension of the clotting time (Table 24). Ascorbic acid, chitosan and ascorbyl chitosans values examined have been shown to delay blood coagulation and might accelerate fibrinolysis process. Therefore, ascorbic acid, chitosan and ascorbyl chitosans may interfere with the blood coagulation due to complex biochemical process in various ways.

Table 25. The percent changes in RBC, WBC, PLT, HCT, MCV and HGB levels in the blood samples of healthy *volunteer 4* by VC, Chi, Chi-VC1, ChiVC-5 after 3 hours of contact *in vitro*.

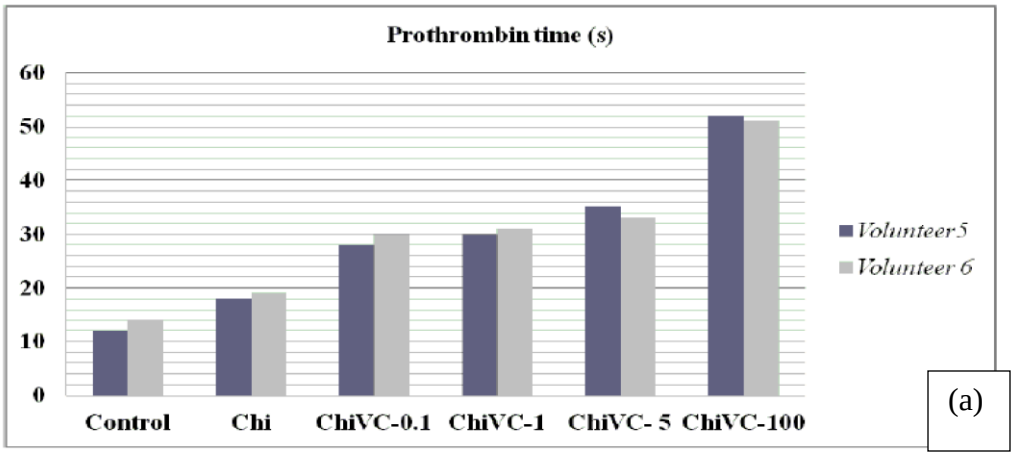
	Normal Ranges	**Blank**	**VC**	**Chi**	**ChiVC-1**	**ChiVC-5**
RBC	*3.5-5.50 (10^6 / mm^3)*	4.97	1.2 (-%75.9)	4.88 (-%0.2)	4.3 (-%13.5)	4.29 (-13.7)
HCT	*35.0-55.0 (%)*	36.20	6.4 (-%82.3)	36.4 (+%0.55)	30.4 (-%16.0)	28.3 (-%21.8)
WBC	*3.5-10.0 (10^3 / mm^3)*	13.90	2.3 (-%83.5)	13.2 (-%5.04)	10.04 (-%27.8)	9.01 (-%35.2)
HGB	*11.5-16.5 (g/dL)*	12.80	1.9(-%85.2)	12.5 (-%2.3)	12.42 (-%3.125)	12.2 (-%4.7)
PLT	*100-400 (10^3 / mm^3)*	295.00	32 (-%89.2)	291(-%1.36)	270 (-%8.5)	265 (-%10.2)
MCV	*75.0-100.0 (µm^3)*	76.70	50(-%34.8)	79.98 (+%4.3)	72.03 (-%6.09)	72.01 (-%6.11)

4.2.2.6.6 Prothrombin time determination in whole blood

The prothrombin time measures the integrity of the extrinsic pathway. The blood samples used were selected from two healthy volunteers and two volunteers using warfarin. The examined samples prolonged the prothrombin time of the analysed blood from healthy and warfarin user volunteers compared to the control as shown in Figure 20 and Table 24. Therefore, chitosan, ascorbic acid and ascorbyl chitosans exhibited anticoagulant effect on the blood and deactivated hemostasis. Ascorbic acid had the strongest blood thinning effect.

According to Table 24, Chi had the minimum anticoagulant effect. It was found that ascorbic acid had the highest anticoagulant activity due to huge increase in prothrombin time. It was observed that increase in ascorbic acid content, prolonged blood coagulation. The increased anticoagulant activity is caused by the removal of calcium ions which are necessary for the extrinsic and intrinsic blood clotting. Due to calcium ion chelating ability of chitosan, ascorbyl chitosans and ascorbic acid, the function of vitamin K is blocked, this leads to a delay or it prevents coagulation completely.

Reduction in PLT prolonged prothrombin time and enlargement in clotting time was measured. The decrease in blood proteins such as Haemoglobin and other proteins that essential for blood clot formation might influence biological process in blood. Moreover, reduction in coagulation factors, which are required for thrombosis, lead to more bleeding and increased prothrombin time.



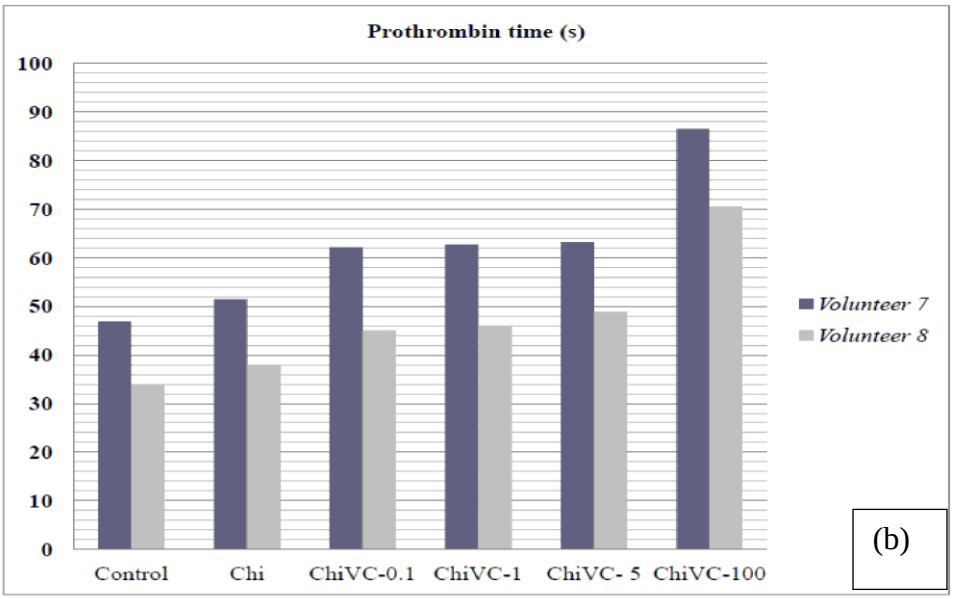


Figure 20. Bar graph of prothrombin time levels after 3 hours of blood contact ascorbyl chitosan samples (a) with two healthy volunteers and (b) with two volunteers using warfarin.

Chapter 5

CONCLUSIONS

In this thesis, some of the biomedical properties of chitosan and its derivatives were explored. Below, two different biomedical applications were discussed that revealed as a result of the thesis; namely 1) antibiotic activity and 2) blood contact applications. Firstly, it was shown that chitosan and some of its derivatives have significantly increased the antibacterial potential of certain commercial antibiotic at low doses. Cell death and cell growth inhibition caused by antibiotic or chitosan, is a complex processes.

Beside the physical interactions with molecules and the specific target in the bacteria, that also involves alterations of the biochemical and molecular levels. Since chitosan is a type of bioadsorbent that reacts with metals, polyanions, lipids and proteins, an inactivation of the biochemical process in the bacteria might have occurred. Overall, it was demonstrated that gentamicin chitosan-based materials exhibited a higher antibacterial activity against gram-positive *S. epidermidis* and gram-negative *E. coli*. This synergistic association of gentamicin with chitosan-based materials against bacteria could lead to new choices for the treatment of infectious diseases. The combination of gentamicin and chitosan-based materials could, at a lower cost, be used to treat bacterial infections, which would otherwise be treated with higher concentrations of conventional

antibiotics. Beside the lower cost, the toxicity to the patients' body would also be reduced. These findings could also prove to be a promising alternative to the treatment of patients for whom existing antibacterial treatment fails. Further studies could be designed to investigate the activity of these antibacterial agents in combination with other antibiotics and against various other gram-positive and gram-negative bacteria. In addition, in-vivo studies are required to confirm these findings. Secondly, a practical and efficient method was developed to prepare Fe^{3+} imprinted chitosan gels by *in-situ* crosslinking. All chitosan gel beads prepared were spherical with a smooth, nonporous and homogeneous surface. Fe^{3+} imprinting resulted in smoother bead surface indicating that complexation with the template ion Fe^{3+} brings polymer chains closer together during bead formation. The chemical treatment carried out on the beads to remove the template ion does not disturb this compact structure formed via *in-situ* crosslinking. The beads proved to be effective bioadsorbents for iron in human blood as well as for haemoglobin. Iron imprinting increases affinity and selectivity towards iron containing species analyzed. The method can appropriately be modified for imprinting other ions on chitosan. This method can be modified to imprint other template molecules to chitosan to obtain more effective and selective bioadsorbents. A more detailed biochemical analysis is needed to understand and explain the blood-chitosan TPP interactions.

This thesis has also potential applications if the examined ascorbyl chitosans facilated to lower lipid levels (such as total cholesterol and LDL cholesterol), and showed a good hemocompatibility without causing adverse reactions (such as thrombosis, haemolysis, or inflammation). Examined ascorbyl chitosan had the ability to lower lipid (total cholesterol, LDL cholesterol, triglyceride) levels in blood. However, their high anticoagulant activity may lead to haemophilia disorders. Additionally,

decreasing HDL cholesterol provided drawbacks for examined ascorbic acid derivatives with chitosan. Further confirmatory *in vivo* studies are required to understand the action of ascorbyl chitosan at the molecular level, and to gain insight into the unknown biochemical functions of these chitosan-based products. Additionally, it is needed to investigate these materials for toxicological evaluation, which is crucial for the safety and biocompatibility before it is used in clinical applications. Moreover, from the obtained observations it could be interpreted that chitosan alone displays a nonspecific binding affinity towards biomolecules used within this study. Therefore, it should be utilized with caution when considered as a bioselective material. Further studies are necessary to improve the utility of ascorbyl chitosan in order to achieve selective binding capacity.

In summary, derivatives of chitosan were investigated in different biomedical domains. The results highlight the importance of the suitability of this particular polymer and its derivatives in biotechnology and biomedical applications.

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