New Wound Dressing Based on Chitosan/Hyaluronan Biopolymers

Ph.D. Thesis

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<tr>
<td>PVB</td>
<td>Poly acrylonitrile</td>
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<td>GlcNAc</td>
<td>acetyl glucosamine</td>
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<td>Static Light Scattering</td>
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<td>Hyaluronic acid</td>
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<td>CD44</td>
<td>cell surface glycoprotein on most cell types</td>
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<td>RHAMM</td>
<td>Hyaluronic acid-mediated motility receptor</td>
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<td>DM</td>
<td>Demineralization</td>
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<td>DP</td>
<td>Deproteinization</td>
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<td>relative light units</td>
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DECLARATION

I developed this work alone. All literary sources and information that I used are listed in the bibliography – reference list. I was aware that my work is to apply the rights and obligations under Act. No. 121/2000 coll. – Copyright law, especially with the fact the University of Pardubice is entitled to the concession contract for this work such as school work according to § 60 par. 1 Copyright law, and that if this work will be used by me or be licensed to use another entity, University of Pardubice entitled to require from me a reasonable allowance to cover costs incurred to create this work, until their actual amount, according to circumstances.

I agree with full disclosure of this work in the Library of University of Pardubice.

Title of Thesis/Dissertation:

New Wound Dressing Based on Chitosan/Hyaluronan Biopolymers

Signature Author

Rasha Abdel-Rahman
TITILE

New Wound Dressing Based on Chitosan/Hyaluronan Biopolymers

ANNOTATION

This dissertation explores strategies for preparing new wound dressings from chitosan / hyaluronan polysaccharides. These wound dressings have excellent healing properties and can be used in different medical application.

This dissertation is organized as follows. Chapter 1 presents an in-depth literature review. Chapter 2 presents the isolation and characterization of chitin and chitosan from Brazilian Atlantic Coast. Chapter 3 focuses on the preparation and characterization of new wound dressing based on hyaluronan and chitosan with using non-woven fabric. Chapter 4 focuses on the conclusion and future work.

**Keywords:** Polysaccharides, wound dressing, chitosan, hyaluronan, diabetic and non-diabetic case.
NÁZEV
Nové kryty ran založené na chitosanovém/hyaluronovém biopolymeru

ANOTACE
Tato disertační práce popisuje strategie pro přípravu nových krytů ran založených na polysacharidech – chitosanu a hyaluronanu. Tyto kryty ran mají vynikající hojivé vlastnosti a mohou být použity v různých lékařských aplikacích.

Tato disertační práce je organizována následovně. Kapitola 1 je hluboká literární rešerše. Kapitola 2 se zabývá izolací a charakterizací chitinu a chitosanu připraveného ze skořápek krevet z atlantického pobřeží Brazílie. Kapitola 3 je zaměřena na přípravu a charakterizaci nových krytů ran založených na hyaluronových a chitosanových netkaných textiliích. Kapitola 4 se zabývá se závěrem a budoucí práce.

Klíčová slova: chitin, chitosan, hyaluronan, kryt rány, netkaná textílie
AIMS OF DISSERTATION WORK

The present work aims at research and development of isolation pure chitin and chitosan with high quality in order to use these biopolymers to prepare new wound dressing with high healing properties that can be used in the field of medical textiles. The main objectives of this study can be summarized as follows.

Isolation of extra pure chitin from new source and preparation of chitosan from it.

Full characterization of isolated chitin and prepared chitosan.

Evaluation of biological activity and cell viability of chitin and chitosan and healing properties of chitosan.

Preparation and characterization of new wound dressing material from non-woven fabrics treated with chitosan and blend of chitosan and hyaluronan.

Evaluation of biological activity, cell viability, fluorescence, phagocytosis, and healing properties of prepared wound dressing on diabetic and non-diabetic rats.
BIOGRAPHY

My name is Rasha Mahmoud Mohammed Abdel-Rahman. I was born in Fayoum, Egypt. I completed my B.Sc. in Faculty of Science-Al-Azhar University at Cairo in Special Organic Chemistry. In 2011, I got my master degree of Organic Chemistry under supervision of Prof. A. Y. El-Kady, Prof. F.K.Mohammed and Prof. R.M.Abdel-Motelab. University of Pardubice, Faculty of Chemical Technology, Czech Republic I joined in December 2011 to pursue the Ph.D. program “Chemistry and Chemical Technologies”, study field “Organic Technology” at Institute of Organic Chemistry and Technology. My dissertation work was on the area of functional materials based on biopolymers under the supervision of Prof. Ing. Radim Hrdina, CSc.
ACKNOWLEDGMENTS

First of all, thanks to ALLAH to whom I relate my success achieving in my work. I would like to sincerely and gratefully thank to my supervisor prof. Ing. Radim Hrdina, CSc. for all of his support, valuable supervision, patience, beneficial discussions, understanding, careful reading and facilities provided for producing this work.

My deep thanks to all my colleagues who cooperated with me in this work.

Additionally, I extend my deep thanks to my brother, my sisters, my children and especial thanks to my husband for all his efforts, encouragement, helps and loving me that much.
TO SPIRIT MY PARENTS
Chapter 1

Theoretical Part
Chapter 1

1. Biomaterials

It can be defined as any substance natural or synthetic can be interact with biological systems in order to use in medical field [1]. The first biomaterials used were gold and ivory and this was done by Egyptians and Romans. In the past, all types of natural materials including wood, glue, rubber, tissues from living forms, and manufactured materials such as iron, gold, zinc and glass were used as biomaterials; but nowadays, a great progress has been made in understanding the interactions between the tissues and the materials. It has been acknowledged that there are differences between living (vital) and non-living (avital) materials. A Biomaterial used for implant should possess some important properties to can be used for long time in the body without rejection, these properties as the Biocompatibility, which can be described as the ability of materials to perform their function with an appropriate host response and the ability to exist in contact with tissues without causing any harm to the body [2]. Also, the biomaterial must not be toxic and must not react with any tissue in the body. The most commonly used biomaterials are biopolymers, composite, ceramics, metals and their alloys. Biomaterials are used in various medical devices and system such as joint replacements, bone plates, artificial ligaments and tendons, heart valves, contact lenses and skin repair devices.

In the last few decades, research has started to move in the direction of renewable raw materials that are environmentally friendly and sustainable. Biopolymers, such as cellulose, chitin, chitosan and starch have been assessed, not only as sustainable resources, but also as attractive materials with interesting properties and functionalities [3]. Biopolymers or their derivatives can be blended with other polymers resulting in a number of new composite materials with enhanced properties and applications in several fields.
1.1. Polymers

Polymers are the most widely attractive materials for biomedical applications [4] as cardiovascular devices, replacement and proliferation of various soft tissues and also used in drug delivery [5]. Polymers are organic materials that form large chains made up of many repeating units. Polymers may be natural or synthetic [6]. Synthetic polymers can be prepared in laboratory by polymerization method such as polyvinyl chloride (PVC or vinyl), polystyrene, polyethylene, polypropylene, poly acrylonitrile, PVB, silicone and many more [2]; natural polymers are derived from sources within the body such as hyaluronic acid, collagen and fibrin (from carbohydrates) or outside such as chitin, chitosan (from exoskeletons of crawfish and shrimp shells) or alginate (from seaweed). There are three main classes of biopolymers: polysaccharides, polypeptides, and polynucleotides. In living cells, they may be synthesized by enzyme-mediated processes, such as the formation of DNA catalyzed by DNA polymerase

1.2. Chitin (Ch)

Chitin is the second most abundant biopolymers found in nature next to cellulose. It is primarily distributed in the exoskeleton of animals such as insects; crustaceans (crab, shrimp, lobsters and etc.) [7, 8]; and also found in microorganisms, e.g. in the cell walls and structural membranes of mycelia of fungi, yeast and green algae in varying proportions from species to species and from region to region [9-11]. Chitin is composed of linear repeating units of N-acetyl-D-glucosamine (GlcNAc), which are linked by β-(1-4)-glycosidic bonds [12, 13]. It is used as a raw material for the industrial production of many products as chitosans, oligosaccharides and glucosamine (GlcN)[14]. The chemical structure of chitin (C$_8$H$_{13}$O$_5$N)$_n$ is similar to cellulose, having one hydroxyl group on each monomer substituted with an acetyl amine group [15-17].
Due to the hydrogen bond, chitin has been found in three polymorphic forms α, β and γ chitin [18, 19], which differ in the arrangement of the chains within the crystalline regions as shown below.

The three chitin types can be differentiated by IR and solid-state NMR spectroscopy together with X-ray diffraction. These types of chitin are mainly differ in the degree of hydration, in the size of the unit cell and in the number of chitin chains per unit cell [20-22]. In α-chitin, the chains are arranged in an antiparallel direction and it is mostly found in crabs, shrimp, insects, fungi and yeast. In β-chitin, all chains are parallel, while in γ-chitin two out of three chains are
parallel with the third oriented in the opposite direction and both $\beta$ and $\gamma$ forms are found in squid pens and cocoons [23].

Like cellulose, chitin is insoluble in water and most organic solvents[24]; the compact structure and the crystallinity of chitin are responsible of this character and this owing to the strong intra- and inter-molecular hydrogen bonds. The solubility of chitin is the main problem inherent with operating difficulties [25, 26]. Chitins exist in nature associated with proteins, minerals, and pigments [27], all these components have to be removed to reach to the highest purity which is necessary for using in bio-medical applications. There are two methods to extract chitin chemical method and biological method [28]. The chemical method performed by using acids and bases[28], while the biological method by using enzymatic or fermentation treatment [29, 30]. In the skeletal tissue, chitin combine with protein and form protein-chitin matrix, which is yield hard shells [31]. Chitin can be isolated through two major steps demineralization and deproteinization; in the first step minerals as calcium carbonates are dissolved by acidic treatment of the shell, the most acidic reagent used in this demineralization process is hydrochloric acid while in the second step the alkaline treatment is used in order to cleavage the covalent chemical bonds between the chitin–protein complexes, alkaline solution from sodium hydroxide is mostly used to remove the proteins, lipids and pigments from the shell [28, 32, 33]. One of the main reactions carried out on chitin is the deacetylation, strong concentration of sodium hydroxide is used to remove the acetyl groups from chitin chain in order to obtain chitosan, the main derivative of chitin[34, 35]. While Chitin has been confirmed as attractive biopolymer with various properties, its applications are limited due to its hydrophobic character [36]. Although, it can be used in different fields such as textiles, water treatment, cosmetics and in biomedical applications [37, 38].
1.3. Chitosan (Cs)

Chitosan, the N-deacetylated form of chitin can be prepared by cleavage of N-acetyl groups of chitin from alkaline or enzymatic deacetylation process [39-41]. It can be described as a copolymer structure comprised of glucosamine and N-acetyl glucosamine. Chitosan is only present in some fungi with small quantities [42]. It exhibits various physicochemical and biological characteristics as antimicrobial [43], antioxidant[44], antitumor [3] and anticancer leading to use in different applications as cosmetics, biotechnology, water purification [45], pharmaceuticals, food additives and agriculture[46, 47].

![Chemical structure of chitosan](image)

**Figure 3**: Chemical structure of chitosan

Study of chitin and chitosan structure showed that these polysaccharides are linear semi-crystalline polymers; and this crystallinity is a function of the degree of deacetylation. Both chitin and chitosan tend to form micro-fibrils that are joined by hydrogen bonds between (NH$_2$ group and C=O group) in order to form supramolecular structure[48 ,17]. Chitosan can be obtained from chitin using high concentrated sodium hydroxide solution [49]; The degree of deacetylation (DDA) and the molecular weight (MW) are the fundamental parameters in characterizing chitosan [50]. The DDA represents the molar % of monomeric unit that have free amino groups, it can be determined by various methods like Infrared spectroscopy, NMR spectroscopy, elemental analysis, and acid-base titration, if the DDA is increased than 40%, the term chitosan is used. Also, different techniques can be used to determine the molecular weight of chitosan like gel
Permeation Chromatography (GPC), Static Light Scattering (SLS), and intrinsic viscosity measurement. The molecular weight can be range from 300 kDa to over 1000 kDa varies from source to source and from place to place [51].

Since chitosan has three functional groups along its chain (hydroxyl group, primary and secondary amino group) this make it highly reactive material which can be used in different application. Also, the cationic character of chitosan (protonation of amino groups NH$_3^+$) is demonstrated highly reactivity of this polysaccharide; the chitosan surfaces support adhesion, proliferation and growth types of cells and this is known as electrostatic interactions [52]. Chitosan is soluble in organic and inorganic acids below pH 6 like hydrochloric, acetic and lactic acid this is because this polymer can be regarded as strong base since it has amino group with pKa 6.3 [36]. Also the DDA and the cationic character of chitosan effect on its solubility, because the protonated amine groups contribute to destroy the hydrogen bonds [53, 54]. On other hand, Chitosan can be used in water treatment [55], in the last years it has gained wide attention as effective bio-sorbent due to low cost and high contents of amino and hydroxyl functional groups which show significant adsorption potential for the removal of various aquatic pollutants [56] Natural chitosan has been modified by several methods in order to enhance the adsorption capacity for various types of pollutants. Different shapes of chitosan, such as membranes, microspheres, gel beads and films have been prepared and examined for the removal of various pollutants from water and wastewater [57, 58].

Chitosan has many advantages for developing micro/nanoparticles, which can be used for preparing scaffolds and for gene and drug delivery [4]. Chitosan’s solubility in aqueous acidic solutions avoids the need for the use of organic solvents when fabricating particulate systems [59]. In addition, the free amino groups become protonated at low pH values which allow the formation
of ionic cross-linking with multivalent anions [60]. Moreover, chitosan surfaces support the
attachment and the subsequent proliferation and growth of different types of cells, which have
been attributed to the high cationic charge density of chitosan [61]. Also, chitosan promotes the
production of transforming growth factor-β-1 (TGF-β1) and platelet-derived growth factor
(PDGF) [62, 63]and maintains the chondrogenic phenotype [64]. Due to their attractive properties,
including non-toxicity, biodegradability, biocompatibility and their natural source [65], Chitin and
chitosan have been investigated thoroughly for different applications, including medical [66],
pharmaceutical [67], textile [68], cosmetics, chemotherapy, wound healing [69], wound dressing
[66], gene delivery [46], tissue engineering and enzyme immobilization [70]. And they can be
easily processed into different products as nanofibers [71], membranes, hydrogels [72], beads,
micro/nanoparticles [73], scaffolds and sponges.

In the past decades, chitosan has been shown highly reactivity in wound healing process;
it can be used in large open skin because chitosan can enhance the functions of inflammatory cells
and promotes granulation and organization [74].

1.4. Hyaluronic acid (HA)

Hyaluronic acid (also known as hyaluronan or hyaluronate) is a naturally occurring
polysaccharide has a linear structure composed of repeat unit of disaccharides N-acetyl
glucosamine and D-glucuronic acid [75-77]. Firstly HA was isolated from the vitreous humor of
bovine; nowadays it can be isolated from many organs and microorganisms [78]. It is the main
component of the extracellular matrix (ECM) of vertebrate tissues, also it present in all blood
vessels, eyes, ligament, umbilical cord, main artery, tendon and skin [79-81], this due to its vital
role in the body as bind water and lubricate movable parts of the body like muscles and joints [82].
Hyaluronan is a high molecular weight (10^5-10^7 Da) and exists as polyanion salt with bioavailable salt as sodium, potassium, calcium and magnesium [83, 84].

Figure 4: Chemical structure of hyaluronic acid

Hyaluronic acid is a promising biopolymer, it has multiple functions in bacteria and animal including human [85]; HA is a biocompatible, biodegradable, anti-inflammatory and non-toxic biopolymer [86, 87]. Also, the oligomer sugars, the degradation products of hyaluronan are considered nontoxic [88]. HA is viscoelastic and its solution is viscous at a low shear rate but become elastic at a high shear rate [86]. In this way, HA keeps skin which contains the major amount of HA, moist and elastic and this elastic properties decrease with age [89, 90]. HA is a hydrophilic biopolymer and has high rate of elimination and turnover depending on its molecular weight and location in the body [91]. When it hydrated it can be swollen to 1,000 times the size of the dry molecule. It can be degraded into smaller oligosaccharides by the action of enzymes such as hyaluronidase (hyase), β-D-glucuronidase [92]. Hyaluronic acid can be prepared with various molecular weights (low and high molecular weight) and can be bind to several other materials in order to use in different field [93]. The mechanical properties of hyaluronic acid could be improved by forming the hydrogel cross-linking of the hydroxyl group and or the carboxyl group of HA
chain; this crosslinking bond has great effect on its properties and on its uses [94, 95]. Furthermore, hyaluronic acid is able to transform into different physical forms as electro-spun fiber [96], fibrillar sponges [97, 98], non-woven meshes [97], scaffolds [99], flexible sheets [100] and nanoparticles fluids [101].

Hyaluronic acid has a diversified in its functions including cell growth, differentiation and migration [102]. The high reactivity of hyaluronan can be attributed to hyaluronan binding receptors as cell surface glycoprotein CD44 and the receptor for hyaluronic acid-mediated motility (RHAMM) [103]. And many tumors like ovarial, colon over express HA-binding receptors CD44 and RHMM and these tumor cells are characterized by enhance by binding and internalization of HA [104]. CD44-Ha interactions play very important physiological roles, including mediation or promotion of macrophage aggregation, cell migration, chondrocyte pericellular matrix assembly, and leukocyte activation [105].

Hyaluronic acid has a wide range of different applications, it can be used in cosmetic field, medical devices as Guidewires, medical and esthetic applications as dermal filling, tissue augmentation and eye surgery [106]. Also HA has found success in nanoparticles as drug delivery agent for different fields including ophthalmic, pulmonary and nasal [107, 108]. Hyaluronic acid plays a vital role in healing process, it helps in formation of connective tissue which is known as a Glycosaminoglycan and it’s able to act as an anti-inflammatory agent by scavenging prostaglandin, metalloproteinases and other bio-active molecules, thus reducing the level of inflammatory mediator’s [107].
2. Wound healing process

Wounds can be defined as any injury, cut, blow, surgery in the protective layer of skin or in the other words it’s a damage in epithelium with or without loss the connective tissue [109, 110]. Some studies have been done on understanding the whole process of the wound healing that has been used by the human body itself [111]. All wounds can be classified into one of the following categories: mechanical, for instance surgical/traumatic wounds; chronic such as leg ulcers or diabetic foot ulcers; burns, which may be chemical or thermal injuries and further classified by depth of injury. Wounds are generally divided into two major types depending on the time of healing: acute and chronic [112]. The acute wounds is a wound that can be healed in the expected time without any problem, passing through all phases of healing process; but the acute wound can be turned into a chronic wound if the healing process is interrupted; so a chronic wound can be defined as any wound fail to heal in the expected time [113, 114]. The failure to heal can be return to different factors as impaired venous drainage; metabolic abnormalities; and genetic disorder, a lack in the oxygen, nutrients and clean environment and there are several types of chronic wounds such as infection wounds, radiation poisoning wounds, surgical wounds, diabetic ulcers and pressure ulcer [115].

Healing of wounds is a complicated process of restoring cellular structures and epidermal tissue in damaged part to its normal condition as closely as possible [116]. The main function of this process is to prevent the body from being infected through wounds, promote wounds to heal with a minimum scar, and return skin functions quickly [117]. This dynamic process consisting of three continuous, overlapping phases, inflammation, proliferation, and remodeling; during these phases a series of reactions are done among the cells and mediators [118, 119]. The first phase is inflammatory phase, which occurs immediately after wounding and continue for 24 to 48 h; the
initial response of the wound is Hemostasis (clotting) which prevents further bleeding, where the damaged blood vessels technically must be sealed\[110\]. The damaged vessels are sealed off by platelet cells that act as “utility workers”. The platelets secrete vaso-constructive substances to provide stable clot sealing of the damaged vessels, epithelial cells have been migrated during a day and new tissue formation happens over two weeks \[120\]. Platelet-derived growth factors are released into the wound that promote the chemotaxis and proliferation of neutrophils in order to clean the wounds from bacteria, dead tissues or debris \[121\]. The proliferation phase is defined as the regenerative phase where the fibroblasts activity produce collagen (the main component of connective tissue it forms about 30 % of the total protein in mammal) which provides structure to the wound and replaces the fibronectin–fibrin matrix \[122, 123\]. This stage can be characterized by the presence of red tissue in the wound base, replacement of dermal tissues and sometimes sub-dermal tissues in the deeper wound \[124\]. The last phase is the remodeling phase which begins at about 2 to 3 weeks and can take more than 2 years \[125\]. In this phase, new collagen forms and wound strength gradually increases \[121\]. Many factor affecting on healing process as the type of damage, the ability of the tissue to repair and the general state of the host’s health \[126-128\].

3. Wound dressing

In the past, health care faced with an increasing number of patients suffering from wounds and burns difficult to treat and heal. During the healing process, the dressing protects the injury and contributes to the recovery of dermal and epidermal tissues \[129\]. Because their biocompatibility, biodegradability and similarity to macromolecules recognized by the human body, some natural polymers as polysaccharides (alginites, chitin, chitosan, heparin), proteoglycans and proteins (collagen, gelatin, fibrin, keratin, silk fibroin, eggshell membrane) are extensively used in wounds and burns management\[130,131\]. The wound dressing can be regarded
as a medical means of cleaning and protecting of wounds against physical, chemical, and microbiological stresses in order to facilitate, accelerate the healing process, decrease the time needed to heal, and reduce the pain and absorb the drainage [132]. Wound dressing products should provide the optimum conditions of wound healing, which has many diverse and interrelated aspects [133]. It should preserve a moist environment at the wound interface, act as a barrier to microorganisms and allow gaseous exchange. Also, the wound dressing material should be nontoxic, non-allergenic, and non-adherent; and made from suitable biomaterial that possesses antimicrobial properties, and promotes wound healing. Achieving this condition has been approached by using either passive or interactive products. Passive products which protect and cover wounds have limited use wherein they impair healing by drying out the wound or adhering to the wound surface [134]. Such products are made of conventional gauzes (yarn-based products consist of viscose rayon, cotton or polyester); paraffin tulle dressings, non-paraffin non-tulle products, and/or a combination of all of them [135]. The main drawback for passive products is that the dressing sticks to the wound resulting in trauma upon removing it. The main focus at this time is on interactive dressings (3rd generation biomaterials) and highly healing materials on nonwoven fabrics have recently been of increasing interest to both the academic and industrial sector [132, 133]. Nowadays many research have been developed in order to make a progress in wound healing agents[136]. Plants with bioactive constituents have been used in treating burns and wounds [137]. When the dermis layer is penterated and blood vessels are destroyed after any cut or injury or etc… wound care and direct treatment of this site of infection is required [138]. Gauze, Films, Hydrogels, Foams, Alginates, Composites, Hydrocolloids and Bioactive/biological dressings are the common types of wound dressings, some of them are made from woven or nonwoven material as gauze [135, 139].
Reference


Chapter 1


Chapter 2

Chitin and Chitosan from Brazilian Atlantic Coast:
Isolation, Characterization and Antibacterial Activity
Abstract

Chitin and chitosan were obtained by chemical treatments of shrimp shells. Different particle sizes (50-1000 µm) of the raw material were used to study its effect on size distribution, demineralization, deproteinization and deacetylation of chitin and chitosan extraction process. The particle size in the range of 800-1000 µm was selected to isolate chitin, which was achieved by measuring nitrogen, protein, ash, and yield %. Hydrochloric acid (5 % v/v) was optimized in demineralization step to remove the minerals from the starting material. Aqueous solution of sodium hydroxide (5 % w/v) at 90 °C for (20 h) was used in deproteinization step to remove the protein. Pure chitin was consequently impregnated into high concentration of sodium hydroxide (50 %) for 3.5 h at 90 °C to remove the acetyl groups under the formation of high pure chitosan. The degree of deacetylation (DDA) of chitosan was controlled and evaluated by different tools. The chemical structure of chitin and chitosan was confirmed by elemental analysis, ATR-FTIR, H/C NMR, XRD, SEM, UV-Vis spectroscopy, TGA, and acid-base titration. The isolated chitin and chitosan from shrimp shell showed excellent antibacterial activity against gram (-ve) bacteria (E.coli) comparing with commercial biopolymers.

Keywords: shrimp shells, chitin, chitosan, isolation, antibacterial activity.

1. Introduction

Chitin is one of the most abundant amino polysaccharides found in nature next to cellulose; it has a wide variety of sources as exoskeleton of crustacean (crab, shrimp crawfish and Oniscus asellus) [1-3], insect cuticles (Melolontha melolontha) [4], Orthoptera species[5,6], wings of cockroach [5], Grasshopper Species [7], Medicinal Fungus [8], Larvae and adult Colorado potato beetle [9], Aquatic Invertebrates [10], Bat guano [11], Resting Eggs of Daphnia longispina [12], spider species [13], Daphnia magna resting eggs [14], and cell wall of fungi and in the green algae[15]. The chemical structure of chitin (C₈H₁₃O₅N)n is similar to cellulose, having one hydroxyl group on each monomer substituted with an acetyl amine groups. Chitin is a linear chain composed of (1-4) linked 2-acetamido-2-deoxy-D-glucosamine while chitosan (the main derivative of chitin) is obtained by removing enough acetyl groups from chitin, the actual difference between chitin and chitosan is the acetyl content of the polymer [1,2,16-21].

Both chitin and chitosan have high nitrogen content varies from (2-8 %) that makes them more attractive to several industrial applications. Chitin is insoluble in water and most organic solvents, this due to its rigid structure and strong intra and inters molecular hydrogen bonds. In contrast, chitosan is readily soluble in diluted acids (as hydrochloric acid, citric acid and acetic acid) with pH below 6 [22-27]. Chitin and chitosan have great economic impact due to their biological activities and their industrial and biomedical applications. Due to the excellent properties of chitosan such as adsorption [28], film-forming and antimicrobial properties [29]. It is used in food preservation [30], cosmetics [31], agriculture [32], biotechnology [33], textiles [1,3,34-37] as well as medical fields [38-40]. Shrimp shell wastes contain about 20–30 % chitin which can be isolated to produce many products such as chitosan, glucosamine and their derivatives, but since chitin is closely associated with proteins, minerals, and pigments, all these components have to be
removed to reach to the highest purity necessary for bio-medical applications [41-53]. The medical applications of chitin and chitosan were limited due to the allergic properties of the both biopolymers [54,56], the phenomenon due to the presence of some impurities like proteins, pigments, and heavy metals attached into the chitin/chitosan chains. In this thesis, I tried to study and optimize all conditions to remove the maximum amount of the hazard material interacted with chitin/chitosan chains.

In this chapter, the acid and base treatments of chitin was fully investigated and optimized, the particle size effect, obtained by sieving of chitin, was evaluated in term of yield (%) of chitin and the residual of protein (%) too. The extraction sequencing of chitin and chitosan was extensively studied which is considered one of the novelty of this work. In addition, the antibacterial assessment of both commercially and isolated chitin and chitosan was evaluated using a novel bioluminescence technique.

2. Experimental

2.1. Materials

Shrimp shells obtained from VCI BrasilIndústriae Commerce de Embalagens Ltda. (Brazil) were selected as a source of raw material used for the extraction of chitin/chitosan. Shrimps were captured in the Atlantic coast of São Paulo, Brazil. Sodium hydroxide, hydrochloric acid, ethanol, isopropyl alcohol and acetone were purchased from Lach-Ner, s.r.o., Czech Republic.

2.1.1. Samples preparation

In order to remove the water-soluble impurities and soluble impurities from the raw material of shrimp, the shrimp shell wastes were washed with tap water and acetone and then dried at 60 °C overnight in dry air oven. The dried material was boiled for 5 h in distilled water and then
refluxed with isopropyl alcohol for 2 h to remove impurities attached into the shrimp surface. Thus, the sample mass decreased after purification by 10-15%. The last step before the extraction process was sieved the shell to different particle sizes from (less 50, 100-200, 200-300, 300-400, 400-500, 500-700, 800-1000 µm).

2.1.2. Sieving shrimp shell to different particle sizes

Particle size was determined on a duplicate 100 g sample with a Bühler laboratory siever MLU 300 (Bühler-Miag, 9240 Uzwil, Switzerland) using a set of woven-wire cloth sieves having a diameter of 26 cm (12 sieves maximum). The sieve openings were chosen according to AFNOR specifications NF X11-501 (AFNOR NF X11-501, 1970) which recommend a geometrical progression of screen sizes in a 50–1000 µm range. The sieving time was 30 min.

2.2. Isolation of chitin and chitosan from shrimp shells

The isolation of chitin and chitosan from shrimp shell begun with the preparation of the samples followed by three classical steps: demineralization (DM), deproteinization (DP) and deacetylation (DA) as described below. Demineralization was carried out by using hydrochloric acid solution. This treatment was done at ambient temperature (25 ± 2 ºC) with varying times (0.5-5 h). The emission of CO₂ gas was observed during the reaction of carbonates with diluted hydrochloric acid [6]. The resulting solid fraction of crude chitin was washed with distilled water until neutral pH was achieved, then the chitin samples were dried at 60°C for 24 h. During this process, the effect of hydrochloric acid with different concentration on the yield, nitrogen and ash content in the product was studied.

Deproteinization (DP) was done by using aqueous sodium hydroxide. Dry crude chitin was dispersed into aqueous solution of sodium hydroxide to remove the non-bounded material like proteins, dyes, lipids and pigments [6]. Thus, crude chitin was stirred in 5% of sodium hydroxide
at 90 °C for 20 h, where the alkaline solution was exchanged every 2 h with fresh solution of sodium hydroxide. The product was filtered off, washed with demineralized water until neutralization after which the sample was dried at 60 °C for 24 h. Deacetylation step was achieved by impeded the chitin sample under reflux with 20 – 60 % of sodium hydroxide [7] at 90 °C with (1/30 w/w) solid to solvent in the time range 0.5–5 h. The product; chitosan was collected, washed to neutrality using demineralized water, rinsed with acetone, and vacuum filtered off and dried at 60 °C for 5 h to remove moisture.

2.3. Characterization of chitin and chitosan

2.3.1. Inductively coupled plasma optical emission spectrometry (ICP-OES)

Elemental analysis was carried out using the sequential, radically observed inductively coupled plasma atomic emission spectrometer INTEGRA XL-2 (GBC, Dandenong Australia), furnished with the ceramic V-groove nebulizers and the glass cyclonic spray chamber (both Glass expansion, Australia). The limits of detection (the concentration equivalent to three times standard deviation of the blank sample in the place of background correction) were 10 μg/L for lines 338, 289 and 328 nm, for analysis of real samples, results from both analytical lines used were averaged.

2.3.2. Scanning electron microscopy (SEM)

All sample images were taken by scanning electron microscope; Pardubice University., Tescan VEGA II LSU electron microscope (Tescan USA Inc.) under the following conditions: high voltage 5 kV, working distance 4.4 mm, display mode secondary electrons, high vacuum room temperature. SC7620 Mini Sputter Coater (Quorum Technologies, UK) applied 15 nm layers of gold particles on the sample. The samples were dusted for 120 s with the current of 18 mA. The pictures were made at these conditions: voltage 2.44–10 kV, detector-SE, the magnification 300–
20000 times, vacuum high, the distance between sample and objective: 4 - 5 mm. The nonwoven dressing sheets were cut with a razor scalpel after being frozen in liquid nitrogen for 10 min.

2.3.3. Elemental analysis

The micro-analyses were performed on an apparatus of FIONS. Instruments EA 1108 C; H; N. The content of proteins (%) was calculated from the nitrogen content by using the following equation

\[ P\% = [N\% - 6.9] \times 6.25 \]  

where P % represents the percentage of proteins remaining in the deproteinized shell, N % represents the percentage of nitrogen measured by elemental analysis, 6.9 corresponds to the theoretical percentage of nitrogen in fully acetylated chitin (this value was adjusted as a function of DA, the degree of acetylation), and 6.25 corresponds to the theoretical percentage of nitrogen in proteins. All the determinations were done in quadruplicate [37].

2.3.4. UV-Vis spectroscopy

UV/Vis spectroscopy was also used to determine the P %. The measurements were carried out on UV-visible spectrophotometer UV-160A, Shimadzu, Japan using quartz cuvette with an optical path of 1 cm. The concentration of the measured solutions was kept at 0.59 mg.ml⁻¹. The protein content in supernatant was calculated from the following equation

\[ P\% = 2.37 \times (A_{564}/W) \]  

where the A₅₆₄ was the absorbance value at 564 nm and W was the weight of sample in mg.

2.3.5. Fourier transform infrared spectroscopy-attenuated total reflection (FTIR-ATR)

Fourier transform infrared spectroscopy (FTIR) was performed by using a Nicolet Impact 400 D FTIR-ATR spectrophotometer (Nicolet CZ, Prague, Czech Republic) equipped with a ZnSe crystal for the FTIR spectroscopy. Transmittance was measured as a function of the wavenumber.
between 4000 cm$^{-1}$ and 600 cm$^{-1}$ with the resolution of 8 cm$^{-1}$ and the number of scans equal to 32. The degree of deacetylation of chitosan was calculated from equation 3 [6,34],

$$DA\% = 100 - [(A_{1655}/A_{3450}) \times 100/1.33] \quad \text{Eq.3}$$

where $A_{1655}$ and $A_{3450}$ were the absorbance at 1655 cm$^{-1}$ of the amide I. The factor 1.33 denoted the value of the ratio of $A_{1655}/A_{3450}$ for fully $N$-acetylated chitosan.

### 2.3.6. Determinations of ash content

Chitin and chitosan ash content were determined by combustion using a constant weight crucible. Chitosan (2–5 g) was subjected to combustion at constant weight crucible in an oven at 550 °C ± 20 °C for 3 h. The crucible was removed, cooled in desiccators for 30 min, and re-weighed ($W_1$). The heating and cooling process were repeated every 1.5 h until a constant weight was established ($W_2$). The ash percentage was calculated by the equation 4 [58],

$$\text{Ash}\% = \frac{W_2-W_0}{W_1-W_0} \times 100 \quad \text{Eq.4}$$

where $W_0$ was the constant weight of crucible, $W_1$ was the weight of sample and crucible, $W_2$ was the weight of ash and crucible.

### 2.3.7. Solid NMR spectroscopy

$^1$D solid-state NMR spectra were measured using a Bruker Avance 500 NMR spectrometer. Magic angle spinning (MAS) frequency of the sample was 11 kHz. Amplitude modulated cross-polarization (CP) with duration 1 ms was used to obtain $^{13}$C CP/MAS NMR spectra with 5 s recycle delay. $^{13}$C scale was calibrated with glycine as external standard (176.03 ppm low-field carbonyl signal).
The degree of deacetylation of chitin and chitosan were calculated from equation 5 [59],

\[
\text{mol}(\%) = \frac{I_{\text{CH}_3}}{(I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6})/6} \times 100 \quad \text{Eq.5}
\]

where \( I_{\text{CH}_3} \) and \( I_{C1} \) corresponds to the integral of the hydrogen atom in \( \text{NHCOCH}_3 \) groups and to the hydrogen atom of \( C_1 \) in glucosamine units respectively.

### 2.3.8. Diffusion NMR spectroscopy

NMR spectra were measured on BRUKER AV 500 MHz Ultra-shield plus with the probe BBOF plus. For all tests except for accuracy, the solutions of chitosan were prepared by stirring at room temperature; 10 mg of chitosan in a solution composed of 1.96 ml of \( D_2O \) and 0.04 ml of \( DCl \) and waiting about 1h to ensure complete dissolution of the polymer. The degree of deacetylation of chitosan was determined according to the following equation 6 [60,61],

\[
\text{DD}(\%) = \frac{H_{\text{ID}}}{H_{\text{ID}} + H_{\text{Ac}}/3} \times 100 \quad \text{Eq.6}
\]

where \( H_{\text{ID}} \) was H-NMR signal for proton at \( C-1 \) at deacetylation monomer; \( H_{\text{Ac}} \) was signal of acetyl group.

### 2.3.9. Acid-base titration

Chitosan sample (0.1 g) was dissolved in 25 ml of 0.1 M HCl aqueous solution. The sample was titrated with 0.1 M NaOH. pH meter was used for pH measurements under continues stirring at ambient temperature (25 ± 2 °C). The titrant was added until the pH value reached to 1.5. The standard solution of sodium hydroxide was added drop-wise and the pH values of the solutions were recorded and a curve with two inflection points was obtained. The degree of deacetylation of chitosan was determined by acid-base titration according to the following equation 7 [62,63],

\[
\text{DDA (\%)} = \frac{C_{\text{NaOH}} \times (V_2 - V_1) \times 161}{m} \quad \text{Eq.7}
\]
Where, $C_{NaOH}$ was the concentration of sodium hydroxide; $(V_2-V_1)$ was the difference between two volume values of sodium hydroxide between the two inflection points; 161 was the molecular mass unit of chitosan; $m$ was the mass of chitosan sample. The average degree of deacetylation (DDA) of chitosan samples could be determined from elemental analysis by using the following equation 8 [7,10,64],

$$DDA \, (\%) = \frac{6.857 - C/N}{1.7143} \quad Eq. \, 8$$

Where, $C/N$ was the carbon/nitrogen ratio measured from the elemental composition of the chitosan samples.

2.3.10. X-Ray diffraction (XRD)

X-ray diffraction was collected on D-8 Advance diffracto-meter (Bruker AXS, Germany) with Bragg-Brentano 0-0 goniometer (radius 217.5 mm) equipped with a secondary beam curved graphite mono-chromator and Na (Tl) I scintillation detector. The generator was operated at 40 kV and 30 mA. The scan was completed at room temperature from 5 to 100° (2θ) in 0.02° step with a counting time of 8 s per step.

2.3.11. Thermal analysis measurements

Samples were studied with regard to the kinetics of thermal decomposition, using different heating rate thermo-gravimeter (TG, Netzsch 209F3 instrument, Al$_2$O$_3$ crucible) and under heating rates of 5 (with data collecting rate of 40 points/K) and 10 °C.min$^{-1}$ (collecting rate data of 60 points/K). The test temperature range for TG was 25– 600 °C with the sample mass of about 10.35–1.45 mg under 30 ml.min$^{-1}$ dynamic nitrogen atmosphere.

2.3.12. Antibacterial activity of chitin and chitosan

Antibacterial effect was examined via bioluminescent (BL) bacteria. Genetically modified $E. \, coli$ K-12 capable of bioluminescence were exposed to three different concentrations of the
isolated chitin and chitosan (5, 10, 15 mg) which led to diminishment of bacterial viability visualized by real time BL measurement [6]. Concentrated stock bacterial suspension was prepared [6], and stored at temperature –80 °C. Final concentration of bacterial cells in applied suspension was set to approximately 380 000 cells per 100 μL. This suspension (200 μL) was mixed with 5, 10 or 15 mg of isolated chitin and chitosan partly suspended in phosphate buffer (100 μL) in wells of a white flat-bottom 96-well μl plates (Thermo Scientific, Czech Republic). MC cellulose samples (5 to 15 mg) were used as an appropriate control. Light (490 nm) generated by enzymatic reaction in bioluminescent bacteria was measured continuously during 60 minutes by luminometer LM-01T (Immunotech, Czech Republic) at laboratory temperature (25 ± 2 °C). Results are expressed in relative light units (RLU). Average integral under the kinetic curve was compared with control and the percentage of bacterial killing was calculated. Experiments were repeated independently three times.

2.3. 13. Statistical analysis

All experiments were carried out in triplicate and the results were expressed with ± S.D (standard deviation).
3. Results and discussion

The present data represents the first attempt to investigate various physicochemical and functional properties of chitin and chitosan from shrimp shell. The shrimp shells were collected from Brazilian Atlantic Coast, washing with water, acetone and isopropyl alcohol then dried at oven at 60 °C overnight (as mentioned before in sample preparation) followed by sieving the shrimp shell to different particle sizes from (less 50, 100-200, 200-300, 300-400,400-500, 500-700, 800-1000 µm) using sieving machine. The variation in chemical, physical, physicochemical and functional properties of isolated biopolymers in the three sequential processes (DP, DM, DA) of the isolation process were investigated.

3.1. Isolation of chitin

In shrimp shell, chitin is found as a part of a complex based on proteins and minerals as calcium carbonate and calcium phosphate deposit to form the rigid shell [65, 66]. Thus The isolation of chitin from Atlantic shrimp shell is summarized in two major steps: removal of calcium salts in demineralization step using diluted hydrochloric acid and second step: removal of proteins in deproteinization process using sodium hydroxide [66].
Figure 1: Isolation process of chitin and chitosan from shrimp shell

3.1.1. Demineralization step (DM)

Shrimp shell contains about 25-40 % of minerals and the huge amount of these minerals are calcium carbonate and calcium phosphate [8, 47]. Hydrochloric acid was used as a reagent in demineralization step in order to remove the minerals from the shell. In this process, minerals were hydrolysed into highly water soluble salts which could be separated by filtration and washing with deionized water for several times. Various concentrations of HCl were used at room temperature with agitation to detect their effects on nitrogen percent, the yield and ash content [67]. In the beginning, studying on the best yield that obtained from different particle sizes (less 50-1000 µm) of shrimp shell by using aqueous solution of hydrochloric acid (5 %) was performed as
in Figure 2A; as expected the yield increased with increasing the size of shell. It was improved slightly with increasing the size of shell from less 50 to 1000 μm, and it could be returned to the bigger size bonded lower impurities. So, the particle size 800-1000 μm, was chosen to study the influence of the isolated chitin and chitosan through isolation sequence process. Figure 2B reveals the effect of different concentration of hydrochloric acid (0.5-5 %) on nitrogen percentage. The treatments were carried out at room temperature with various time intervals (0.5-5 h). At zero time treatment, the nitrogen percent was about 4, and increased slowly by increasing the concentration of HCl (0.5 to 2 %) and there was no significant differences in the treatment time (Figure 2B). By increasing the concentration of hydrochloric acid from 2-5 %, the nitrogen percent value was raised from 6 to 8.5 and this could be confirm the presence of large amount of chitin. Thus, the 5 % of hydrochloric acid produced higher percent of chitin.

![Graph showing yield percent vs particle size](image)

**Figure 2A**: Effect of particle size of shrimp shell on the yield percent after sieving step. **Experimental conditions**: different sizes of dry shrimp shell, 5 % aqueous hydrochloric acid, RT (22±2 °C), for 2h.
Figure 2B: Demineralization step-effect of hydrochloric acid on nitrogen percent of dry shrimp shell with particle size 800-1000 µm. **Experimental conditions:** shrimp shell with particle size 800-1000 µm, ratio of solid (shrimp shell) to aqueous solution of hydrochloric acid 1:20 (w/v), RT (22± 2 °C).

Figure 2C shows the effect of concentrations of hydrochloric acid on the yield percent during the treatment of the shrimp shell under different time. It’s obviously clear that, the yield percentage decreased with increasing the concentration of hydrochloric acid; this is due to the removal higher percentage of mineral salts (calcium carbonate and/or phosphate) during the treatment [8]. The yield percentage decreased rapidly in the first 1 h then the rate became constant as the composition of minerals decreased with demineralization time.
Figure 2C: Demineralization step-effect of hydrochloric acid on yield percent of shrimp shell with particle size (800-1000 µm). **Experimental conditions**: shrimp shell with particle size 800-1000 µm, ratio of solid (shrimp shell) to aqueous solution of hydrochloric acid 1:20 (w/v), RT (22±2 °C).

Figure 2D shows the effect of different concentrations of hydrochloric acid on ash content varying with treatment time. Based on the obtained results, the ash content was decreased rapidly with increasing the concentration of hydrochloric acid, then it started to be more constant, this could be explain in term the fast treatment and fast removal of the impurities and the metal salts in the first hour. The decrease of ash content percentage reflect the purity of chitin. The ratio of solid to solvent was an important factor affecting in yield percent and nitrogen percent of shrimp shell.
Figure 2D: Demineralization step - effect of hydrochloric acid on ash content of shrimp shell with particle size 800-1000 µm. Experimental conditions: shrimp shell with particle size 800-1000 µm, ratio of solid (shrimp shell) to aqueous solution of hydrochloric acid 1:20 (w/v), RT (22±2 °C).

In Figure 2E, the demineralization of shrimp shell with 5 % hydrochloric acid for 2 h at room temperature with different liquor ratio between solid to solvent 1:5, 1:10, 1:15, 1:20, 10:30:1:40, and 1:50 (w/v) were effective in decreasing the yield % and nitrogen % by increasing the liquor ratio between solid to solvent. According to our result the mineral content obtained from Atlantic Ocean shrimp shell had lower percentage yield (8-10) which was lower when comparing with the different sources of chitin in the previous literature as shown in Table S2 [24, 68-71].
Figure 2E: Effect of liquor ratio of hydrochloric acid on yield percent and nitrogen percent of shrimp shell with particle size 800-1000 µm. **Experimental conditions:** 5% hydrochloric acid, shrimp shell with particle size 800-1000 µm, RT (22± 2°C).

3.1.2. Deproteinization step (DP)

In deproteinization step the chemical treatment is used to destroy the covalent chemical bonds between the chitin-protein complexes (Proteins are bound by covalent bonds to the chitin through aspartyl or histidyl residues or both forming stable complexes such as glycoproteins) [72, 73]. Alkaline solution from sodium hydroxide was used to remove the proteins, lipids and pigments from the shrimp shell [8, 74]. Effect of particle sizes and the time of chemical deproteinization process influence the properties of the chitin product. Figure 3A shows the effect of different particle sizes of the shell on the yield percentage and the residual protein percent. The particle sizes play an important role in the purity of chitin. (Figure 3A) reveals the relation between particle size and protein content. The amount of protein percent decreased sharply at higher particle
size of the shell. In addition; bigger particle size effect the reaction efficiency of shell with hydrochloric acid (demineralization step) and/or with sodium hydroxide (deproteinization step). Based on figure 3A the protein content decreased to 0.005 for particle size 800-1000 µm in comparing with 0.317 for particle size less 50 µm.

![Figure 3A](image)

**Figure 3A**: Effect of particle size of demineralized shell on protein percent of demineralized shell.

**Experimental conditions**: ratio of solid of demineralized shell to solvent (aqueous NaOH) 1:20 (w/v), RT (22±2 °C), 2h.

Figure 3B shows the relation of treatment time with the protein percent measured by various two methods. In Figure 3B, the protein percent decreased in the shell by increasing the time of treatments from (2-20 h) confirmed by elemental analysis. In the same time the percentage of protein increased in the supernatant by increasing the time of treatment from (2-20 h) confirmed by UV/Vis spectroscopy. The reason of protein decrease, was the reaction of sodium hydroxide with the residual impurities [75]; the protein percent was very low comparing with different sources of shrimp shell in previous literature as shown in table S1[24, 65, 69-71].


**Figure 3B:** Relation between time of treatment of demineralized shell and protein percentages of demineralized shell. **Experimental conditions:** DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 °C), solid to liquid (1: 20 w/v); DP-deproteinization: 5 % sodium hydroxide, 90 °C, solid to liquid (1:20 w/v).

### 3.1.2.1. Scanning electron microscopy of pure chitin

To better understand of chitin morphology, some chitin flakes were isolated from different places of the exoskeleton and were observed by SEM. Figure 4A shows scanning electron microscopy of the raw material of shrimp shell after washing and drying process. As shown (Figure 4A), there was heterogeneous in particle size, beside some impurities are attached on the shrimp surface. Also, a roughness surface of the raw material without porosity was observed which might be related to their high molecular packing, with inter- or intramolecular hydrogen bonds, imparting a high crystalline degree to chitin. Figure (4B-H) shows the different particle sizes (less 50-1000 μm) of chitin after deproteinization and demineralization treatments. It was observed that, independent of part of the shrimp exoskeleton, the isolated chitin retains its fibrils character. After
separation, different particle sizes (Figure 4B-H), the surface morphology of the chitin was changed due to the treatment with acid and basic agents. The mobility or diffusion of the acid or basic inside chitin structure was crucial and inhabited by low porosity and high crystallinity of chitin. Due to the sieving process, each sample has approximately the same particle size, and it easy to detect the surface changes due to the treatment process. Different sizes of chitin has porous structure after the extraction process which somehow could significantly reduce the time of treatments. The surface of the isolated chitin from shrimp shell was found to have smooth surface structure similar to morphology of chitin isolated from spider species [14]. It was very clear that the particle size 800-1000 µm gave the best clear morphology, smooth, and high porosity than other sizes. Therefore to perform all experiments in our study.
3.2. Isolation of chitosan from chitin (Deacetylation step)

Chitosan was isolated from chitin by removing the acetyl groups from the chain of chitin using strong concentration of sodium hydroxide in process called deacetylation. Different parameters could be effect the deacetylation step like concentration of sodium hydroxide, time and temperature of the reaction medium. In order to determine the effect of sodium hydroxide on the degree of deacetylation of chitosan (DDA % calculated by Solid NMR), different concentrations of sodium hydroxide solution (30 %, 40 %, 50 %, 60 %) were used to replace the acetyl group of chitin with free amino groups. Figure 5A, demonstrated the great influence of NaOH in removing the acetyl groups along the chitin Chain. The DDA values of chitosan increased gradually as the
sodium hydroxide concentration increased up to 30 % and increased rapidly when the concentration was higher than 50 %. The DDA of chitosan was lower than 45 % when the concentration of NaOH was lower than 30 %. However, the DDA of chitosan increased dramatically up to 95.5 when the concentration of NaOH was between 50-60 %. Figure 5B shows the influence of time on the DDA of chitosan (calculated by elemental analysis). Chitin was refluxed with 50 % of sodium hydroxide at 90 °C for different treatment time (0.5-4 h). In the first hour of the treatment the degree of DDA reached to 40 %; then increased gradually with the time till 3.5 h and then the DDA became 95 %.

**Figure 5A**: DA-deacetylation step - effect of the sodium hydroxide on the degree of deacetylation of formed chitosan (DDA). **Experimental conditions**: DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 °C), solid to liquid (1:20 w/v); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C, solid to liquid (1:20 w/v); DA-deacetylation: 2 h, 90 °C, chitin to liquid (1:30 (w/v).
**Figure 5B:** DA-deacetylation step - effect of time on the degree of deacetylation of chitosan. **Experimental conditions:** DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 °C), solid to liquid (1:20 w/v); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C, solid to liquid (1:20 w/v); DA-deacetylation: 50 % sodium hydroxide, 90 °C, solid to liquid (1:30 w/v).

**3.3. Isolation sequences of chitin and chitosan**

Figure 6 shows the extraction sequence process of the isolated chitin and chitosan by demineralization (DM), deproteinization (DP) and deacetylation steps (DA); in obviously clear the sequence treatment steps effect on the yield percent of chitin and chitosan. The yield percent of chitin and chitosan was higher by the following sequence DMPA>DPMA>DAPM>DAMP, respectively. Based on Figure 6, the demineralization, deproteinization, and deacetylation sequence were the best sequence for isolation of pure and high yield percent of chitin and chitosan from shrimp shell.[76, 77].
Experimental conditions for chitin; DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 °C), solid to liquid (1:20 w/v); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C, solid to liquid (1:20 w/v). Experimental conditions for chitosan; DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 °C), solid to liquid (1:20 w/v); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C, solid to liquid (1:20 w/v); DA-deacetylation: 50 % sodium hydroxide, 3.5 h, 90 °C, solid to liquid (1:30 w/v), degree of deacetylation DDA (95%, measured by NMR).

3.4. Thermal stability of chitin and chitosan

Thermal gravimetric analysis of chitin and chitosan has been used to investigate the thermal degradation and crystallization of the polymers. Figure 7A shows the TGA curves and the corresponding derivate-grams (DTG) of the isolated chitin and chitosan. The isolated chitin (Figure 7A) shows two major peaks on the DTG curves. The first, which appeared around 50-100 °C, could be explain as the evaporation of physically adsorbed and strongly hydrogen boned water to the complex and alcohol solvent, respectively [14, 15, 78]. Second peaks appeared at 330 °C was related to cleavage of glyosidic amine units by dehydration or deamination. Commercial chitin
had three peaks on the DTG curve. At 50-100 °C, could be explained as the evaporation of physically adsorbed and strongly hydrogen boned water to the chitin [78]. The second peaks were appeared at 230 °C, which may corresponding to the residual of proteins, pigments encored to chitin chains. The third peak appeared at 300 °C, related to cleavage of glyosidic amine units by dehydration or deamination [79]. Based on the TGA and DTG data of both isolated and commercial chitin, the isolated chitin from Brazilian Atlantic Ocean was more pure and more thermal stable than the commercial chitin. Figure 7B showed the TGA and DTG of isolated and commercial chitosan. The isolated chitosan had two different peaks appeared in DTG curve. The first peak appeared at 50-90 °C, which was related to the evaporation of residual of water[12]. The second decomposition peak was appeared at 290 °C, may be related to decomposition of glucosamine and residual acetyl glucosamine in chitosan chain[80]. In commercial chitosan, there was two different peaks appear at 50-80 °C and at 270 °C for evaporated water and decomposition of the glucosamine and residual of acetyl glucose amine units in chitosan chains [80]. From the comparison data between isolated chitin, chitosan and commercial product chitin, chitosan, the isolated chitin and chitosan were more thermal stable, pure than commercial products.
Figure 7A: TGA and DTG of chitin

Experimental conditions: DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 °C); DP deproteinization: 5 % sodium hydroxide, 20 h, 90 °C. Degree of deacetylation DDA (5 %, measured by FTIR).
**Figure 7B**: TGA and DTG of chitosan

**Experimental conditions**: DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 °C); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C; DA-deacetylation: 50 % sodium hydroxide, 3.5 h, 90 °C, degree of deacetylation DDA (95 %, measured by NMR).

### 3.5. ATR-FTIR of chitin and chitosan

Figure 8 shows the FTIR-ATR of the isolated biopolymers (chitin, chitosan) comparing with the commercial one. Figure 8A shows the FTIR-ATR of chitin isolated from shrimp shell and commercial chitin, respectively; both commercial and isolated chitin showed similar peaks there were not identical. Different peaks appeared at 3470-3390 cm\(^{-1}\) for stretching vibration of hydroxyl and secondary amine groups and at 2880 cm\(^{-1}\) for methylene groups in chitin. Within the FTIR-ATR spectrum of isolated β-chitin, bands were observed at 1650 and 1550 cm\(^{-1}\) [6], the peaks were appeared at 1620 cm\(^{-1}\) and 1590 cm\(^{-1}\) for amide I and amide II, respectively [6, 7]. Beak at 1050
cm⁻¹ correspond to the C-O-C in chitin chain. isolated chitosan (Figure 8B) has a broad band about 3200-3374 cm⁻¹ for stretching vibration of OH and NH groups, 1661 cm⁻¹ for (amide I), 1595 cm⁻¹ for (amide II), 1380 cm⁻¹ for (amide III)[5, 7]. The intensity of the absorption band at 1595 cm⁻¹ for very short due to the highly degree of deacetylation of the isolated chitosan (95 %) comparing with the commercial chitosan. Asymmetric stretching of (C-O-C bridge) and 1080 cm⁻¹ for (C-O stretching) [11] characteristic of its polysaccharide structure (Figure 8B) in comparing with the commercial chitosan. Higher similarity was recorded between the commercial and the chitin and chitosan isolated from Brazilian Atlantic Ocean.

**Figure 8A:** FTIR-ATR of chitin  **Experimental conditions:** DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 °C); DP-deproteinization: 5 %sodium hydroxide, 20 h, 90 °C, Degree of deacetylation DDA (5 %, measured by FTIR).
Experimental conditions: DM-demineralization: 5% hydrochloric acid, 2 h, RT (22±2 °C); DP-deproteinization: 5% sodium hydroxide, 20 h, 90 °C; DA-deacetylation: 50% sodium hydroxide, 3.5 h, 90 °C, degree of deacetylation DDA (95%, measured by FTIR).

3.6. Solid NMR of chitin and chitosan

Solid-state NMR spectroscopy has been identified as the most useful and accurate tool for structurally analyzing insoluble complex biomaterial [81]. Figure 9 shows solid-NMR spectra of different deacetylated products prepared from chitin with various time using sodium hydroxide. The deacetylation temperature and concentration of sodium hydroxide were consistently set at 90 °C 50%, respectively. Each spectrum included seven well-defined resonances of C1-C6 and acetyl. They were ε = 173.7 ppm for C=O, ε = 102.8 ppm for C1, ε = 82.5 ppm for C4, ε = 74.5 ppm for C5, ε = 61.3 ppm for C6, ε = 56.1 ppm for C2, and ε = 23.2 ppm for CH₃. In highly deacetylated
chitosan, there was no any appearance for \( \varepsilon = 73.7 \), and 23.2 for carbonyl and acetyl groups in chitin, respectively. The DDA increased by increasing the treatment times, \( \text{DDA} \) (\%) = 30 \%, 67 \%, 93 \% and 95 \% after 1, 2, 3, and 4 h. treated with 50 \% sodium hydroxide at 90 °C, respectively, were illustrated in Figure 9. Different peaks were related to protein residual appeared at \( \sim 181.5, 128.7, 32.9, 30.0 \) and 14.2 ppm [82-84] which were not appeared in our products spectra this indicated that there was no any residual of protein interconnected to Atlantic source of purified chitin and chitosan compared with different other sources [82, 85-87].

![Figure 9](image)

**Figure 9**: Solid \(^{13}\)C CP/MAS NMR spectra of chitin and chitosan with different degree of deacetylation after various time. (a) DDA 30 % after 1h ; (b) DDA 67 % after 2h ; (c) DDA 93 % after 3h; (d) DDA 95 % after 4h. **Experimental conditions**: DM-demineralization: 5 \% hydrochloric acid, 2 h, RT (22±2 °C); DP-deproteinization: 5 \% sodium hydroxide, 20 h, 90 °C.
3.7. Residual of metals on chitin and chitosan after isolation process

Figure 10 shows the amount of minerals of the isolated chitin and chitosan in comparison to their source (shrimp shell). The concentrations of calcium, potassium, sodium, magnesium, aluminum, copper, manganese and titanium was decreased after the treatment with hydrochloric acid in demineralization step and sodium hydroxide in deproteinization and deacetylation steps of the extraction chitin and chitosan from the shrimp shell. Comparing with commercial different sources of chitin and chitosan, the metal residual in commercial products were 20 times higher than the isolated chitin and chitosan, indicated that the Brazilian Atlantic Ocean biopolymers were more pure than the commercial biopolymers (Table S1).

**Figure 10:** Residual metals in the extracted chitin and chitosan in the comparison with shrimp shell. **Experimental conditions:** DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22 °C±2); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C, DA-deacetylation: 50 % sodium hydroxide, 3.5 h, 90 °C, degree of deacetylation DDA (95 %, measured by NMR).
3.8. X-ray diffraction of chitin and chitosan

The XRD spectra of the isolated chitin and chitosan comparing with commercial biopolymers were shown in Figure 11. Diffraction pattern of isolated chitin (Figure 11a) exhibits ten markable diffraction peaks on angle $2\theta = 9.3^\circ, 19.3^\circ, 20.9^\circ, 23.5^\circ, 26.5^\circ, 34.8^\circ, 38.9^\circ$, positions of peaks corresponds to inter-planar distances 9.5 Å, 4.6 Å, 4.2 Å, 3.8 Å, 3.4 Å, 2.3 Å which in accordance with the literature data from different sources of chitin and chitosan [70, 88-90]. The commercial chitin exhibits also 10 remarkable diffraction peaks on the same position peaks comparing with isolated one. Only one more beaks at $2\theta = 12.7^\circ$ corresponds to inter-planar distances 7 Å which was corresponding to residual of impurities in commercial chitin like protein and pigments [91]. Some recent studies also reported similar crystalline peaks in the XRD measurements of $\beta$-chitin [76, 92]. From comparable intensities of corresponding peaks concluded that the crystallinity of isolated chitin and commercial chitin were similar. The Diffraction patterns of the isolated and commercial chitosan (Figure 11b) shows comparable data. Isolated chitosan and commercial chitosan had two broad beaks at $2\theta = 9.5^\circ$, and $21.5^\circ$ corresponded to inter-lattice distances 4.6 Å and 2.6 Å [6]. From XRD data both isolated and commercial chitin and chitosan (shrimp shell sources) had the same crystalline and amorphous properties.
Figure 11: XRD of chitin and chitosan

(a) **Experimental conditions**: DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22 °C±2); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C. (b) **Experimental conditions**: DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22 °C±2); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C; DA-deacetylation: 50 % sodium hydroxide, 3.5 h, 90 °C; degree of deacetylation DDA (95 %, measured by NMR).
3.9. Scanning electron microscopy of chitosan

The morphology of the isolated chitosan sample was studied using a scanning electron microscope. SEM images with different magnifications 1000, 500, 200, 50 µm (Figure 12A-D), respectively; of chitosan sample were presented in Figure 12. It was observed that the biopolymer surface became very smooth as comparing with the chitin sample before deacetylation step [93, 94].

Figure 12: Scan electron microscope of pure chitosan extracted from shrimp shells with particle size 800-1000 µm at different magnification (A) 1000 µm; (B) 500 µm; (C) 200 µm; (D) 50 µm, respectively. Experimental conditions; DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 ºC); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 ºC; DA-deacetylation: 50 % sodium hydroxide, 3.5 h, 90 ºC, degree of deacetylation DDA (95 %, measured by NMR).
3.10. Deacetylation degree (DDA) measurements of chitosan by different techniques

Chitosan was obtained from chitin using high concentrated sodium hydroxide solution. The degree of deacetylation (DDA), was an important parameter that indicated the molar % of monomeric units that have amino groups and vary from zero (chitin) to 100 (fully deacetylated) chitosan. DDA of chitosan was measured and compared between the data as described in Table 1. As shown in Table 1 the DDA of chitin was very low after treatment with 5 % of sodium hydroxide for 20 h. DDA was 35, 30, 23, and 25 by ATIR-FTIR, H/C-NMR, elemental analysis and acid-base titration, respectively. After removing the acetyl groups from chitin by using high concentration of sodium hydroxide for 3.5 h at 90 °C, the degree of deacetylation was measured and confirmed by ATR-FTIR, H/C-NMR, elemental analysis, and acid-base titration and it was 65, 95, 85, 75, respectively. From the data in Table 1, we could conclude that, the DDA of chitosan have different variable values due to the different techniques used.

Table 1: Determination of degree of deacetylation (DDA) by different analytical techniques

<table>
<thead>
<tr>
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<th>Degree of deacetylation (%)</th>
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<tr>
<td></td>
<td>FTIR</td>
</tr>
<tr>
<td>Chitin</td>
<td>35±2</td>
</tr>
<tr>
<td>Chitosan</td>
<td>65±3</td>
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*mean (standard deviation)

3.11. Antibacterial activity of chitin and chitosan

Figure 13, shows the antibacterial activity of chitin and chitosan isolated from shrimp shell by chemical treatments. The biological activity of chitin and chitosan was evaluated against gram negative bacteria (E. coli) by chemo-luminescence technique. This technique is more accurate
comparing with the other techniques like disc diffusion method. As described in (Figure 13), three different concentrations (5, 10, 15 mg) of chitin, chitosan, isolated from Atlantic Ocean and commercial were measured and evaluated to compare the biological activity of both material. As shown in Figure 13, the antibacterial activity of the isolated chitin increased by increasing the concentration of chitin (5, 10, 15 mg). However, with lower reduction rate value (10, 15, and 20 %, respectively). This could be explain by the blocking of the amino groups (low deacetylation degree, DDA = 5 % confirmed by NMR data). On the other hand the antibacterial activity of isolated chitosan increased (reduction rate; 60, 80, 90 %) dramatically by increasing the concentration of chitosan (5, 10 15 mg), since in this case chitosan had highly DDA=95 % confirmed by NMR data. There was relations between the antibacterial activity of chitosan and the free amino groups (DDA). The mechanism of the antibacterial activity of chitin and chitosan are still well unknown. The antibacterial activity of chitin and chitosan has been correlated with its degree of deacetylation (DDA), purity and molecular weight [1, 13, 18, 21, 95-97]. The antibacterial or antifungal activity of chitosan depends on the presence of positive charge numbers created on chitosan chains by DDA step, could be expected to have a more potent antibacterial activity. From the obtained results, this concluded that the biological activity of both chitin and chitosan isolated from Atlantic ocean as a source after DM, DP, DA steps had very high antibacterial activity in comparison with biopolymers (chitin and chitosan) from different other sources [98-101].
Figure 13: Kinetic curve of the bioluminescence of *E.coli* within sixty minutes in the presence of extracted chitin and chitosan. **Experimental conditions for chitin:** DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22±2 °C); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C. **Experimental conditions for chitosan:** DM-demineralization: 5 % hydrochloric acid, 2 h, RT (22 °C±2); DP-deproteinization: 5 % sodium hydroxide, 20 h, 90 °C; DA-deacetylation: 50 % sodium hydroxide, 3.5 h, 90 °C, degree of deacetylation DDA (95 %, measured by NMR).
4. Conclusions

Highly pure chitin and chitosan were isolated from Brazilian Atlantic Coast shrimp shell by the chemical treatment method. Furthermore, chitosan of different DDA and zero % of protein is obtained by alkali treatment. The isolated biopolymers were characterized and confirmed by different analytical tools like ATR-FTIR, TGA, XRD, elemental analysis, NMR, SEM, acid-base titration and compared with the commercial available chitin and chitosan. DMPA>DPMA>DAPM>DAMP had the heights value of chitin and chitosan. The isolated chitin and chitosan showed excellent antibacterial activity against gram-negative bacteria (E. coli). In addition, the high pure chitin and highly DDA of chitosan were obtained by Demineralization, deproteinization and deacetylation sequences steps. Finally we can conclude that, chitin and chitosan from Brazilian Atlantic Ocean had low protein, minerals percentage, high DDA, thermal stable as well as excellent antibacterial activity compared with the other sources of chitin and chitosan. According to this finding, Atlantic chitin and chitosan can be used for different medial application especially for tissue engineering and drug carrier purposes.
References

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Chapter 3

Wound Dressing based on

Chitosan/hyaluronan/Nonwoven Fabrics: Preparation,

Characterization and Medical Applications
Abstract

Thin layers of chitosan (positively charged) / sodium hyaluronate (negatively charged) / nonwoven fabrics were constructed by polyelectrolyte multilayer pad-dry-cure technique. Pure chitosan (CS) was isolated from shrimp shell and immobilized onto nonwoven fabrics (NWFs) using citric acid (CTA) as cross linker and solvent agents through a pad-dry-cure method. The prepared thin layer of chitosan citrate/ nonwoven fabrics (CSCTA/NWFs) were consequently impregnated with hyaluronan (CSCTA/HA/NWFs) in the second path through a pad-dry-cure method. Chitosan/hyaluronan/nonwoven fabrics wound dressing were characterized by different tools such as FTIR-ATR, TGA and SEM. The antibacterial activity and the cytotoxicity of the dressing sheets were evaluated against Escherichia coli (E.coli) and Streptococcus aureus (St. aureus), mouse fibroblast (NIH-3T3) and keratinocytes (HaCaT) cell lines, respectively. The cell-fabrics interaction was also investigated using fluorescence microscope, based on live/dead staining assay of 3T3 cells. The healing properties of the new wound dressing were evaluated and compared with the control sample.

Keywords: Hyaluronan, chitosan, nonwoven fabrics, cytotoxicity, healing properties
1. Introduction

Healing of wound is a complicated process of restoring cellular structures and epidermal tissue in damaged part to its normal condition as closely as possible [1]. This dynamic process consisting of three continuous, overlapping phases, the first phase is the inflammation phase which occurs immediately after wounding and continue for 24 to 48 h, hemostasis is the beginning of this phase which leads to the inflammation. Platelet-derived growth factors are released into the wound that promote the chemotaxis and proliferation of neutrophils in order to clean the wounds from bacteria, dead tissues or debris [2-6]. The second phase is fibroblast proliferation; in this phase, collagen is produced in order to supply the structure to wounds and replaces the fibronectin–fibrin matrix. The last phase is the remodeling which approximately starts after two or three weeks and can take more than two years. In this phase, new collagen forms and wound strength gradually increases. Many factors affecting on healing process as the type of damage, the ability of the tissue to repair and the general state of the host’s health [7-9]. Layer-by-layer (LbL) assembly is a technique that was used in large scale due to its extraordinary advantages in the preparation of multilayer films [10-14]. Multilayer films were formed as the result of impregnation procedure, different alternating polyelectrolyte layers of oppositely charged were successfully assembled on flat surfaces. Also, these films were easy synthesized on different substrates. On other hand, these layers had varies features as uniform, continuous, easily tailored, and resistant to protein adsorption in especial cases [15-27].

Chitosan is a cationic biopolymer, owing to its characteristic as nontoxicity, biodegradability, biocompatibility, antimicrobial activity and improving the healing of wounds; chitosan has been widely used as topical dressing in wound management [28-35].
Chitosan is characterized by three functional groups, an amino, acetamido and hydroxyl groups. Cationic character of amino group (after its potentization) gives good opportunities for it to react with other compounds, and the key properties of chitosan includes bactericidal, fungicidal, and it has immune-enhancing properties [28, 36, 37]. Also, chitosan is water soluble in acidic media due to the protonation of amino group on the second carbon of glucosamine [37]. Recently, chitosan has received extensive attention because it is a promising natural substances used in the biomedical, food, and chemical industries [36]. Chitosan and its derivatives have been shown an excellent antimicrobial activities towards most of living microorganisms as yeast, fungi and bacteria, this make many scientists be attention by this biopolymers [38, 39]. The anti-bactericidal activity of chitosan is related to its cationic character with high affinity for the microbial cell wall [28, 38, 40-42].

Hyaluronic acid (HA) is a natural biopolymer found in all living organisms; the major component in the extracellular of many organs in all body fluids as eyes, skin, joints and hyaline cartilage [43, 44]. HA has various applications especially in medical field, it can be used as tumor marker in different types of cancer such as prostate and breast cancer. Also, it’s used in wound healing repair and improve the biomechanical properties of tissues. HA interact with many cell surface receptor as CD44, and ICAM-1 causing cellular processess including morphogenesis, wound repair, inflammation and metastasis [45-47]. Furthermore, HA has a main role in the viscoelasticity of bio-fluids, it can control the hydration of tissue and transport of water [48-50]. The characteristic of hyaluronic acid as the biocompatibility and the hydrophobicity makes it an excellent component in cosmetics[50]. Nowadays HA has been used as drug delivery agent in various fields as ophthalmic, topical and parental [51].
This chapter describes the preparation of novel wound dressing sheets based on chitosan/hyaluronan/nonwoven fabrics and their physical, chemical and biological properties were evaluated.

2. Experimental Section

2.1. Materials

Chitosan was isolated from shrimp shell of Brazilian Atlantic Ocean obtained from VCI Brasil Indústria e Comércio de Embalagens Ltda. (Brazil). Citric acid was purchased from Sigma Aldrich (Germany) and hyaluronic acid (sodium salts) high molecular mass (1.5-1.7 MDa, determined by SEC-MAALS) was purchased from Contipro Biotech Ltd., Dolni Dobrouč, Czech Republic. Viscose (100 %, 40 g/m²) nonwoven cotton fabrics and samples (produced by random distribution for web and spun bonded thermally) were used in this study and kindly provided from El-Nasr Company for Spinning, Weaving and Dyeing–El-Mahalla El-Kubra, Egypt. These fabrics were cut into identical sheets (10 × 10 cm) and used for all experiments as described below.

2.2. Isolation of chitosan from shrimp shell

Pure chitosan (CS) was isolated from Brazilian Atlantic ocean as described in chapter 2 [28]. Briefly, shrimp shells were washed with water, acetone and isopropyl alcohol to remove sand, salts and other impurities. Washing was followed by three classical steps: the first step was demineralization which was carried out by 5 % hydrochloric acid solution for 2 h; the second step was deproteinization step (DP) which was done by the use of 5 % aqueous sodium hydroxide for 20 h; finally, the crude chitin was stirred in 50 % sodium hydroxide for 3.5 h to obtain pure chitosan. The isolated chitosan was fully characterized
by FTIR, X-ray diffraction, NMR, TGA-DTG and elemental analysis. The deacetylation degree of chitosan was 95% (determined by NMR and FTIR-ATR techniques).

2.2.1. Immobilization of chitosan citrate/hyaluronan onto nonwoven cotton fabrics

Cotton nonwoven fabrics (NWFs) were used as substrates for immobilization of chitosan citrate (CSCTA) and sodium hyaluronate (HA). Firstly, cotton nonwoven fabrics were washed with hot water for 30 min then with 0.1% sodium bicarbonate to activate the fabric surface. The nonwoven fabrics (10 × 10 cm) were immersed in chitosan citrate (CTA was 5 wt. %) of 0.5, 1, 2% solutions for 30 min, then squeezed at constant pressure using padding machine. The CSCTA/NWFs (coded as CS\textsubscript{0.5}CTA/NWFs, CS\textsubscript{1}CTA/NWFs and CS\textsubscript{2}CTA/NWFs) were dried at 80 °C for 10 min in thermal fixation machine. The CSCTA/NWFs were rinsed for three times with ultrapure water to remove nonattached (non-bonded) materials from the surface of nonwoven sheets and then dried at 80 °C for 10 min. The washing step was repeated three times to obtain layer of chitosan citrate on cotton layer (CSCTA/NWFs).

The CSCTA/NWFs (coded as CS\textsubscript{0.5}CTA/NWFs, CS\textsubscript{1}CTA/NWFs, CS\textsubscript{2}CTA/NWFs) were immersed in the second bath contained 0.5 wt. % of sodium hyaluronate, then CS\textsubscript{0.5}CTA/HA/NWFs, CS\textsubscript{1}CTA/HA/NWFs, CS\textsubscript{2}CTA/HA/NWFs were dried at 80 °C for 10 min, rinsed with ultrapure water to remove nonattached (non-bonded) materials. The washing step was repeated three times to obtain layer of hyaluronan layer onto chitosan citrate layers. Keep the wet pick up 100% in immersing step in each step. All the prepared layers assembled nonwoven fabrics were dried at 150 °C for 5 min. The CSCTA/HA/NWFs were code as CS\textsubscript{0.5}CTA/HA/NWFs, CS\textsubscript{1}CTA/HA/NWFs and CS\textsubscript{2}CTA/HA/NWFs.
2.3. Wound dressing characterization

Attenuated total reflectance Fourier transforms infrared spectroscopy (ATR-FTIR) was performed by the spectrophotometer Nicolet Impact 400 D FTIR-ATR (Nicolet CZ, Prague, Czech Republic) equipped with a ZnSe crystal for the ATR-FTIR spectroscopy. Transmittance was measured as a function of the wavenumber (cm⁻¹) between 4000 cm⁻¹ and 650 cm⁻¹ with the resolution of 8 cm⁻¹ and the number of scans equal to 128. Thermal decomposition was measured on thermogravimetric TG, Netzsch 209F3 instrument (Al₂O₃ crucible) with the heating rate 10 °C min⁻¹ (with data collecting rate 40 points per Kelvin), which was performed in the dynamic nitrogen atmosphere with the pressure of 0.1 MPa. The sample mass for TGA was about 0.9 mg, TGA temperature range 25-800 °C. The morphology of nonwoven fabrics was studied by the scanning electron microscopy (SEM). The nonwoven dressing sheets were cut with a razor scalpel after being frozen in liquid nitrogen for 10 min. X-Ray diffraction (XRD) data were collected utilizing the D8 Advance diffractometer (Bruker AXS, Germany) with Bragg-Brentano θ-θ goniometer (radius 217.5 mm) equipped with a secondary beam curved graphite mono-chromator and Na(Tl) I scintillation detector. The generator was operated at 40 kV and 30 mA and the scan was performed at room temperature from 2 to 50° (2θ) in 0.02° steps with a counting time of 8s per step.

The antibacterial activity of the wound dressing sheets was evaluated by the following way: Muller-Hinton agar (which consists of 3 g beef extract powder, 17.5 g hydrolyzed casein, 1.5 g starch and 17 g agar) was suspended in one liter of distilled water and heated to the formation of clear solution. The prepared medium was sterilized by autoclaving at 121 °C for 15 min [52, 53]. The nonwoven fabrics were divided into three groups. The first group
was control group (CTA/NWFs; treated with 5 % of citric acid). The second group were
nonwoven fabrics (NWFs) treated with 0.5 %, 1 % and 2 % of chitosan citrate forming
CSCTA/NWFs coded as (CS_{0.5}CTA/NWFs, CS_{1}CTA/NWFs, CS_{2}CTA/NWFs). The third
group were CSCTA/NWFs immersed in 0.5 % sodium hyaluronate solution forming
CSCTA/HA/NWFs assembly coded as (CS_{0.5}CTA/HA/NWFs, CS_{1}CTA/HA/NWFs, and
CS_{2}CTA/HA/NWFs). The all prepared nonwoven fabrics (untreated/treated) were sterilized
by autoclaving at 121 °C for 20 min. All treated and non-treated wound dressing code as in
table S1.

The antibacterial activity of the wound dressing nonwoven fabrics was performed
as follow [54, 55]; 1 cm of each wound dressing nonwoven fabric were prepared in sterilize
manner. Each sample was placed in a sterile vial and the dressing nonwoven fabrics were
pre-treated with 5 ml distilled water for 30 min. Tryptone soy broth (3 ml). 10 µl of
Escherichia coli (E. coli) and Staphylococcus aureus (St. aureus) suspension was added to
each nonwoven dressing fabric-containing vial (1.6 × 10^3/ml). Control agar with/without
bacterial inoculation was also included. The vials were incubated with agitation at 37 °C at
500 rpm. Aliquots of 10 µl broth were sampled at 24 h and serial dilution for the aliquots
was prepared in broth. Duplicate aliquots (50 µl) of the serially diluted samples were spread
onto plates. The plates were incubated at 37 °C and bacterial counts were performed. The
bactericidal activity was evaluated after 24 h and the Antibacterial efficacy of bacteria was
calculated by the following equation 1:

\[
\text{Antibacterial efficacy (\%)} = \frac{(A - B)}{A} \times 100
\]

Where, \(A\) = the number of bacterial colonies from untreated nonwoven fabrics, and \(B\) = the
number of bacterial colonies from treated wound dressing nonwoven fabrics. Each
measurement was carried out in triplicate and the arithmetic means are reported for all experiments.

The swelling ratio of nonwoven fabrics (NWFs), fabrics immobilized with chitosan citrate (CSCTA/NWFs), and fabrics immobilized with chitosan citrate/sodium hyaluronate (CSCTA/HA/NWFs) was measured under the physiological conditions. Thus, the samples were immersed in phosphate buffer solution (pH 7.5) at 37 °C for different times (0.5 - 72 h). The swelling percentage of the wound dressing was calculated by the following equation 2;

\[
\text{Swelling percentage} \, (\%) = \frac{(W_f - W_i)}{W_i} \times 100 \quad (1)
\]

where, \(W_f\) was weight of sample after certain swelling time and \(W_i\) was weight of sample in the dry state.

Cell viability assays are commonly used to measure the response of cells to toxic substances. Mouse fibroblast cell line (NIH 3T3) was cultured till 20\textsuperscript{th} passage. Cells of 5\textsuperscript{th}-20\textsuperscript{th} passage were used in the following experiments. NIH-3T3 were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10 \% FBS, glutamine (0.3 mg/ml), penicillin (100 \mu/ml) and streptomycin (0.1 mg/ml) in 7.5 \% CO\textsubscript{2} at 37 °C in 75 cm\textsuperscript{2} culture flask as recommended by the supplier. Spontaneously immortalized human keratinocyte cell line (HaCaT) was grown in DMEM supplemented with 10\% FBS, glutamine, and gentamycin in 5\% CO\textsubscript{2} at 37 °C as described previously [56-59]. All nonwoven cotton fabric (NWFs) treated with citric acid 5 \% (CTA/NWFs), nonwoven cotton fabric (NWFs) treated with chitosan citrate 1 \% (CS\textsubscript{1}CTA/NWFs) and nonwoven cotton fabric treated with 1 \% chitosan citrate then treated with 0.5 \% hayluronan (CS\textsubscript{3}CTA/HA/NWFs) samples were sterilized by autoclave at 120 °C for 20 min.
3000 (3T3) and 4000 (HaCaT) cells/well were seeded to wells of 96-well test plates. The cells were cultured for 24 h before treated with the suspended solutions. Then, the suspended solutions were added to each well using as diluent medium. Cell viability was measured 0, 24, 48, 72 h after treatment using the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) assay. In the assay, MTT was reduced by viable cells to a colored formazan salt, which was later released from the cells and determined spectrophotometric [59, 60]. When cells die, they lose the ability to convert MTT into formazan, thus color formation express of only the viable cells [61]. MTT stock solution was added to the cell culture medium and plates were incubated at 37°C for 2.5 h. The supernatant was discarded and cells were lysed in lysis solution for 30 min on a shaker. The optical density was measured in 96-well plate in triplicate by a versamax microplate reader at a wavelength of 570 nm. All experiments were carried out at least in four independent repeats.

In vitro qualitative analysis of cell viability as performed with live/dead assay. The samples of differently treated nonwoven fabrics (0.5 x 0.5 cm) were sterilized using UV light for 30 min. Before seeding with NIH-3T3 cells, the fabrics were incubated in culture medium (DMEM with L-glutamine, 10 % FBS, 1 % penicillin/streptomycin) one hour at 37 °C. Cells were seeded on fabrics at a density of 10⁵ /area in 24-well plate. Samples with seeded 3T3 cells were cultivated at 37 °C and 5% CO₂ for 3, 24, 48 and 72 h. Fluorescence microscopy and live/dead staining (calcein-AM/ propidium iodide) were used to determine cell viability 3, 24, 48 and 72 h after seeding. The mix of calcein-AM (2 μM) and propidium (1,5 μM) in PBS was added to fabrics containing seeded cells and incubated for 15 min at 37 °C and 5 % CO₂ for live/dead cell detection. Afterwards, fabrics were rinsed twice in PBS and visualized using a Zeiss Axio Imager 2 microscope.
The healing properties of control sample (CTA/NWFs), CS_{0.5}CTA/NWFs, CS_{1}CTA/NWFs, CS_{2}CTA/NWFs, CS_{0.5}CTA/HA/NWFs, CS_{1}CTA/HA/NWFs, and CS_{2}CTA/HA/NWFs were measured by using phagocytosis test [62,63]. Untreated/treated wound dressing fabrics were dispersed in phosphate buffer HBSS (pH 7.4) and each sample was analyzed in the following concentrations: 1.6, 0.8 and 0.4 mg/100 μl. PBS (178 μl) in case of spontaneous phagocytosis and 158 μl of HBSS in case of activated phagocytosis were added into micro-wells. The content of micro-wells was mixed with 20 μl of 0.01 M luminophor and 2 μl of whole human blood. Zymosan (20 μl) particles suspension (2.5 mg/ml) were added into micro-wells with activated phagocytosis and the control micro-wells did not contain analyzed substances, otherwise the contents was identical. Light (490 nm) emitted during reaction was measured continuously (1 h) by luminometer LM-01T. Results were expressed in relative light units (RLU). Experiments were repeated independently twice with the same results. The images of samples were done at the scanning-electron microscope. Tuscan VEGA II LSU electron microscope (Tuscan USA Inc.) under the following conditions: high voltage 5 kV, working distance 4.4 mm, display mode secondary electrons, high vacuum room temperature. SC7620 Mini Sputter Coater (Quorum Technologies, UK) applied 15 nm layers of gold particles on the sample. The samples were dusted for 120 s with the current of 18 mA. The pictures were made at these conditions: voltage 2.44–10 kV, detector: SE, the magnification 300–20000 times, vacuum high, the distance between sample and objective: 4 - 5 mm.

Fifty male rats (Division of veterinary medicine, National Research Centre, Cairo, Egypt) weighing (150 ± 20 g) were used as the test animals. The animals were maintained at normal room temperature (25–29 °C) on a 12 h. Light/dark cycle, with free access to a
commercial pellet diet and water. After the wound procedure, the animals were kept in individual cages. All experiments were conducted according to the National Communities Council directives on animal care. The dorsal hair of control and treated rats were shaved and (2.5 × 2.5 cm) diameter full thickness wounds were created with a biopsy punch. The wounds were cleaned with normal saline and disinfected by 75 % ethanol. The test wounds (n=3) were then covered with the sterile chitosan/hyaluronan nonwoven fabrics and fixed with sterile gauze and plasters. Similarly, control wounds (n=3) were covered with sterile nonwoven cotton fabrics and fixed with plasters. On 0, 3rd, 7th, and 14th, postoperative days, the dressings were removed and the appearance of the wound was photographed. The wound area was estimated by outlining the wound area. New dressings were applied to the wound sites after they were cleaned with normal saline. Photographs of wounds were taken every one to three days. If the bandage was applied, photographs were taken at each bandage change. When photographing the wound, a millimeter ruler was placed approximately 5 mm from the wound edge. Wound surface and ruler were in one plane. The images of the wound were open in Image J software. The image calibrated using a known length (2–4 cm): The rate of wound closure was determined by the following equation 2;

\[
\text{Amount of wound healing (\%)} = \frac{\text{wound area on } A_0 - \text{Ax}}{A_0} \times 100
\]

(2)

Where, \( A_0 \) wound area at day 0, \( Ax \) wound area at specified time of surgery, respectively. The Student (− t) test for two samples was used to analyze the significance of the experimental data (\( p \leq 0.05 \)). IFS Services Statistical tests on-line service was utilized.
3. Results and discussion

3.1. Immobilization of chitosan citrate/ hyaluronan onto nonwoven cotton fabrics

Pure chitosan (particle size 800-1000 µm) was used in this work by the method described in previous chapter [28]. Thus, the isolation of chitosan (CS) was done by four consequent steps. Firstly, shrimp shells were washed for many hours under reflux with hot water to kill some microorganisms and to remove the organic and inorganic impurities. In demineralization process (second step) the shrimp shell was treated with aqueous hydrochloric acid (5 % v/v) for 2 h at room temperature (22 °C) to remove the minerals (mainly CaCO₃) from the shells. The third step was deproteinization step which was done by aqueous solution of sodium hydroxide (5 % w/v) at 90 °C for 20 h in order to remove the proteins. The last step was deacetylation, where highly concentrated sodium hydroxide (50 %) was used for 3.5 h at 90 °C in order to hydrolyze the acetamido groups under the formation of high pure chitosan. The chemical structure and degree of deacetylation of chitosan were measured and confirmed by different techniques like ATR-FTIR, TG-DTG, XRD, NMR (Fig.S1). The degree of deacetylation of chitosan was found 95 %. Citric acid was used as dual functional material for dissolution of chitosan and as cross-linker agent between nonwoven fabrics (NWFs)/chitosan (CS) and chitosan/nonwoven fabrics (CSCTA/NWFs)/ hyaluronan (HA). Citric acid have three carboxylic groups which can prepare anhydride cycle under higher temperature [30]. Layer by layer (LbL) assembly is mainly occurred by the electrostatic interactions. This process consist of adsorption and deposition of different charged constituents and washing step removed the non-reacted specimens [62]. Citrate was improved the solubility of chitosan compared with other organic solvent for LbL assembled with sodium hyaluronate. Layer by layer technique itself
was an effective way produced functionalized materials. In fact, both CSCTA and HA are pH-sensitive. It can be expected that the multilayers agglomerated of CSCTA–HA also responsive to the change of environmental pHs.

Nonwoven cotton fabrics (NWFs; 10×10 cm, 0.45 g) were first impregnated in a solution of chitosan citrate solutions (CS$_{0.5}$CTA, CS$_1$CTA, CS$_2$CTA) prepared different layer of chitosan citrate onto NWFs surface coded as CS$_{0.5}$CTA/NWFs, CS$_1$CTA/NWFs, CS$_2$CTA/NWFs and the molar ratio between CS and CTA was (1:8; 1:4; 1:2), respectively (Fig. 1A). During the activated step of NWFs surface the hydroxyl groups in cellulosic fabrics convert to salt forms (ONa$^+$) can easily interact with carboxylic anhydride in the citric acid generated during the increased the reaction temperature in curing step. Citric acid/anhydride citric acid was used as cross-linker between NWFs and CS by amidation or esterification reactions. CS$_{0.5}$CTA/NWFs, CS$_1$CTA/NWFs, and CS$_2$CTA/NWFs were dried at 80 °C for 10 min in thermal fixation machine. The samples were rinsed for three times with ultrapure water to remove nonattached (non-bonded) materials from the surface of nonwoven sheets, then dried at 80 °C for 10 min. this step was repeated three times to obtain LBL (multi-layer) of chitosan citrate. The CS$_{0.5}$CTA/NWFs, CS$_1$CTA/NWFs, CS$_2$CTA/NWFs were immersed in the second bath contained 0.5 wt. % of sodium hyaluronate, coded as CS$_{0.5}$CTA/HA/NWFs, CS$_1$CTA/HA/NWFs, CS$_2$CTA/HA/NWFs (molar ratio between CS0, 5, 1, 2 %) and HA was 2:1, 4:1, 8:1, respectively. The dressing materials were dried, washed with water to remove the non-bonded hyaluronan then immersed again in HA solution, then dried at 80 °C for 10 min. All the prepared nonwoven fabrics were dried at 150 °C for 5 min. Different layers of hyaluronan were generated on the surface of CSCTA/NWFs by pad-dry cure technique. Figure 1B shows the proposed
mechanism of interactions between NWFs, CS and CTA. CTA could interact with hydroxyl groups on NWFs surface formed ester bond as well as with CS formed ester/amide bonds with hydroxyl and amino groups of chitosan chains under higher temperature. During the layers preparations efficiency of interactions was improved. Hyaluronan layer was generated during the impregnated process of CS$_{0.5}$CTA/NWFs, CS$_1$CTA/NWFs, and CS$_2$CTA/NWFs into sodium hyaluronan solution. Hyaluronan have different functional groups like coo$^-$ and -OH which could interact and/or assembled with the chitosan layers on the surface of nonwoven fabrics formed new ester or amide bonds between CSCTA/NWFs and HA (Fig.1B). During dissolution of hyaluronan in alkaline solution, the deacetylation process was partially done and few free amino groups [57, 63] were generated, and the pH of final product was 4.7.

Fig.1A: Immobilization of chitosan citrate Layers, sodium hyaluronate layers onto nonwoven fabrics.
**CS=chitosan; HA=hyaluronan; NWFs=nonwoven fabrics**

**Figure 1B:** Proposed mechanism of layer by layer assembled of chitosan citrate (CSCTA), sodium hyaluronate (HA) onto nonwoven fabrics.
3.2. Physical characterization of the wound dressing

Figure 2A shows ATR-FTIR spectra of nonwoven fabrics (NWFs), chitosan citrate/nonwoven (CS_{0.5}CTA/NWFs, CS_{1}CTA/NWFs, CS_{2}CTA/NWFs), and chitosan/hyaluronan/nonwoven fabrics (CS_{0.5}CTA/HA/NWFs, CS_{1}CTA/HA/NWFs, CS_{2}CTA/HA/NWFs) sheets prepared by layer by layer assembled technique. Polycarboxylic acid such as citric acid (CTA), butane-tetracarboxylic acid (BUTCA) could form a five member cyclic anhydride as reactive intermediate by dehydrate of two carboxylic groups bound to adjacent carbons in its backbone, which could reacted with a hydroxyl groups in nonwoven cellulosic fabrics and/or hydroxyl/ amino groups in the backbone of chitosan chains[30, 32, 64-67]. New band was appeared at 1715-1720 cm\(^{-1}\) due to the overlapping band between carbonyl, carboxyl and ester bonds formed between cellulosic nonwoven fabrics (hydroxyl groups) and chitosan (hydroxyl groups). The intensity of the carbonyl/carboxylic/ ester decreased by increase the concentrations of chitosan citrate [68-70]. Due to the presence of chitosan (free hydroxyl, amino groups), it could easy interact with a cyclic anhydride of citric acid and prevented appearance of anhydride cyclic ester band at 1852 and 1785 cm\(^{-1}\), respectively[64]. It was seen in figure 2Aa that the intensity of amide bond at 1650 cm\(^{-1}\) was slightly increased by increase of chitosan concentrations (0.5-2 wt. %). by addition of hyaluronan layers interacted with chitosan/nonwoven fabrics layers (CSCTA/CNFs), the intensity of the band 1715 cm\(^{-1}\) was slightly increased due to the formation of ester bonds between chitosan citrate and hyaluronan (carboxyl, Hydroxyl) groups. (Fig. 2A). It was seen in figure 2Aa that the intensity of amide bond at 1650 cm\(^{-1}\) was slightly increased by increase of chitosan concentrations (0.5-2 wt. %) after addition of hyaluronan layer generated. From figure 2,
we could conclude that citric acid can work as cross-linking agent between nonwoven cellulosic fabrics/chitosan, and sodium hyaluronate generated ester amide, and/or electrostatic hydrogen bonds between the different functional groups of the biomaterial and fabric matrix.

**Figure 2A:** FTIR-ATR of chitosan citrate/nonwoven fabrics (CS<sub>0.5</sub>CTA/NWFs, CS<sub>1</sub>CTA/NWFs, CS<sub>2</sub>CTA/NWFs); chitosan/sodium hyaluronate/nonwoven (CS<sub>0.5</sub>CTA/HA/NWFs, CS<sub>1</sub>CTA/HA/NWFs, CS<sub>2</sub>CTA/HA/NWFs), nonwovens (CTA/NWFs), respectively.
Figure 2B shows the thermogravimetric analysis (TGA-DTG) of wound dressing chitosan/hyaluronan/ nonwoven fabrics prepared by layer by layer technique. Three different concentrations of chitosan citrate were used (CS\textsubscript{0.5}CTA/NWFs, CS\textsubscript{1}CTA/NWFs, CS\textsubscript{2}CTA/NWFs) for preparation different layers on the surface of nonwoven fabrics. Chitosan citrate/nonwoven fabrics layers had three different stage were appeared. The first stage was related to evaporate the non-bonded and/or bonded water/solvent solution at decomposition at 75 °C and the second stage around 250 °C corresponded to decomposition stage of chitosan citrate. The last stage around 320 °C was associated with the oxidative degradation of chitosan backbone [30, 32, 71].

Hyaluronan layers were generated onto chitosan citrate/nonwoven fabrics in (Figure 2B) only two different stages were appeared after CSCTA/NWFs impregnated with HA solution and formed HA layers (CS\textsubscript{0.5}CTA/HA/NWFs, CS\textsubscript{1}CTA/HA/NWFs, CS\textsubscript{2}CTA/HA/NWFs). There were two different stages distinguished, the first stage was appeared in the same area of chitosan citrate layers at 75 °C related to the evaporations of non/bonded water and solvent from the dressing sheets. The stage was appeared when NWFs was treated with CSCTA solution, it means the hyaluronan assembled on the surface of chitosan layers improved the stability of wound dressing layers. Furthermore, the second stage (appeared at 330 °C) was related to decomposition of chitosan/hyaluronan layers, it was shifted comparing with only chitosan layers. Thus, a assembled of chitosan citrate and hyaluronan layers provided–strong chemical interactions reflected in improved thermal stability of the new wound dressing sheet.
Figure 2B: TGA-DTG of chitosan citrate /nonwoven fabrics (CS$_{0.5}$CTA/NWFs, CS$_{1}$CTA/NWFs, CS$_{2}$CTA/NWFs); chitosan/sodium hyaluronate/nonwoven (CS$_{0.5}$CTA/HA/NWFs, CS$_{1}$CTA/HA/NWFs, CS$_{2}$CTA/HA/NWFs), nonwovens (CTA/NWFs), respectively.

Figure 2C shows the swelling percentage of nonwoven fabrics (NWFs), chitosan citrate/nonwoven fabrics with different concentrations of chitosan (CS$_{0.5}$CTA/NWFs, CS$_{1}$CTA/NWFs, CS$_{2}$CTA/NWFs) and chitosan citrate/sodium hyaluronate/nonwoven fabrics with different chitosan concentrations (CS$_{0.5}$CTA/HA/NWFs, CS$_{1}$CTA/HA/NWFs, CS$_{2}$CTA/HA/NWFs). The layers of chitosan citrate (CSCTA) improved the swelling percentage of nonwoven fabrics (NWFs) compared with the non-treated sample. The swelling percentage of nonwoven fabrics was increased with the decrease the concentration of chitosan from CS$_{0.5}$CTA/NWFs to CS$_{2}$CTA/NWFs. This decreasing may be due to the hydrophobicity of chitosan chains, affecting the swelling of NWFs. After layers of sodium
hyaluronate were immobilized onto chitosan citrate/nonwoven fabrics (CSCTA/NWFs) surface, the swelling percentage (Figure 2C) was increased comparing with CSCTA/NWFs and plain NWFs and the percentage of swelling was raised by the increase the concentrations of chitosan (CS_{0.5}CTA/HA/NWFs to CS_{2}CTA/HA/NWFs). Due to the amphiphilic properties (hydrophobic of chitosan and hydrophilic of hyaluronan) of the immobilized layers onto NWFs, the swelling percentage was improved.

![Swelling percentage of wound dressing fabricated from chitosan citrate/nonwoven fabrics without/with hyaluronan (CS_{0.5}CTA/NWFs, CS_{1}CTA/NWFs, CS_{2}CTA/NWFs); (CS_{0.5}CTA/HA/NWFs, CS_{1}CTA/HA/NWFs, CS_{2}CTA/HA/NWFs), and nonwovens (CTA/NWFs) under physiological conditions (PBS, pH 7.5; 37 °C).](image)

**Figure 2C:** Swelling percentage of wound dressing fabricated from chitosan citrate/nonwoven fabrics without/with hyaluronan (CS_{0.5}CTA/NWFs, CS_{1}CTA/NWFs, CS_{2}CTA/NWFs); (CS_{0.5}CTA/HA/NWFs, CS_{1}CTA/HA/NWFs, CS_{2}CTA/HA/NWFs), and nonwovens (CTA/NWFs) under physiological conditions (PBS, pH 7.5; 37 °C).
Figure 3 shows scanning electron microscopy of wound dressing nonwoven fabrics impregnated with chitosan citrate different concentrations (0.5, 1, 2 wt.%) and sodium hyaluronate solution (0.5 wt.%). Layer thickness of chitosan citrate onto the nonwoven fabrics surface was increased by increase the chitosan concentration for 0.5 % (a-c), 1.0 5 1 2 % for (d-f) and 2 wt. % for (g-h). From the cross-section (Figure 3 c,f,i), the chitosan citrate coated the nonwoven fabrics and homogenous layer with aggregations appeared on the monofilament surface and between the fabrics matrix. The hyaluronan layer was assembled on the chitosan/nonwoven fabrics surface by chemical / physical interactions (Figure 3k-s). The hyaluronan solution was coated the chitosan citrate layer onto nonwoven fabrics and the coated layers thickness was increased and CSCTA concentration increase. From the cross-section of CS CTA/HA/NWFs (Figure 3m), CS CTA/HA/NWFs (Figure 3p), CS CTA/HA/NWFs (Figure 3s), the layers of chitosan/hyaluronan were surrounded the 0.5 1 2 monofilament formed coated layer on fiber surface. Figure 3t-z, the plain nonwoven fabrics with clean surface and circular shape from cross-section, respectively.
Figure 3: Scanning electron microscopy of layer by layer wound dressing sheet

CS$_{0.5}$CTA/NWFs (a,b,c); CS$_{1}$CTA/NWFs (d,e,f); CS$_{2}$CTA/NWFs (g,h,i); CS$_{0.5}$CTA/HA/NWFs (k,l,n); CS$_{1}$CTA/HA/NWFs (m,o,p); CS$_{2}$CTA/NWFs (q,r,s); CTA/NWFs (t,x,z). (a, d,g,k,m,q,t) scale bars were 100 µm; (b,e,h,l,o,r,z) scale bars were 10 µm; (c,f,i,n,p,s,z) scale bars were 5 µm.
Fibers and fabrics materials were carriers of different microorganism like pathogenic bacteria, odor-producing bacteria, and fungi due to the adhesion of these microorganisms to the fibers/fabric surface. Most nonwoven fabrics recently used in operation rooms and hotels were good environment to cross-infection or transfer of diseases occurred by microorganisms. Bacteria attached on fabrics tend to pass through fabric, especially the fabric is wet [32]. Research revealed that ordinary hospital textiles could not prevent bacterial transmission from person in surgical rooms [30]. The innovate of new wound healing dressing products (dressing) fabricated from immobilization of antibacterial and highly healing materials on nonwoven fabrics have recently increasing the importance to both the research and production sectors. Nowadays, a huge range of biological materials with different chemical structures were immobilized on the fibers/fabrics, bringing new unique performance to the final fibers/fabrics products [72, 73]. Chitosan, was used as an antimicrobial agent, have recently received renewed interest [72, 73].

3.3. **Antibacterial activity of chitosan/hyaluronan wound dressing**

The antibacterial activity of chitosan, chitosan/hyaluronan immobilized onto nonwoven cotton fabrics against different types of bacteria was evaluated as shown in figure 4. The antibacterial activities of chitosan were remarkably affected by the concentration of it, (CS0.5CTA/NWFs, CS1CTA/NWFs, and CS2CTA/NWFs) and the type of bacteria. CS1CTA/NWFs has high reduction rate (RD % = 95, 92 for –G and +G) than CS0.5CTA/NWFs (RD % = 85, 80 for -G and +G) and for CS2CTA/NWFs (RD (%) = 90, 75 for –G and + G) on the growth of both types of bacteria *Staphylococcus aureus* (*S. aureus*) as Gram positive bacteria (+G) and *Escherichia coli* (*E.coli*)as Gram negative bacteria (-G), respectively. The antibacterial activity of chitosan clearly decreased with low
concentration of its solution and it higher concentrations of chitosan (CS₂CTA/NWFs) the diffusion of chitosan inside the medium was hard and inhibited the interaction of chitosan and bacteria. Chitosan/hyaluronan/nonwoven cotton fabrics have also highly antibacterial activity \( (\text{Table S2}) \) against \( E.\text{coli} \) and \( S.\text{aureus} \) compared with plain chitosan coated nonwoven fabrics. The reduction rate was 88, 84 % for \( \text{CS}_0.5\text{CTA/HA/NWFs} \), 98, 92 % for \( \text{CS}_1\text{CTA/HA/NWFs} \), and 80, 70 % for \( \text{CS}_2\text{CTA/HA/NWFs} \) against \( \text{E.coli} \) and \( \text{St. aureus} \), respectively. The antibacterial activity of chitosan/hyaluronan was higher than plain chitosan against both types of bacteria due to hyaluronan has antibacterial activity against different types of microorganisms [72, 74].

In figure 4, chitosan possess high antibacterial activity against both types of bacteria especially with gram-negative bacteria; and the mechanisms were proposed that, the chemical interaction between positively charged chitosan and negatively charged microbial cell membranes this could be explain as the electrostatic forces between the protonated \( ^+\text{NH}_3 \) groups and the negative residues or the positively charged \( ^+\text{NH}_3 \) in glucosamine unit inhibit the growth of bacteria and this was occurred by binding the negatively charged cell wall [9, 75, 76].
Figure 4: Antibacterial activity of nonwoven wound dressing fabrics

CTA/NWFs (a); CS_{0.5}CTA/NWFs (b); CS_{1}CTA/NWFs (c); CS_{2}CTA/NWFs (d) against \((E. coli)\). CTA/NWFs (e) CS_{0.5}CTA/HA/NWFs (f); CS_{1}CTA/HA/NWFs (g); CS_{2}CTA/HA/NWFs (h) against \((E. coli)\). CTA/NWFs (i); CS_{0.5}CTA/NWFs (j); CS_{1}CTA/NWFs (k); CS_{2}CTA/NWFs (i) against \((St. aureus)\), CTA/NWFs (m); CS_{0.5}CTA/HA/NWFs (n); CS_{1}CTA/HA/NWFs (b); CS_{2}CTA/HA/NWFs (q) against \((St. aureus)\).

3.4. Cytotoxicity of the wound dressing

Viability assays were studies aimed to detect the cellular response to a toxic materials.

From the evaluation of cytotoxicity, the information about metabolic activity and the death
of cell can be obtained from it [77]. There were many of helpful assay technologies that use to evaluate metabolic markers to estimate the number of viable cells. The MTT 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide has been widely used to detect the viable cells; and this assay is based on the reduction of tetrazolium salt (yellow color) to insoluble formazan crystals (purple color) by metabolically viable cells [60, 61]. The MTT viability test was carried out to obtain the main information about the metabolism and proliferation influenced by CS1CTA/NWFs and CS1CTA/HA/NWFs wound dressing. This assay is rapid and very effective method for testing for both NIH 3T3 and HaCaT cell line activity [78, 79]. On the basis of obtained results in figure 5A (a), CS1CTA/NWF did not induce any changes in the cell growth for both NIH-3T3 and HaCaT cell line and the cell viability of tested biopolymers demonstrating the nontoxic effect (Fig. 5Aa,d). The viability of cells after the treatment with chitosan had small increases changed in whole tested time 0-72 hours but still in safe values. Figure 5A (b,e) shows the toxicity of sodium hyaluronate immobilized into nonwoven fabrics with different concentrations (100-1000 µg/ml) under different time treatments. Interestingly, chitosan/hyaluronan nonwoven fabrics (CS1CTA/HA/NWFs) enhanced and accelerated the cell growth with all concentrations ranges (100-1000 µg/ml) and with different treatment times (0-3 days). This result agrees very well with the good biocompatibility properties of chitosan/hyaluronan with different types of cells.
Figure 5A: Cytotoxicity measurements of plain chitosan (CS), plain hyaluronan (HA) and chitosan/hyaluronan treated nonwoven fabrics (CS1CTA/HA/NWFs) with different concentrations (100-1000 µg/ml), 3T3 viability, MTT, n=6; nonwoven complex sheets with different concentrations (100-1000 µg/ml), 3T3 viability, MTT, n=4 (a, b, c) respectively. Toxicity measurement plain chitosan (CS), plain hyaluronan (HA) and chitosan/hyaluronan treated nonwoven fabrics (CS1CTA/HA/NWFs) with different concentrations (100-1000 µg/ml) on the keratinocytes cell line (HaCaT), (d, e, f) respectively. Cell viability was measured at 0-3 days. The data were expressed as mean ± SEM (n= 6). Statistically significant differences (Student’s t-test, p≤0.05) to the non-treated sample (control).
Figure 5B shows fluorescence images of CS1CTA/NWFS; a-d, CS1CTA/HA/NWFS; e-h and cell-wound dressing fabrics interaction (V,VI) after 3 and 72 h of treatments with mouse fibroblast cell lines (NIH-3T3). From our observation the cells prefer treated fabrics without any evident differences between CS1CTA/NWFs and CS1CTA/HA/NWFs treated surfaces; that could be explain as the more biocompatible treatment of fabrics for cell adhesion. However, it seems that the cells did not show the character of well spread cells. By contrast, they were round, captured in the fibers and just a couple of cells were spread and interact with the surface of fabrics. This interaction was observed only for CS1CTA/HA/NWFs wound dressing treated fabrics (Figure 5Bm) higher magnification of interacting cells in Figure 5Bp,q marked with red arrows in CS1CTA/HA/NWFs and detailed cell-fiber interaction in Figure 5Bn). The low spreading density could be the reason why the cells did not exhibit extensive proliferation as just a slight increasing number of cells is evident for treated fabrics and a constant number for the control. Further, for all samples the decreasing number of cells was observed 72 h of cultivation. This could imply the washing cells out from the fabrics due to the poorer adhesion in time. In summary, the treatment of nonwoven fabrics with different concentrations of chitosan and chitosan/hyaluronan ratio were promising as new wound dressing/healing materials and correspond with our results obtained from the cytotoxicity assay.
Figure 5B: Fluorescence microscopy of nonwoven cotton fabrics (CTA/NWFs) immobilized by chitosan citrate (CS$_1$CTA/NWFs), chitosan citrate/hyaluronan layer (CS$_1$CTA/HA/NWFs) and 3T3 cells after staining with calcein and propidium iodide. (a-d) nonwoven cotton fabrics treated by chitosan citrate (CS$_1$CTA/NWFs); layered hyaluronan (HA) assembled on the chitosan/nonwoven fabrics layers (e-h; CS$_1$CTA/HA/NWFs); nonwoven fabrics (control; i-l) and cell dressing interaction (m-q) after different seeding time (3, 24, 84, 72 h).
Phagocytosis measurements were performed to measure the level of reactive oxygen species (ROS) in blood due to the increase consumption of oxygen by phagocytic cells [80]. During phagocytosis process, oxygen is converted into different types as hydrogen peroxide, monomolecular oxygen and hydroxyl radicals by several kinds of phagocytic cells [81]. These reactive oxygen species (ROS) cause different biological effects as parasites and tumor cells, destruction of bacterial cells, promoting inflammation and modulating the immune reaction [82]. The generation processes of reactive oxygen species can be monitored using luminescence analysis.

Figure 6 shows that chitosan did not change the spontaneous production of ROS by intact phagocytes it means that the isolated chitosan was safe for cells while hyaluronan immobilized nonwoven fabrics demonstrated slightly release of free radical but still safe and there was no damages on cells (Figure 6). On the other hand zymosan-induced ROS production was significantly decreased by all concentrations of chitosan/hyaluronan nonwoven fabrics (1.6, 0.8 and 0.4 mg/µl) in comparison with control. It was suggested that the presence of chitosan/hyaluronan composite couldn’t be responsible for the inhibition of zymosan-activated oxidative burst of phagocytes and this is very important since phagocyte derived ROS has harmful effect in healing wound.
Figure 6: Effect of chitosan citrate (CS/CTA/NWFs), sodium hyaluronate (HA/NWFs) and chitosan/hyaluronan nonwoven fabric sheets (CS1CTA/HA/NWFs) on the spontaneous and zymosan-activated oxidative burst of blood phagocytes. Oxidative burst of phagocytes was analysed chemiluminometrically. Light emission expressed as relative light units (RLU) was recorded continuously for 90 min at 37°C and corrected to the number of polymorph nuclear cells.

3.5. Wound healing measurements

Wound healing is stepwise process which consists of various phases as inflammation, proliferative and remodeling [7, 9, 80]. The genetic response regulating (normal state) cellular resistance mechanisms contributes to the wound and its repair. The damaged tissues requires biocompatible materials like chitosan on which cells may adhere and proliferate. Chitosan prepared from natural biopolymer chitin has been tested as wound dressing at the skin-shaving site in rats [7, 81-83]. Figure 7Aa induced the results of nondiabetics rats wounds treated with control nonwoven fabric (without any treatment with chitosan or chitosan/hyaluronan) at 0, 3, 7, and 14 days and for wounds treated. There was
slightly evident effect of non-treated nonwoven fabrics (control sample) onto nondiabetic rats after two weeks of treatments and the wound closer percentage was 40% (Fig. 7B). Comparing with chitosan/hyaluronan composite wound dressing, the healing properties of nondiabetic rates (Fig. 7Ab) was enhanced and accelerated after wound treated with biomaterials and the wound closer rate after two week was 95% (Fig.7B).

In figure 7Ac control diabetic’s rat treated with nonwoven cotton fabrics (treated with 5 wt. % CTA). The closer percentage after two week of treatments was 25% comparing with control diabetic rats. Figure 7Ad present the CS1CTA/HA/NWFs treated the diabetic rats. The wound closure was 60% after 14 days of the treatment. It was suggested that their wound-healing properties of chitosan were due to their ability to stimulate fibroblast production by affecting the fibroblast growth factor [39, 84-88]. The results from figure 7A, B that, chitosan/hyaluronan was highly efficient accelerated and enhancement the healing properties of diabetics and nondiabetics rats comparing with control sample. Chitosan/hyaluronan composite had very high healing properties compared between plain chitosan or plain hyaluronan (data not shown), due to healing properties of both chitosan and hyaluronan together. Enhanced tissue repair ability of CS/HA immobilized into nonwoven cotton fabrics in the early phase of inflammatory response could be reasonably ascribed to the recapitulation of induction signals exerted by HA. Indeed, HA naturally interacts with signaling receptors (primarily CD44) to initiate inflammatory response, to maintain structural cell integrity and to promote recovery from tissue injury [89]. Furthermore, a possible activity of HA on peroxisome proliferator-activated receptors (PPARs) during wound healing could not be excluded [90]. The formation of a highly hydrated HA-rich matrix could be supposed to facilitate cell migration into the provisional
wound matrix fostering proliferation and in turn regeneration process [91]. Although wound contraction is the primary mechanism underlying healing process of closure in the murine model adopted, re-epithelialization of the wound site, which is prevalent in human skin, and suggested by in vitro results, can well take place and contribute to wound closure as observed elsewhere [92].

Figure 7A: Visual observation of the surface healing dressed with nonwoven sheet (control-non-diabetic) at 0th, 3th, 7th and 14th days after operation (a-d), respectively; (one of three same cases in each group was shown); healing dressed with chitosan/hyaluronan nonwoven dressing sheet (non-diabetic) at 0th, 3th, 7th and 14th days after operation (e-h), respectively; surface healing dressed with nonwoven sheet (gauzy) from cellulosic material (control diabetic) at 0th, 3th, 7th and 14th days after operation (i-m), respectively; (one of three same cases in each group was shown); healing dressed with chitosan/hyaluronan/nonwoven fabrics sheet (diabetic) at 0th, 3th, 7th and 14th days after operation (n-r), respectively.
Figure 7B: Wound contraction as a function of postoperative time for (non-diabetic and diabetics) wounds treated with chitosan/hyaluronan nonwoven fabric (CS1CTA/HA/NWFs) sheets and control (nonwoven cellulosic fabrics- gauze), indicated by the percentage of wound size vs healing dates.
4. Conclusions

New wound dressing materials based on chitosan/hyaluronan composite/nonwoven fabrics were prepared and evaluated. Pure chitosan was isolated from shrimp shell with high degree of deacetylation (95%) and non-protein residual percent. Nonwoven viscose fabrics were used for immobilization of biologically active biopolymers (chitosan, hyaluronan) by pad-dry-cure method. Chitosan/hyaluronan/composite showed well distribution of chitosan and hyaluronan on the surface of viscose fibers confirmed by scanning electron microscope. The prepared wound dressing material showed excellent antibacterial activity against two types of bacterial *E. coli* and *S. aureus* as gram negative and positive, respectively. The cytotoxicity of the new dressing materials was revealed by using mouse fibroblast cell line (*NIH-3T3*) and keratinocytes cell lines (*HaCaT*) with different times and concentration of treatments and showed that there was not any toxicity of the prepared dressing material. From the healing measurements, chitosan/hyaluronan viscose nonwoven fabrics composite accelerated and enhanced the healing properties of diabetics and nondiabetics rats compared with control sample. In summary, this novel chitosan/hyaluronan/nonwoven fabrics wound dressing will open new doors for realizing potential using the dressing as carrier of drugs to better treat wound defects and promote the wound healing.
Chapter 3

References
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61. A. Lojek, L. Kubala, H. Čížová, M. Číž, Luminescence, 17 (2002) 1.4-
Chapter 4

Conclusion and future work
Dissertation work conclusion

At the end of this thesis can be stated that despite all doubts can chitin and chitosan from shrimp shells to obtain such purity to be used in medicine and in textile applications where the fabric comes into contact with the human skin and possibly infected wounds. Based on the positive results presented in this work the following research and development will be done in the future:

Chitin, the second most abundant biopolymers found in nature next to cellulose and chitosan (the N-deacetylated form of chitin) could be isolated from Brazilian Atlantic Coast shrimp shell by the chemical treatment method in highly pure form. Demineralization, deproteinization and deacetylation processes were the main steps in the isolation process. The particle size in the range of 800-1000 µm was selected to isolate chitin, which was achieved by measuring nitrogen, protein, ash, and yield %. Pure chitosan was isolated from shrimp shell with high degree of deacetylation (95 %) and non-protein residual percent. The isolated biopolymers were characterized and confirmed by different analytical tools like ATR-FTIR, TGA, XRD, elemental analysis, NMR, SEM, acid-base titration and compared with the commercial available chitin and chitosan. The best sequences in isolation chitin and chitosan was DMPA>DPMA>DAPM>DAMP. The isolated chitin and chitosan showed excellent antibacterial activity against gram-negative bacteria (E. coli). New wound dressing materials based on chitosan/hyaluronan composite/ nonwoven fabrics were prepared and evaluated. Thin layers of chitosan (positively charged) / sodium hyaluronate (negatively charged) / nonwoven fabrics were constructed by polyelectrolyte multilayer pad-dry-cure technique. Chitosan/hyaluronan/composite showed well distribution of chitosan and hyaluronan on the surface of viscose fibers confirmed by
scanning electron microscope. The prepared wound dressing material showed excellent antibacterial activity against two types of bacterial *E. coli* and *S. aureus* as gram negative and positive, respectively. The cytotoxicity of the new dressing materials was revealed by using mouse fibroblast cell line (NIH-3T3) and keratinocytes cell lines (HaCaT) with different times and concentration of treatments and showed that there was not any toxicity of the prepared dressing material. From the healing measurements, chitosan/hyaluronan viscose nonwoven fabrics composite accelerated and enhanced the healing properties of diabetics and nondiabetics rats compared with control sample. Finally this novel chitosan/hyaluronan/nonwoven fabrics wound dressing will open new doors for realizing potential using the dressing as carrier of drugs to better treat wound defects and promote the wound healing.

**Future work**

- Isolation of chitin and chitosan in nano-fibrilis structure with control size and length of the nano-fibrous.

- Preparation of hydrogel from chitin/chitosan/hyaluronan and study the physical and chemical properties of the hydrogel.

- The chitin and chitosan nano-fibrous will be used as blood clotting in 3D scaffold shape.
Supporting Information
Supporting Information 1

Chitin and Chitosan from Brazilian Atlantic Coast: Isolation, Characterization and Antibacterial Activity

<table>
<thead>
<tr>
<th>Components</th>
<th>Compositions of Shrimp shell from Atlantic coast</th>
<th>Compositions of Shrimp shell from other sources(Ref.1-5)</th>
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</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
<td>30</td>
<td>52.03[162];48.97[215]; 40.6[216]; 48.1[217]; 48.8[218]</td>
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<tr>
<td><strong>Mineral</strong></td>
<td>8-10</td>
<td>28.84[162]; 29.5[215]; 20.6[216]; 14[217]; 29.93[218]</td>
</tr>
<tr>
<td><strong>Chitin</strong></td>
<td>40-53</td>
<td>19.1[162]; 21.53[215]; 16.2[216]; 38[217]; 20[218]</td>
</tr>
<tr>
<td><strong>Chitosan</strong></td>
<td>30-43</td>
<td>10[162]; 7.11[215]; 9[216]; 20[217]; [218]</td>
</tr>
</tbody>
</table>

Table S1: Comparison among the components of shrimp shell and other sources

References

## Supporting Information 2

**Wound Dressing via Layer by Layer Assembly of Chitosan Citrate /Hyaluronan/ /Nonwoven Fabrics: Preparation, Characterization, *In Vitro* and *In Vivo* Evaluations**

<table>
<thead>
<tr>
<th>Sample codes</th>
<th>Treatment code</th>
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<tbody>
<tr>
<td><em>CTA/NWFs</em></td>
<td>Nonwoven cotton fabric (NWFs) treated with citric acid (5%).</td>
</tr>
<tr>
<td><em>CS</em>₀·₅<em>CTA/NWFs</em></td>
<td>Nonwoven cotton fabric (NWFs) treated with chitosan citrate (0.5 %).</td>
</tr>
<tr>
<td><em>CS</em>₁<em>CTA/NWFs</em></td>
<td>Nonwoven cotton fabric (NWFs) treated with chitosan citrate (1 %).</td>
</tr>
<tr>
<td><em>CS</em>₂<em>CTA/NWFs</em></td>
<td>Nonwoven cotton fabric (NWFs) treated with chitosan citrate (2 %).</td>
</tr>
<tr>
<td><em>CS</em>₀·₅<em>CTA/HA/NWFs</em></td>
<td>Chitosan citrate (0.5 %) nonwoven cotton fabric (NWFs) treated with hyaluronan (0.5 %).</td>
</tr>
<tr>
<td><em>CS</em>₁<em>CTA/HA/NWFs</em></td>
<td>Chitosan citrate (1 %) nonwoven cotton fabric (NWFs) treated with hyaluronan (0.5 %).</td>
</tr>
<tr>
<td><em>CS</em>₂<em>CTA/HA/NWFs</em></td>
<td>Chitosan citrate (2 %) nonwoven cotton fabric (NWFs) treated with hyaluronan (0.5 %).</td>
</tr>
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</table>

**Table S1:** Code of treated and untreated cotton fabrics with different chitosan citrate/hyaluronan concentrations.
<table>
<thead>
<tr>
<th>NONWOVEN TREATMENTS</th>
<th>E. coli</th>
<th>St. aureus</th>
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<tr>
<td>CTA/NWFS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CS₀.₅CTA/NWFS</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td>CS₁CTA/NWFS</td>
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<td>92</td>
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<tr>
<td>CS₂CTA/NWFS</td>
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<td>75</td>
</tr>
<tr>
<td>CS₀.₅CTA/HA/NWFS</td>
<td>88</td>
<td>84</td>
</tr>
<tr>
<td>CS₁CTA/HA/NWFS</td>
<td>98</td>
<td>92</td>
</tr>
<tr>
<td>CS₂CTA/HA/NWFS</td>
<td>80</td>
<td>70</td>
</tr>
</tbody>
</table>

**Table S2.** Reduction rate percentage of chitosan and chitosan/hyaluronan/nonwoven cotton fabrics
**Fig.S1.** FTIR-ATR (A); TGA-DTG (B); H-NMR (C); XRD (D) of the isolated chitosan from shrimp shell (our work).
Curriculum vitae

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- M.Sc. in Organic Chemistry
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  In titled "Synthesis and Some Reactions of Heterocyclic Compounds Containing Coumarin Moiety"

- Bachelor of Science
  Faculty of Science, Al-Azhar University, [Very Good with Class Honor].

Current Research

✔ Extraction processes of biopolymers (chitin, chitosan).
✔ Biological activity of biopolymers.
✔ Preparation of different metal nanoparticles

List of Patent


**Poster papers in international and national conferences**


6. **Rasha M. Abdel-Rahman**, Radim Hrdina; Finishing of cellulosic fabrics with Chitosan/polyethylene glycol-siloxane to improve their Performance and antibacterial properties;5th EUCHEMS Chemistry Congress 31 August-4 September 2014 in Istanbul, Turkey. ISSN: 1614-7499.
