CHITOSAN-DERIVATIVES AS HEMOSTATIC AGENTS: THEIR ROLE IN TISSUE REGENERATION

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ABSTRACT

In a recent discovery in the field of tissue regeneration, chitosan, a natural polysaccharide, received attention as a hemostatic agent due to its character to function independently on platelets to achieve hemostasis. In our present review, we highlight the composition and chemical structure of chitosan and its application in the current medical breakthrough, its reactions on erythrocytes and platelets, and its use as a wound dressing to promote tissue regeneration.

1.0 Introduction

Regenerative medicine is a noteworthy field that faces many challenges from the life sciences. In scientific terms, regeneration is the action of replacement, refurbishment, and growth of genomes, cells, organs, and organisms, caused by damage to the human body. It is interlinked with the fields of tissue engineering, stem cells, gene therapy and therapeutic cloning[1]. The study of tissue regeneration helps experts to explore the restoration of the function of impaired organs and tissues such as bones, cartilage, blood vessels and the pancreas. Ongoing scientific research on cell growth and tissue replacement seeks to develop relevant breakthroughs in the medical field. Tissue engineering is a novel field in biomaterial science that intends to fully imitate the original processes to fix damaged tissues and cells.

Hemorrhage is the most common cause of death for severely injured patients when prompt action was not taken within a critical time period. Over the past 3 decades, since the Vietnam War, improved methods have been widely introduced in the civilian setting. Battlefield wounds differ from other usual injuries in terms of epidemiology, mechanism of injury and pathophysiology of the body's response[2]. Forty percent of traumatic mortality deaths[3] and up to 90% of all civilian deaths took place in pre-hospital settings[4]. At the same time, 50% of combat deaths have been reported due to massive blood loss resulting from hemorrhage.

Post-mortem studies of casualties in Operation Iraqi Freedom (OIF) and World War II suggested that 24% of all battlefield mortalities could have been prevented with improved methods in hemorrhage control, and 85% of them were due to uncontrolled hemorrhage[5]. The discovery of new methods and

devices for hemorrhage control are emphasized to reduce future hemorrhage morbidity and mortality[6]. Therefore, attention has been focused on the development of alternative methods for controlling hemorrhage including topical and surgical hemostatic agents[7].

Hemostatic agents

There are a large number of hemostatic agents on the market, such as oxidized regenerated cellulose, absorbents, hemostats, sealants, starch-based powder, collagen-based dressings, topical thrombin, thrombin-fibrinogen dressings, light-cured gelatin hydrogel, chitin and chitosan-based hemostatic agents, cyanoacrylate adhesives, polyethylene glycol polymers, bovine albumin and glutaraldehyde, microporous polysaccharide hemospheres, microcrystalline collagen, gelatin sponge, fibrin glue and poly(L-glutamic acid) (PLGA). There are various types of hemostatic agents and respective configurations available. Each of these agents has its own advantages and limitations.

Composition and chemical structure of chitosan

Chitosan is composed of randomly distributed β(1-4) linked D-glucosamine and N-acetyl-D-glucosamine[24]. Chitosans are the major elements derived from the shells of arthropods, such as crabs, shrimps, lobsters, and insects, and are also produced extracellularly by the cell wall of fungi and brown algae[25]. Chitosan is rarely found in nature but does occur in dimorphic fungi, such as *Mucor rouxii* by the action of the deacetylase enzyme on chitin[26]. The amines on chitosan become protonated at acidic pH and transmit a positive charge to the chitosan chains. Chitosan was selected as a hemostatic agent due to its cationic nature. Most biological cell surfaces are anionic, and chitosan was thought to strongly adhere to the tissue at the site of a wound via electrostatic interactions.

Besides resembling novel hemostatic agents, it also has anti-microbial properties that prevent infection at the wound site. Generally, chitosan has three kinds of reactive functional groups.

Advantages	Disadvantages
• Sustain hemostasis for at least several hours	• Incapable of stopping bleeding and can cause the formation of thrombi
• Reduce blood loss and decrease mortality	• Can cause an allergic reaction
• Seal wound site	• A short shelf life and has to be prepared just before use
• Induce vasospasm	
• Absorb water, accelerate the concentration of red blood cells, clotting factors, and platelets at the bleeding site	• Very specific handling instructions, difficult to utilize in certain environments
• Induce platelet activation and form clot	• Costly

Table 1 Advantages and disadvantages of hemostatic agents[8-18]

Chitosan

Recently discovered, chitosan-based hemostatic agents appear to function independently on platelets rather than following the normal clotting mechanism. Chitosan is an aminopolysaccharide molecule with a strong positive electrical charge, which strongly attracts and bonds to negatively charged molecules. In this fast paced world, chitosan received considerable attention as a functional, renewable, nontoxic, biocompatible, bioabsorbable and biodegradable biopolymer agent[19-21]. Its ingredients have been reported to be dependent on the chemical structure, molecular size, and enzymatic hydrolysis tailored for its potential pharmaceutical and medical application[22]. Nevertheless, it is crucial to note that chitosan has different structures with different biological activities. Not all biological activities are found in one kind of chitosan. Each special type of bioactive chitosan has been developed by chemical modification and enzymatic hydrolysis[23].

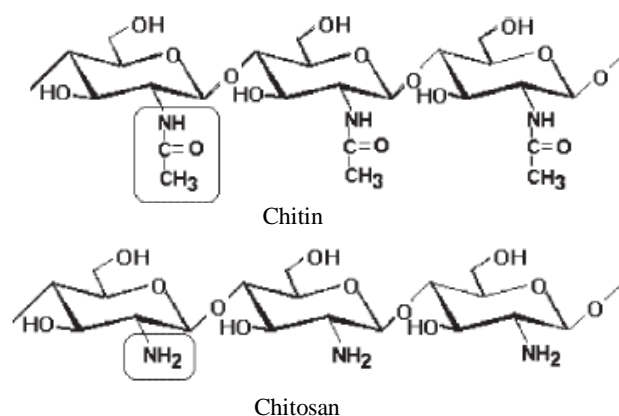Its amino groups have both primary and secondary hydroxyl groups at the C(2), C(3), and C(6) positions[23].

Chitin

Chitosan

Fig. 1 The chemical structure of chitin and chitosan[27]

These groups allow the modification of chitosan-like graft copolymerization for specific applications that are useful in tissue regeneration. The chemical nature of chitosan provides many possibilities for covalent and ionic modifications which allow extensive adjustment of mechanical and biological properties.

Each special type of bioactive chitosan has evolved by altering the chemical ingredients and enzymatic hydrolysis[21]. Chitosan components insoluble in most of solvents but were slightly soluble in diluted organic acids, such as acetic acid[28].

Table 2 Types of chitin and chitosan-derivative hemostatic agents[6,8,31-37]

Types	Mode of Action
HemCon®	Freeze-dried chitosan acetate salt, mainly used for emergencies to stop blood loss, enhance platelet function
Chitoflex®	Antibacterial and biocompatible wound dressing designed to reduce moderate to severe bleeding by adhering strongly to tissue surfaces, forming a flexible barrier that seals off and stabilizes the wound surface
Chitoseal®	Supported with a cellulose coating for hemorrhage wounds, reduces compressible timing.
Clo-Sur®	Used topically to stimulate wound healing at sites of vascular injury
TraumaStat®	Freeze-dried chitosan containing highly porous silica, proposed for external temporary use to control moderate to severe bleeding.
Syvek-Patch®	Achieves faster hemostasis by agglutinating red blood cells, activates platelets, controls bleeding following catheter removal in diagnostic operations
BST-CarGel®	Chitosan-glycerophosphate hydrogels, biodegradable gel used to repair cartilage impairment
Remedium's Hemogrip™	Adheres to tissues in a very effective manner, soft tissue sealant from transudation and microbial intrusion, able to treat injuries ranging from normal to life-threatening arterial punctures.
Celox	Used in lethal bleeding, is very effective in just 30 seconds, does not generate heat, forms a robust plug when red blood cells react with the agent.
Celox-A	Applicator and plunger delivery system, able to react deep into a small penetrating wound.
Celox Trauma Gauze	Controls traumatic bleeding and helps cool and protect first and second degree burns.
Chitipack S	Widely used for traumatic wounds and surgical tissue defects, reported with no retractive scar formation upon usage, supported on polyethylenetherephthalate, for the treatment of large skin defects, suitable for defects that are difficult to suture.
Chitipack P& C	Cotton-like chitosan obtained by spinning chitosan acetate salt into a coagulating bath of ethylene glycol, ice, and sodium hydroxide, can be used for whole set reconstruction of body tissues by rebuilding normal subcutaneous tissues and regular regeneration of skin
Tegasorb	The dressing contains chitosan particles that swell while absorbing exudates. producing a soft gel
Tegaderm film dressing	A layer of waterproof dressing covers the hydrocolloid, used for leg ulcers, sacral wounds, and chronic wounds
Chitodine	Chitosan powder with adsorbed elementary iodine, for the disinfection and cleaning of wounded skin and surgical dressings
QuikClot®	Adsorbent hemostatic agent that speeds up the coagulation profile, stops blood loss, and is very suitable for larger wounds.

The usage and benefit of chitosan is limited due to its insolubility in water, high viscosity and aggregation of the protein molecules at high pH levels.

Chitosan as a hemostatic agent used in the medical field

Chitosan can be used medically by directly applying it to bleeding surfaces in various physical forms, such as powder, solution, coating, film, hydrogel, and filament composite. Currently, there are a variety of products designed to serve as hemostatic agents that can stop blood loss[8, 29-30]. Chitin and chitosan-based hemostatic agents that have been approved and applied medically to accomplish hemostasis are presented in Table 2.

Previously, Malette *et al*. reported the hemostatic activity of chitosan. Generally, the hemostatic activity of chitosan does not rely on a normal hemostasis cascade. Rather, it depends on the molecular weight, degree of deacetylation, and characteristics of chitosan primarily its polycationic properties that interfere with negatively charged molecules at the cell surface[38]. Lee *et al*. reported that low molecular weight polymers of chitosan were incapable of promoting effective coagulation[39]. Olsen *et al*. elucidated some possible mechanisms of blood coagulation with the various physical forms of chitosan salts.

Their results summarized the experiments on animals that further defined the efficacy of various chitosan compositions. These compositions included chitosan glutamate lyophilized films, chitosan glutamate or collagen lyophilized pads and chitosan glutamate aqueous solutions. The commercially available hemostatic agents, include crosslinked and non-crosslinked collagen pads and oxidized cellulose woven pads [40]. Hoekstra *et al*. used microcrystalline chitosan (MCCh) as a sealant for arterial puncture sites and found that it was safer due to its character as a biocompatible biopolymer. Meanwhile, the modified chitosan patch (MCP) showed significant reduction in the post-treatment of hemorrhage and sustained the mean arterial pressure[34]. Photocrosslinkable chitosan is a chitosan hydrogel that carries lactose moieties and photoreactive azide groups. The chitosan hydrogel had a similar outcome to that of fibrin clots[41]. In another study, chitosan and water-soluble chitin (WSC) solutions had the most prominent tensile strength and enhanced wound healing characteristics relative to other compounds due to their biodegradable properties and the hydrophilic nature of WSC [42].

Chitosan and erythrocytes

The specific mechanism of action of chitosan remains undiscovered but researchers have shown that erythrocytes coagulate in the presence of chitosan[43-46]. The agglutination of erythrocytes was elevated in the presence of chitosan due to crosslinking of the erythrocytes. They were bound together by chitosan polymer chains and repolymerized to form a lattice that captured cells creating an artificial clot[47]. The changes in erythrocyte morphology, upon adherence of chitosan, were also elucidated in this *in vivo* study. The cells linked to form aggregates to prevent blood loss. Klokkevold *et al*. revealed that erythrocytes from anesthetized rabbits lost their typical biconcave morphology and appeared to have an unusual affinity towards one another. Additionally, chitosan-treated incisions formed erythrocyte clots or plugs. The morphological features of these clots were viewed through scanning electron microscopy (SEM), which showed that the resulting blood clots did not demonstrate any affinity towards one another[46].

Kind *et al*. using a liver laceration model, reported that chitosan failed to improve blood coagulation in control and heparinized rats. This unfavorable outcome was due to the method used. The cotton applicators used were pulled away from the laceration during the period of clot formation. This decreased the influence of chitosan, and the bleeding tendency was maintained. Upon contact with blood, chitosan stopped the bleeding by absorbing water and changing into an adhesive material that was attached to the underlying damaged tissue[48]. Fukasawa *et al*. elucidated that the fibrinolytic activity of rabbits became limited upon the adherence of chitosan onto peritoneal abrasions[49]. Chitosan's effect on erythrocytes, arresting the loss of the formed clot, may only explain part of its hemostatic function[46].

Chitosan's effect on platelet adhesion, aggregation and activation

T.C. Chou *et al*. reported that chitosan is capable of inducing platelet adhesion and aggregation at 5 and 30 minutes, in a concentration-dependent manner. In a rabbit model, chitosan time-dependently enhanced platelet adhesion and dose-dependently enhanced platelet aggregation. These researchers washed the rabbit's platelets and pre-warmed them at 37°C for 3 minutes. Varying concentrations of chitosan-derivatives were added to stimulate platelet aggregation. Their results could explain the interaction of chitosan with platelets in damaged tissues[50].

It was demonstrated that chitosan films can induce platelet adhesion, aggregation and the activation of intrinsic blood coagulation[51]. Shen *et al*. discovered that chitosan induced platelet adhesion and aggregation by noting that the level of aggregation was related to the concentration of the platelets in the plasma[52]. Okamoto *et al*. stated that chitosan acted more effectively than chitin, though chitin was found to aggregate platelets more than chitosan in blood coagulum. Platelets aggregation did not directly reflect blood coagulation. The effects of chitin and chitosan on the coagulation profiles were elucidated by their physical properties and were associated

with their chemical structure, particularly the amino residue. Based on scanning electron microscope morphological evaluation, platelets were more strongly attached on the surface of chitin and chitosan particles with an elongated process. Platelets were attached and bound to each other by forming an aggregated mass in irregular shapes[53].

Chitosan was able to promote platelet adhesion and to generate intracellular signal reactions that activate Glycoprotein IIb/IIIa and discharge thromboxane A2/ADP. These signals elevate platelet spreading and strengthen the stability of adhesion[54]. Qing He et al. measured platelet adhesion and activation using chitosan and Chitosan-Heparin (Chi-Hep) composite films. Scanning electron micrographs of the films after contact with the platelet rich plasma (PRP) showed that many platelets formed pseudopodia shapes. Chi–Hep composite films inhibited platelet adhesion. For the platelet activation test, the percentage of positive P-selectins induced by the Chi–Hep composite scaffold was significantly decreased relative to those induced by chitosan scaffold. This indicated an improved platelet compatibility of the composite matrices[55]. M.S Lord et al. investigated the actin cytoskeleton of adhered platelets in the presence of chitosan. Platelets adhered to chitosan at concentrations ranging from 0.01 to 1% (w/v) and resulted in a rounded morphology with no apparent development of actin-rich stellate shaped pseudopodia. Platelet adhesion to plasma coated chitosan resulted in a more spread morphology with radial actin-rich protrusions. There were no differences in the morphology of platelets exposed to chitosan-coated in either 0.1% plasma or 10% plasma. For platelet activation test, increased levels of P-selectin were detected on platelets exposed to chitosan compared to platelets isolated from whole blood. An increased level of integrin $\alpha 2\beta 3$ was found to be expressed by platelets that adhered to chitosan at concentrations of chitosan in the range of 0.001 to 1% (w/v). Platelets were found to be activated by chitosan alone, and the extent of activation was found to be modulated by the presence of proteins adsorbed by chitosan[56].

Chitosan and wound healing

The novel use of chitosan and its derivatives as a biocompatible material in wound healing applications was referenced to as early as 1977. Muzzarelli highlighted the properties of chitin derivatives in human wound healing. Reports concluded that chitosan has a stimulatory effect on fibroblasts and activated macrophages[57-59]. Chitosan was found to interact with the basic fibroblast growth factor (bFGF) to increase the rate of healing[60]. Conversely, other reports showed that chitosan suppressed the role of fibroblast cell growth, makes it unable to trigger the activation of macrophages[61-62].

Nevertheless, chitin and chitosan do ease wound recovery through the reformation of granulation tissue. Kuo et al.

studied β-tricalcium phosphate (β-TCP)/chitosan composite membranes that were applied to rabbit models to study the generation of new bone. Based on their research, all three prepared chitosan membranes were effective in maintaining the membrane integrity on bone defects and the observed healing rate. Under these conditions, cell division improved, and a higher percentage of bone appeared after 4 weeks. They concluded that the β-TCP/chitosan membranes prepared in this study had a positive result[63]. Wang et al. evaluated the in vivo bioadhesive strength of chitosan relative to collagen under wet conditions. Based on their outcome, chitosan sponges promoted better adhesion over collagen sponges, and no breakages were present. They implanted collagen and chitosan sponges, subcutaneously in rats for time periods of up to 8 weeks. After one week of implantation, the chitosan sponges were fully absorbed and a layer of purulent cells penetrated the external side of the chitosan sponges. Acute inflammatory cells infiltrated the chitosan sponges, and there were no signs of biodegradation. At 4 weeks the implants still had their porous structure. A much thicker purulent layer and more acute inflammatory cells were found around or in the chitosan sponges. At 6 weeks after implantation, chitosan maintained its scaffold integrity, and an increase in pus cells was observed. At 8 weeks the infiltrated purulent cells were greater in the chitosan sponge relative to the collagen sponge[51]. It was reported by Cai et al. that chitosan improved the proliferation, differentiation and mineralization of osteoblast cells to form new bone[64]. Okamoto et al. evaluated platelet aggregation and noticed that chitosan group also induced the release of PDGF-AB and TGF-β1 from platelets. The levels of PDGF-AB in the chitin and chitosan groups were significantly higher than in the control phosphate buffer saline (PBS) group and increased to 130 and 190%, respectively. The results also indicate that chitin and chitosan enhance the release of PDGF-AB and TGF-β1 and play important roles in the wound healing process. These results support combined use of chitin and chitosan for biomedical applications due to their effectiveness as a wound healing treatment for hemostasis[53]. The results presented by M.S Lord et al. stated that, in the presence of chitosan, the role of plasma and extracellular matrix proteins in promoting platelet adhesion has important positive consequences on wound healing[56].

Chitosan as a wound dressing towards tissue regeneration

Fibroblasts and keratinocytes are the primary cell components of the dermal and epidermal layers and can be cultured to study the biocompatibility of chitosan-derivatives as wound dressings. According to Lim et al. newly formulated chitosan derivatives in the form of porous skin regenerating templates (PSRTs) were examined for biocompatibility with a direct-contact method with primary normal human epidermal keratinocyte (pNHEK) cultures. Cytotoxicity, genotoxicity

and inflammation were assessed using MTT assay. A comet assay and analysis were performed to measure the pro-inflammatory cytokines TNF-α and IL-8. Based on the outcomes, PSRT 82, 87 and 108 were all cytocompatible for 3 days at the cellular level *in vitro*. PSRTs 87 and 82 exhibited a genotoxic effect at 24 hours post-treatment and evoked better inflammatory reactions than PSRT 108 upon the detailed examination of the molecular weight. Their study concluded that PSRT 108 was the most biocompatible wound dressing, followed by PSRT 87 and 82[65]. Tissue engineering employs polymer scaffolds to improve cell adhesion, proliferation and differentiation *in vitro*. Due to its intrinsic wound-healing abilities, chitosan has been widely used to form, films, pastes, sheets and porous templates of biocompatible wound dressings[66-69]. In another study, a naturally derived, biomedical-grade bilayer chitosan porous skin regenerating template (CPSRT) with pore sizes ranging from 50 to 150 *μm* was used as a dermal scaffold for skin tissue engineering. The favorable outcome of the study was supported by the cytokine expression profiles of keratinocytes. The pro-inflammatory cytokines IL-8 and TNF-*α* were expressed in the negative control cultures[70]. Patients with keratinocyte lesions were observed to have a high expression of TNF-*α* indicating that the activation of this cytokine pathway was associated with inflammation[71].

According to Shah *et al*. chitosan used in the form of a bilayer skin regenerating system was comparable to Integra, an artificial synthetic skin substitute developed for use in burn patients. An animal model study was performed to evaluate and compare the biocompatibility of the chitosan skin regenerating template (SRT), Integra, and human skin allograft (HSA) as skin replacements. The chitosan SRT possessed similar inflammatory, angiogenesis, and tissue growth properties as Integra. Chitosan SRT showed statistically significant differences ($P < 0.005$) in the degree of angiogenesis, due to it being 89% deacetylated. Based on this study, chitosan SRT could serve as an alternative to Integra and HSA as a skin substitute because it does not stimulate any negative side effects due to its biocompatibility[72].

The cytocompatibilities of the novel chitosan derivatives oligo-chitosan (O-C), NO-carboxymethyl-chitosan (NO-CMC) and N-carboxymethyl-chitosan (N-CMC) in the form of sheets and pastes were acceptable due to their mechanical strength and biocompatibility. They were also able to reduce fluid and heat loss from the wound sites. Based on these results, the tested material's cytocompatibility and cell viability were suitable for hypertrophic scars and normal fibroblasts. Metalloproteinase-13 (MMP-13) expression was determined to be expressed in hypertrophic scar samples that were treated with chitosan. This expression may degrade the collagen that was overexpressed in the hypertrophic scars by inhibiting the hypertrophic scar cell properties and

manipulating the overproduction of collagen in hypertrophic scars during the wound healing process[73]. Previous reports also stated that chitosan-based wound dressings have been shown to inhibit scar tissue by reducing fibrin formation in wounds due to their hemostatic properties. These properties resulted in the formation of a safety film coating on the wound[74]. Koyano *et al*. stated that blending poly(vinyl alcohol) (PVA) with chitosan would produce a highly elastic hydrogel and improve attachment and growth of cells. PVA and chitosan were blended in different ratios. Cell culture methods were used to study the attachment and growth of fibroblast cells (L-929) on the resulting hydrogels. Upon addition of chitosan to PVA, increased cell attachment and growth were observed on the hydrogels. The PVA/chitosan-blended hydrogels were biocompatible materials due to their high water content level, which was associated with a high degree of cell attachment and a high growth rate[75]. Mao *et al*. conducted a study on the co-culture of keratinocytes and fibroblasts in chitosan-gelatin scaffolds to develop a bilayer skin substitute *in vitro*. The goal was to produce a novel scaffold for skin tissue engineering. The scaffolds utilized in this study were asymmetric in structure because one side of the container was in direct contact with the cooling plate. The outcome of this *in vitro* fibroblast cultivation indicated that thinner scaffolds have faster growth rates. Keratinocytes were found to form a thick layer on the surface of chitosan-gelatin scaffolds after 1 week of co-culture *in vitro*. Their incredible results showed that chitosan-gelatins scaffolds used for skin tissue engineering would be a better substitute to some collagen materials used to accelerate wound healing[76].

5.0 Conclusion

There are various hemostatic agents available, but an ideal topical and surgical hemostatic agent is still unavailable for treating abrupt hemorrhages. An ideal hemostatic agent must possessed native clotting mechanisms that do not pass through the salvage filtration systems[77], must be inexpensive, must be easy to apply and require minimal supervision, must be long lasting with low risk, must be able to accomplish hemostasis within the necessary time period, and does not cause allergic reactions[8]. Chitosan-based hemostatic agents are becoming one of the most promising agents in reducing preoperative and postoperative bleeding. Various forms of chitins and chitosans have been used to promote hemostasis in experimental studies. Researchers are still in the process of developing improved chitosan-based hemostatic dressings. In our review, we presented several types of chitin and chitosan-based dressings that are commercially available. We noted in detail chitosan's effect on erythrocytes, platelet aggregation and adhesion in assessing its wound healing effectiveness for tissue regeneration. Future studies, should be focused on understanding the detailed mechanism of action and cell

signals involved in chitosan's effects on the hemostasis pathway.

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