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Reference

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Chitosan as a Starting Material

for Wound Healing Applications

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Abstract:

Chitosan and its derivatives have attracted great attention due to their properties beneficial for application to wound healing. The main focus of the present review is to summarize studies involving chitosan and its derivatives, especially N,N,N-trimethylchitosan (TMC), N,O-carboxymethyl-chitosan (CMC) and O-carboxymethyl-N,N,N-trimethyl-chitosan (CMTMC), used to accelerate wound healing. Moreover, formulation strategies for chitosan and its derivatives, as well as their in vitro, in vivo and clinical applications in wound healing are described.

Keywords: chitosan, chitosan derivatives, carboxymethyl chitosan, trimethyl chitosan, biopolymers, wound healing, wound dressings, skin regeneration, drug delivery, polyelectrolytes.

Abbreviations: CMC, carboxymethyl-chitosan; CMTMC, O-carboxymethyl-N,N,N-trimethylchitosan; EGF, Epidermal Growth Factor; FGF, Fibroblast Growth Factor; HMWC, high molecular weight chitosan; LMWC, low molecular weight chitosan; PDGF, platelet-derived growth factor; TMC, N,N,N-trimethylchitosan.
1. Introduction

Wound healing is a spontaneous process, which might be impaired in large or difficult-to-heal, chronic wounds. In such cases, and when autologous skin grafts are not available, biopolymers may be proposed to promote and initiate the normal dermal and epidermal wound healing process (Barrientos et al., 2008). Dermal wound healing is a complex biological process, which includes four overlapping steps. These are the inflammatory phase immediately after the lesion has occurred, the migratory, proliferative and maturation phase resulting in remodeling (Dreifke et al., 2015). Among other factors involved in wound healing, the extracellular matrix (ECM) has a key role in orchestrating and guiding cell phenotype, adhesion, migration and proliferation. The ECM comprises proteins synthesized by fibroblasts, including proteoglycans (e.g., chondroitin sulfate), keratin sulfate, heparin sulfate and fibrous proteins like laminin, type IV collagen and elastin. In addition, the ECM serves as a deposit for growth factors, proteases, cytokines and chemokines (Hynes, 2009). ECM proteins have an important role during the proliferative phase by providing the mechanical support necessary for angiogenesis in the newly forming granulation tissue (Folkman, 2007). Beside the formation of granulation tissue in the dermis, wound re-epithelialization is another important process during which keratinocytes and fibroblasts migrate into the wound area (Glim et al., 2014).

Skin wounds are the result of disruption of normal tissue anatomy and may be classified by the type of repair process involved as acute or chronic (Boateng et al., 2008). Acute wounds originate from superficial scratches to deep injuries and heal completely with minimal or no scars within a timeframe of 3 weeks. Chronic wounds, e.g., certain types of ulcers or diabetic wounds (Dreifke et al., 2015) start to develop when the acute wound fails to heal after a minimum time period of 3 months. Based on etiology, type, depth and clinical appearance, wounds are classified as shown in Figure 1 (Boateng et al., 2008; Kyriacos et al., 2008).
The treatment of chronic or bacteria-infected wounds necessitates new technologies. In this view, the use of biocompatible, absorbable polymers such as pectin (Ninan et al., 2014), chitin and its derivative chitosan (Muzzarelli, 2009), gelatin (Ulubayram et al., 2001), polycaprolactone (Bui et al., 2014), hyaluronic acid (Estes et al., 1993), and others have been described.

Having a unique set of biological properties including biocompatibility, biodegradability and low to absent toxicity (Baldrick, 2010), chitosan has been found to be an attractive material for wound healing applications. In addition, chitosan has antibacterial, haemostatic and mucoadhesive properties (Muzzarelli et al., 1999), and may act as a wound healing accelerator (Yilmaz, 2004).

Chemically, chitosan is a linear aminopolysaccharide, composed of glucosamine and N-acetyl glucosamine units linked by β (1→4) glycosidic bonds formed by N-deacetylation of chitin (its parent polymer). Chitin, the second most abundant biopolymer, is mainly found in exoskeletons of crustaceans and cell walls of fungi (Senel & McClure, 2004). Chitosan is a polycationic polymer featuring free acetamide groups.
and hydroxyl functions linked to the glucopyranose rings that are susceptible to react through nucleophilic attack (Berger et al., 2005). A wide range of chitosan functionalization can thus be performed through selective modification of the free amino groups (Delgadillo-Armendariz et al., 2014; Florea et al., 2006). According to the Chemical Carcinogenesis Research Information System (CCRIS, 2014), chitosan has no mutagenic effects, which makes it a candidate for biomedical application.

Chitosan has been used in gels, micro- or nanoparticles, and films. Its physical and biochemical properties can be further tailored to meet conditions in wound healing applications (Alves & Mano, 2008; Berger et al., 2004; Florea et al., 2006; Patois et al., 2009; Schuetz et al., 2008). Beside numerous chitosan derivatives, trimethyl chitosan (TMC), N-carboxymethyl chitosan (CMC) and O-carboxymethyl-N,N,N-trimethyl chitosan (CMTMC) have been synthesized, raising increasing interest due to their enhanced solubility, antibacterial activity, ability to complex drugs or DNA (Thanou et al., 2002), and good biocompatibility (Chen et al., 2006; Hansson et al., 2012a; Janvikul et al., 2006). The mechanism of action in the promotion of wound healing by chitosan derivatives is still debated but is suggested to depend on the type of functionalization.

This review will describe the uses of chitosan as a starting material and dressing for wound healing applications, with a specific focus on clinical studies performed to date. Specific derivatives based on trimethylated and carboxymethylated chitosan will be described as well. We focused on chitosan derivatives, which are known to promote wound healing. Other chitosan derivatives developed for other purposes have also been described in literature in the context of wound healing (Alves & Mano, 2008; Casettari et al., 2012; Francesco & Tzanov, 2011; Park et al., 2010). Specifically, for diabetic wounds, where healing fails, other chitosan derivatives have been proposed (Moura et al. 2013) to increase fibroblast migration and collagen deposition in diabetic mice (Moura et al. 2014).
2. Chitosan as wound healing promoter

2.1. Chitosan history

Chitosan, a chitin derivative, was discovered in mushrooms in 1811 by the French chemist Henri Braconnot and named in 1859 by C. Roget (Dodane & Vilivalam, 1998). Following D-glucosamine synthesis in 1903 by Fischer and Leuchs, Karrer decomposed chitin with chitinase in the year 1929. The absolute configuration of glucosamine was determined by Haworth in 1939 (Irvine & Hynd, 1912). Only in 1970, Prudden’s research concluded that glucosamine and N-acetyl-D-glucosamine accelerated wound healing processes (Prudden et al., 1970).

Chitosan and its derivatives were first used for skin and wound healing in the 1980’s. A chitin material, Beschitin®, was used for human application as dressing for skin and nasal wounds (Kifune, 1992). From then on chitin and chitosan were consistently shown to enhance wound healing in animals (Okamoto et al., 1993) and human subjects (Azad et al., 2004).

2.2. Chitosan toxicity

2.2.1. In vitro toxicity of chitosan

In vitro toxicity of chitosan is closely related to both its degree of deacetylation (DD) and to its molecular weight (MW) (Schipper et al., 1997). The effect of two types of micron-sized chitosan particles with different molecular weight - low (LMWC; 50 - 190 kDa) and high (HMWC; 310 - 375 kDa) molecular weight chitosan - was studied on a keratinocyte cell line (HaCaT) using the MTT assay. Both chitosan species promoted proliferation of HaCaT cells, with HMWC inducing a stronger, almost 2-fold increased proliferation at 300 µg/mL when compared to LMWC. However, both chitosan species inhibited proliferation at concentrations exceeding 1 mg/mL (Wimardani et al., 2012). In a subsequent study, the IC50 value for LMWC was determined with 800 µg/mL (Wimardhani et al., 2014). Scarce data are available for different salts of chitosan such as hydroglutamate, glycol, hydrochloride and hydrolactate. All of these compounds were shown to have some cytotoxicity against B16F10 (murine melanoma) cells, with the
highest toxicity being exerted by chitosan hydrochloride salt (IC₅₀ = 0.21 ± 0.04 mg/mL, MW > 100 kDa) (Carreño-Gómez & Duncan, 1997).

Ribeiro et al., 2009 studied the in vitro cytotoxicity of chitosan hydrogels when tested with dermal fibroblasts obtained from rat skin. MTT results suggested that chitosan exerted no acute toxicity. Summarizing these results, chitosan can be considered as a promising candidate for successive in vivo studies.

2.2.2. In vivo toxicity of chitosan

In vivo, the extent of chitosan acute toxicity appears to be correlated to the administration route. Upon oral administration, Arai et al. reported a lethal dose of orally administered chitosan at LD₅₀ > 16 g/kg in mice, exceeding the LD₅₀ for sucrose (Arai et al., 1968). Costa et al. reported LD₅₀ values for orally administered chitosan in rats at > 1500 mg/kg; intraperitoneally administered in rats: 3000 mg/kg and in mice: 5200 mg/kg; subcutaneously administered in mice: > 10000 mg/kg (Costa et al.). According to these studies, chitosan does not show significant acute toxicity. In addition, the absence of local irritation following topical application in both rabbits and guinea pigs was demonstrated (Rao & Sharma, 1997).

In vivo chronic toxicity studies reported by Carreño-Gómez and Duncan (Carreño-Gómez & Duncan, 1997) have shown that intravenously administered chitosan (4.5 mg/kg/day for 11 days) to rabbits did not lead to any abnormal changes, while doses of 50 mg/kg/day induced death after the third day of exposure to chitosan. However, oral administration of chitosan at doses of 700-800 mg/kg/day to rabbits or hens during 34 weeks produced little toxic effects (e.g., loss of appetite in hens). These data suggest low acute and chronic toxicities for chitosan.

However, a dose-dependent immunostimulation potential was observed after 3 mg chitosan orally administered to rats. Porporatto et al. observed that a dose of 1 mg chitosan had no significant effect, while a single dose of 3 mg stimulated the expression of IL-10 in mononuclear cells of rats fed (Porporatto et al., 2005).
Ueno et al. reported some adverse effects of chitosan in dogs after high chitosan doses of 200 mg/kg were subcutaneously injected. Chitosan stimulated interleukin 8 (IL-8) secretion from fibroblasts, resulting in angiogenesis and migration of neutrophils. The dogs died from respiratory distress after the injection, due to high levels of IL-8 and successive activation of neutrophils accumulating in the lungs (Ueno et al., 2001).

In addition, Rao and Sharma reported the absence of pyrogenic effects of chitosan and almost no toxicity in mice, and no skin and eye irritation in guinea pigs and in rabbits (Rao & Sharma, 1997). Most of the studies described chitosan as a safe material, inducing low or minimal toxic effects. Chitosan is therefore generally recognized as safe (GRAS) for food application and is considered as suitable and safe pharmaceutical excipient for parenteral route (Baldrick, 2010).

Data on chitosan toxicity from human studies in general are quite limited. Gades and Stern reported that quantities exceeding 4.5 g chitosan taken daily by human volunteers did not result in toxic effects (Gades & Stern, 2003). In addition, human trials up to twelve weeks showed no toxic effects, no allergies, and only nausea symptoms and constipation in 2.6 - 5.4% of subjects (Ylitalo et al., 2002).

2.3. Chitosan formulations

Chitosan-based wound dressings possess a set of unique properties, including haemostatic (Janvikul et al., 2006), biodegradable (Patois et al., 2009), and antibacterial properties (Agnihotri et al., 2004) that make them useful for wound healing. Chitosan's antibacterial activity was already observed at low concentrations against a variety of pathogens such as \textit{E. coli} or \textit{S. aureus} (Felt et al., 2000) and may be used in various formulation types such as gels (Yan et al., 2010), films (Mizuno et al., 2003) or nanoparticles (Hansson et al., 2012a). As a consequence, it is widely used in medical and veterinary areas as a wound healing promoter (Ishihara et al., 2001).
2.3.1. Dressings

Dressings should keep the wound moist to favor healing, ensure solubilization of growth factors and/or antimicrobial agents, and support fibroblast growth. Low adhesion to the wound surface, absorption of exudate and ability to exchange oxygen also favor healing. Chitosan in the form of a hydrogel does meet these requirements.

Chitosan interacts with many cellular processes during wound healing. Chitosan dressings were found to provoke minimal adverse reactions with little or almost absent fibrous encapsulation, and were shown to provide protection against bacterial infections. Chitosan is also able to accelerate wound healing when applied as powders, nano- and microparticles, granules, sponges or as composites with other materials (Shigemasa & Minami, 1996). Several studies reported that chitosan promotes migration of polymorphonuclear neutrophils (PMNs) (Ueno et al., 1999), and promotes granulation by inducing proliferation of dermal fibroblasts (Howling et al., 2001). It was shown that chitosan is involved in all stages of wound healing. During the initial healing phases, chitosan shows its unique hemostatic properties and promotes infiltration and migration of neutrophils and macrophages (Park et al., 2009; Simard et al., 2009). Thereby wounds are cleaned from foreign agents and granulation tissue is formed allowing fibrous tissue formation and re-epithelialization. In the case of formation of hypertrophic scars, which is caused by excessive collagen production in the remodeling phase, chitosan is able to decrease scar tissue, allowing for a good re-epithelialization (Howling et al., 2001). Chitosan also affects the expression of growth factors implied during the healing process. As shown in burn wounds in mice, chitosan increases the expression of TGF-β1 and collagen production in the early post-injury phase (day 3), facilitating tissue regeneration. In the late post-injury phase (day 7), chitosan decreases TGF-β1 expression, which would otherwise promote scar formation (Baxter et al., 2013). Such modulation is thought to accelerate the wound healing process.
2.3.2. *Hydrogels and growth factor delivery*

Hydrogels are appropriate for wound healing due to their ease of administration, wound protection, water retention and oxygen permeability. Chitosan may conveniently be applied as a viscous liquid undergoing gelation upon application on the wound surface. In order to trigger in situ gel formation, parameters such as ionic charge, pH and temperature have to be investigated (Berger et al., 2004). Chitosan’s ability to undergo hydrophobic interactions and hydrogen bonding have also been used to produce thermally triggered, in situ gel formation (Patois et al., 2009; Schuetz et al., 2008).

Hydrogels may serve as reservoirs for local release of proteins such as growth factors. In addition, chitosan bioactivity may contribute to directing the healing response (Luca et al., 2011). The typically fast growth factor release – within hours - may be delayed by physical interactions (e.g., ionic or hydrophobic) of the protein with the gel. The ability of growth factors to strongly promote cell migration, differentiation and proliferation has spurred the effort to combine such factors with the advantages of a chitosan-based formulation. Useful growth factors for wound healing are epidermal growth factor (EGF) (Alemdaroğlu et al., 2006), basic fibroblast growth factor (bFGF) (Mizuno et al., 2003; Park et al., 2009), fibroblast growth factor-2 (FGF-2) (Obara et al., 2005), transforming growth factor-beta (TGF-β) (Gopal et al., 2014), platelet-derived growth factor (PDGF) (Judith et al., 2010), and vascular endothelial growth factor (VEGF) (Frank et al., 1995).

Alemdaroğlu et al. (Alemdaroğlu et al., 2006) developed a chitosan gel for topical administration of EGF in the treatment of second degree burns. The gel released EGF completely over 24 hours. Daily application of chitosan-EGF gel for 14 days on rat second degree burns resulted in shorter healing times compared to wounds treated with chitosan gel in the absence of EGF. Increased re-epithelialization and accelerated formation of granulation tissue for wounds treated with chitosan-EGF gel was likewise reported. Chitosan gels without EGF lead to incomplete wound healing without cell differentiation, while chitosan-EGF gels lead to almost complete wound healing.
A chitosan photocrosslinkable hydrogel with lactose and azide moieties was used for controlled release of fibroblast growth factor-2 (FGF-2). Chitosan hydrogel was used as dressing to accelerate wound healing using both healing impaired diabetic (db/db) mice and their normal (db/+) littermates. Chitosan hydrogel induced wound closure. The addition of FGF-2 to the chitosan hydrogel further accelerated wound closure, a substantial formation of granulation tissue and re-epithelialization only in db/db mice (Obara et al., 2003). Such properties support the use of chitosan as a carrier for growth factors with a gradually controlled release.

Judith et al. (Judith et al., 2010) evaluated the effect of the formulation of PDGF in a collagen-chitosan gel matrix system on excision wounds (1.5 cm long × 1.5 cm width) during the healing process. They could assess the rate of wound contraction and the concentration of growth factors in the granulation tissue in rats. The in vivo results showed that adding PDGF leads to increased fibroblast migration and proliferation. After excision at day 10, they observed that the newly, continuously formed epidermis contained many capillaries.

Sponge-like dressings based on chitosan glutamate and sodium hyaluronate were studied for platelet lysate delivery to chronic wounds. Platelet lysates are a good source for many growth factors necessary for wound healing. In vitro tests on human fibroblasts proved that such systems accelerated cell proliferation. The drawback of this formulation is that it may break at an elongation exceeding 30-40% of its original length (Rossi et al., 2013). These data strongly support possible clinical applications of growth factors to a chitosan matrix, and confirm that chitosan hydrogels act as a protective wound environment having beneficial effects on healing of skin wounds.

2.3.3. Film, sponge and powder

Chitosan may conveniently be applied on the wounds in dry form of sponges, films or powders that will hydrate rapidly by exudate absorption forming a chitosan hydrogel at the wound surface. Mizuno et al. evaluated bFGF incorporated into chitosan films on
diabetic mice wounds. Accelerated angiogenesis and granulation tissue formation was reported. They further suggested that bFGF-chitosan films may be used for chronic ulcers (Mizuno et al., 2003). In another study, chitosan acetate dressing was tested on a third-degree burn in mice heavily contaminated with an aggressive and invasive strain of *P. aeruginosa*. The results showed that chitosan is an effective topical bactericide dressing for wound healing as the rate of mice survival was 73.3% compared to nanocrystalline silver dressing at a significantly lower rate (27.3%) of survival (Dai et al., 2009). Another study used a porous chitosan sponge for absorption of amikacin and vancomycin antibiotics in solution over 72h. The *in vitro* elution results showed that chitosan sponge loaded with an antibiotic can be used as drug carrier for treating and protecting wounds against infections (Noel et al., 2010).

Jin et al. compared the effect of chitosan, applied in a powder form, with or without heparin on deep partial-thickness (loss of epidermis and dermis, see Fig. 1) burns. The injuries were done on the dorsum of rats. After 72 h, histological results showed that chitosan powder prevented the early extension of burns, while heparin, whether alone or in combination with chitosan, was less effective (Jin et al., 2007). In contrast, heparin complexed with chitosan showed positive effects on later healing stages. Kratz et al. prepared heparin-chitosan membranes and reported that the wound healing activity of heparin-chitosan complexes was dependent on heparin concentration. While 1.9% heparin was not sufficient to induce re-epithelialization, 7.7% of heparin included in the chitosan complexes led to almost complete re-epithelialization (Kratz et al., 1997). This suggests that, to reach therapeutic efficacy, a timely, controlled delivery of heparin at an appropriate dose is needed, which is achieved by ionic complexation with chitosan. Furthermore, heparin-chitosan scaffolds showed no tissue ingrowth, with the formation of a highly vascularized granulation layer (Chupa et al., 2000), suggesting its potential to induce an angiogenic response.

2.4. **Clinical studies**

In clinical studies, chitosan dressings (Hyphecan®) were shown to be efficient and easy to apply, maintain and remove after healing. Stone et al. (Stone et al., 2000)
stimulated healing at the split skin graft donor site with chitosan. A total of 11 female and 9 male patients were enrolled in the study during 7 months. Chitosan dressings were compared to commercial alginate dressings (Kaltostat	extsuperscript{®}), or silicone net gauze (Mepitel	extsuperscript{®}). Half of the wound was dressed with a Kaltostat	extsuperscript{®} or Mepitel	extsuperscript{®} as controls and the other half with chitosan dressing. Kaltostat	extsuperscript{®} and Mepitel	extsuperscript{®} dressings were found to adhere to the donor site while chitosan dressings were easy to remove resulting in less pain to the patient. Chitosan biopsies revealed an increased number of dermal nerves and a dermis richer in glycosaminoglycans and capillaries compared to control dressings at day 11. At the split skin donor site, no infections or other adverse reaction were observed (Stone et al., 2000). Another study on 35 burn patients showed a similar healing time when using the chitosan dressing in comparison with Kaltostat	extsuperscript{®} (Ho et al., 2001).

Another study performed by Azad et al. (Azad et al., 2004) evaluated chitosan membranes (meshed and non-meshed) as wound dressings. The clinical data showed chitosan membrane promotes adhesion, hemostasis, healing and re-epithelialization after application to the fresh skin wound. For each patient, half of the wound was treated with a chitosan membrane and the other half with the conventional dressing, Bactigras	extsuperscript{®}. The application of non-meshed membrane lead to accumulation of blood under the membrane, while meshed chitosan membranes promoted a faster healing, a better organization of the repaired tissue including re-epithelialization, and a cosmetically acceptable outcome (Azad et al., 2004). This confirms the importance of porosity when designing scaffolds for wound healing. In addition, Kratz et al. (Kratz et al., 1998) studied the effect of chitosan-heparin membrane on wound healing in human skin. Chitosan-heparin complex showed faster and complete re-epithelialization after 12 days, compared to the donor untreated sides where an incomplete re-epithelialization was observed even after 15 days.
Table 1. Selected studies on human cells *in vitro*, preclinical and clinical studies of chitosan as a wound dressing biomaterial.

<table>
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<th>Delivery strategies</th>
<th>Results</th>
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<td>Hydrogels</td>
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<tr>
<td>Films</td>
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<td>Preclinical studies</td>
<td></td>
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<td>Hydrogels</td>
<td>Rapid hemostasis in mice</td>
<td>(Ishihara et al., 2001)</td>
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<td></td>
<td>Accelerated wound healing and bleeding stopped in mice</td>
<td>(Ishihara et al., 2001)</td>
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<td></td>
<td>Accelerated wound healing and bleeding stopped in mice</td>
<td>(Ishihara et al., 2002)</td>
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<td>Skin reconstruction after third-degree burns on a pig back skin</td>
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<td>Decreased healing time and accelerated reepithelialization in rats</td>
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<td>Gel</td>
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<td>Chronic udder trauma near nipples in cows</td>
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<tr>
<td>Artificial skin</td>
<td>Burn damage much less severe in rats</td>
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<td></td>
<td>Almost complete vascularization and colonization by fibroblasts in rats and mice</td>
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<td>Dressing material</td>
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<td>(Azad et al., 2004; Stone et al., 2000)</td>
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<td>Membranes</td>
<td>Shortens re-epithelialization in human subjects</td>
<td>(Kratz et al., 1998)</td>
</tr>
<tr>
<td>Artificial skin</td>
<td>Faster vascularization and colonization with fibroblasts in mice, rats and human subjects</td>
<td>(Vescovali et al., 1989)</td>
</tr>
<tr>
<td></td>
<td>Complete colonization by fibroblasts in human subjects</td>
<td>(Damour et al., 1994)</td>
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First clinical reports of dermal substrate for dermis replacement were reported by Damour group in the 1980s (Vescovali et al., 1989). They tested collagen-chitosan-glycosaminoglycans as artificial dermis first in rats and mice and then in humans on full-thickness wounds. First animal experiments revealed a relatively poor vascularization and some fibrosis. To further improve these results, they evaluated the overgrafted cultured epidermis made from collagen-chitosan-glycosaminoglycans on deep burn wounds from autologous cultured autograft after earlier excision in four patients. After 10 days of grafting, histological results on biopsies showed a homogenous partial colonization by fibroblasts, which turned into a complete colonization at day 21. Furthermore, they showed that using their dermal substrate, vascularization and colonization with fibroblasts could be easily controlled (Damour et al., 1994).

The studies listed in Table 1 support chitosan as one of the most suitable biopolymers for wound healing. Still, the exact mechanisms of chitosan action need to be investigated in more depth.

3. Chitosan derivatives for wound healing

Depending on the proposed goals (e.g., increased solubility, cellular uptake, cell targeting ability), a large family of chitosan derivatives may be produced through chemical modifications, as reviewed elsewhere (Casettari et al., 2012; Kean & Thanou, 2010). Since chitosan application is limited by its poor solubility at physiological pH (Sieval et al., 1998), we will focus on water-soluble chitosan derivatives and their application in wound healing, specifically tri-methyl chitosan (TMC), carboxymethyl-chitosan (CMC) and carboxymethyl-trimethyl-chitosan (CMTMC). Indeed, aqueous solubility of CMTMC allows for moisture retention properties that are beneficial in cosmetic applications (Muzzarelli et al., 2002). Trimethylation, through cationic properties, confers properties ranging from mucosal adhesion to the ability to carry drugs or oligonucleotides (Borchard, 2001). The derivatized biopolymers still share with chitosan some interesting properties such as antimicrobial activity.
3.1. TMC

Chitosan methylation leads to N,N,N-trimethyl derivatives, which have permanent positive charges at the C-2 position in the chitosan backbone (de Britto & Assis, 2007). The first studies on chitosan methylation were done by Wolfrom et al. (Wolfrom et al., 1964). The strategy was developed after the observation that after 45 times successive methylation of chitin, only one methoxyl group for each monosaccharide unit was grafted. Wolfrom et al. investigated chitosan methylation with dimethyl sulfate. Only 29% of methoxyl groups were grafted and, upon changing dimethyl sulfate for dimethylformamide and adding methyl iodide, the contents of methoxyl groups raised to 66%. Repeating methylation and acetylation raised the degree of substitution to 92% of 3,6-O methylated chitosan (Wolfrom et al., 1964). In 1975, Nud‘ja developed N-alkyl-chitosan with 78% of substitution in the presence of triethylamine (Nud‘ja et al., 1975). In 1985, Muzzarelli and Tanfani studied chitosan trimethylation with methyl iodide in a three step-reaction, which lead to N,N,N-trimethylchitosan (TMC). However, according to Muzzarelli et al., TMC at a 60% methylation degree was not water-soluble (Muzzarelli & Tanfani, 1985). In 1986, Domard et al. brought an important contribution by treating chitosan with methyl iodide, N-methyl-2-pyrrolidone (NMP) and sodium hydroxide, resulting in a water-soluble TMC, as shown in Figure 2 (Domard et al., 1986).

![Figure 2. Synthesis of quaternized chitosan as performed by Domard et al. (Domard et al., 1986).](image)

Generally, during chitosan trimethylation synthesis, O-methylation at C-6 and C-3 may occur, leading to uncontrolled trimethylation. Moreover, O-methylation leads to chain scission resulting in reduced polymer solubility (Verheul et al., 2008). In this case,
treating chitosan first with formic acid and formaldehyde and secondly with methyl iodide, would help to overcome this issue of uncontrolled trimethylation (Patrulea et al., 2015). Briefly, chitosan is treated with formaldehyde in the presence of formic acid at 70 °C for 118 h, which leads to the formation of N-dimethyl chitosan (DMC) by Eschweiler-Clarke reaction. In this case, the third and sixth position of the chitosan chain is protected. Subsequently, DMC is treated with methyl iodide in the presence of NMP at 70 °C for 140 min and further washed and dried to obtain the final TMC. The protective step during trimethylation allows not only to achieve high degree of substitution (46.6%), but also leaves third and sixth positions free at the chitosan backbone for further peptide grafting or other chemical modification.

3.1.1. TMC toxicity

Currently, studies investigating the effect of chitosan derivatives on human skin cells are limited. Thanou et al. investigated TMC with different degrees of methylation as potential absorption enhancer for drug delivery purposes. In vitro studies on Caco-2 and COS-1 cells showed no significant toxicity (Thanou et al., 2002). Murata et al. measured TMC toxicity against HeLa uterocervical carcinoma cells in vitro and as expected, toxic effects were increasing with TMC concentration. No survival was found at TMC concentrations higher than 10 mg/mL (Murata et al., 1996).

TMC toxicity strongly increases with trimethylation degree – i.e. ionic charge – and decreases with molecular weight. Low molecular weight TMC (3-6 kDa) has relatively low in vitro cytotoxicity (IC$_{50}$ > 10 mg/mL) for a degree of trimethylation < 55% against MCF7 (human breast cancer) and COS7 (monkey kidney fibroblast) cells. However, a high degree of trimethylation (94%) of the same low molecular weight chitosan lowered IC$_{50}$ to 1.4 mg/mL (MCF7) and 2.2 mg/mL (COS7) (Kean et al., 2005). Different MW values of chitosan (400, 100, 50, 25 and 5 kDa) with the same degree of trimethylation lead to IC$_{50}$ values of 30, 70, 90, 270 and > 1000 µg/mL, respectively against L929 (mouse fibroblasts) cells (Mao et al., 2005). These results demonstrate that TMC cytotoxicity is strongly correlated to molecular weight and to the degree of trimethylation.
TMC toxicity has been attributed to electrostatic interactions of TMC with the plasma membrane. However, with respect to another positively charged polymer, polyethyleneimine (PEI), TMC showed much lower toxicity (Kean et al., 2005). TMC, despite some toxicity related to free cationic charges, has therefore a key role to play in wound healing as a drug delivery material, most likely when complexed with anionic drugs such as RNA or DNA.

Based on the work of Domard et al., Murata et al. measured the toxicity of TMC (degree of quaternization not reported) and formation of chitosan-DNA polyelectrolyte complexes for gene delivery. The cytotoxicity was measured against HeLa uterocervical carcinoma cells \textit{in vitro} and, as was expected, toxic effects were linearly increasing with TMC concentration. Almost all of the cells were dead at TMC concentrations higher than 10 mg/mL (Murata et al., 1996).

\subsection*{3.1.2. TMC in wound healing applications}

So far, few studies on TMC for wound healing have been reported. Guo et al. used TMC as a DNA delivery vector for burn wounds in a pig model. They used a bilayer dermal equivalent (collagen-chitosan) sponge with TMC complexed with plasmid DNA encoding for EGF or VEGF. Higher angiogenesis and number of mature blood vessels were observed for the TMC/pDNA-VEGF group. TMC helped the regeneration of full-thickness defects of skin (Guo et al., 2011). TMC was able to condense and protect DNA against degradation by nucleases for further endocytosis and growth factor expression (Thanou et al., 2002). A similar approach on incisional wounds revealed an even higher number of new and mature blood vessels, which was attributed to the more severe injury of surrounding cells and blood vessels found in burn wounds (Guo et al., 2010).
3.2. **CMC and CMTMC**

Carboxymethyl-chitosan (CMC) is a very important water-soluble and biocompatible chitosan derivative. These enhanced properties are attributed to the carboxymethyl groups on the copolymer backbone (Pang et al., 2007). In this work, CMC was obtained in a one-step reaction, directly from chitosan. Briefly, chitosan was treated with sodium hydroxide in the presence of isopropanol and water followed by adding chloroacetic acid to the reaction mixture. CMC is soluble over a wide pH range and exhibits good biocompatibility, as well as gel-forming capacity. It is able to interact with different drugs making it attractive for wound healing. Furthermore, CMC was shown to promote skin fibroblasts proliferation (Chung et al., 1994). CMC antibacterial activity is affected by its molecular weight, solution pH, concentration and degree of deacetylation (Fei Liu et al., 2001). Liu et al. reported that highest antibacterial activity against *E. coli* was found in N,O-carboxymethylated chitosan followed by chitosan and then O-carboxymethylated chitosan (Fei Liu et al., 2001). Moreover, the cytocompatibility of CMC on human skin fibroblasts and its promotion of keloid fibroblasts proliferation was shown (Chen et al., 2002).

To synthesize O-carboxymethyl-N,N,N-trimethyl chitosan, Hansson et al. treated TMC with chloroacetic acid in the presence of NMP under basic conditions (pH 10.0) (Hansson et al., 2012b). Under these conditions, a low degree of substitution of carboxymethyl groups (27%) was achieved. This yield is not only affected by the nature of the solvent, but also by O-methylation arising from the previous step of trimethylation. As mentioned before, O-methylation blocks potential sites for carboxymethylation at C-3 and C-6 on the chitosan backbone, impeding subsequent carboxymethylation and leading to low degrees of substitution. Therefore, each synthesis step needs to be tightly controlled. Using a protective method for trimethylation allows to achieve a high carboxymethylation (Patrulea et al., 2015). More specifically, NMP was replaced by isopropanol in order to have better access to the free –OH groups of chitosan. Another important parameter for achieving a high degree of substitution is to treat TMC with a sufficient base concentration (NaOH 50%) that allows chloroacetate to access chitosan chains. Then, TMC is treated with chloroacetic acid at 60 °C for 3 h, washed and
lyophilized to obtain the final product. This CMTMC polymer can serve for further peptide grafting or, thanks to its positive charges on C-2, to prepare nanoparticles by complexation combining CMTMC with anionic polymers.

3.2.1. CMC and CMTMC toxicity

No CMC cytotoxicity was found in vitro by MTT assay on hepatic L02 cells. Moreover, CMC increased TGF-α secretion of L02 cells at a concentration of 300 mg/kg (Zheng et al., 2011). Administered intraperitoneally in rats, CMC (1350 mg/kg) had no significant toxicity on the blood system after being absorbed from the abdominal cavity (Yang et al., 2012). Rasad et al. evaluated in vitro both N,O-CMC and N-CMC for its possible application as wound dressing on human dermal cells and hypertrophic scar dermal cells (Abdull Rasad et al., 2010). Both chitosan derivatives exhibited good cytocompatibility on both normal and hypertrophic scar cells. However, it was observed that after 24 and 48 h, chitosan derivative sheets and pastes showed growth effects on normal dermal cells, while after 72 h they inhibited the growth of hypertrophic scar cells only.

Lim and his team evaluated in vitro the cytotoxicity of different N,O-CMC concentrations (1 and 5%) on human epidermal keratinocytes using MTT assay. The viability was higher than 70% for both CMC concentrations applied (Lim et al., 2007). Recent in vitro studies on human dermal fibroblasts (HDF) showed no cytotoxicity for a concentration of 0.1 mg/mL of CMTMC suspension in Dulbecco Modified Eagle’s Medium (DMEM) and a minor decrease of viability (about 82% viability) for concentrations of 1 and 0.5 mg/mL after 10 days of exposure and changing polymer suspension every 3 days (Patrulea et al., 2015).
Table 2. Delivery strategies of chitosan derivatives and their effects on wound healing.

<table>
<thead>
<tr>
<th>Chitosan derivatives</th>
<th>Effect</th>
<th>References</th>
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<td><strong>In vitro results</strong></td>
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<tr>
<td>TMC nanoparticles</td>
<td>TMC 60 is more effective in modulation of tight-junction barriers than TMC 20 and both TMC 20 and TMC 60 enhance bronchial epithelial permeation</td>
<td>(Florea et al., 2006)</td>
</tr>
<tr>
<td>CMC</td>
<td>High cytocompatibility and accelerated fibroblasts proliferation on human skin; inhibition of the proliferation of keloid fibroblast by regulation the ratio of collagen I/III</td>
<td>(Chen et al., 2002)</td>
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<tr>
<td>CMTMC and SDGRG complexes</td>
<td>Induction of phenotype and promotion of wound healing on human dermal cells</td>
<td>(Hansson et al., 2012a)</td>
</tr>
<tr>
<td>Chitosan-RGDSGGC scaffold material</td>
<td>Strong cell adhesion and proliferation in chondrocytes and fibroblasts</td>
<td>(Masuko et al., 2005)</td>
</tr>
<tr>
<td>N,O-CMC films</td>
<td>Decreased blood clotting time and fibrin formation in human blood</td>
<td>(Janvikul et al., 2006)</td>
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<td>CMC pastes and sheets</td>
<td>High cytocompatibility on human skin cells and on hypertrophic scar cells</td>
<td>(Abdull Rasad et al., 2010)</td>
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<tr>
<td>CMC suspension</td>
<td>High viability on human dermal fibroblasts</td>
<td>(Patrulea et al., 2015)</td>
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<td><strong>In vivo results</strong></td>
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<td>TMC/DNA-EGF and TMC/DNA-VEGF complexes</td>
<td>Enhanced angiogenesis for TMC/DNA-VEGF compared to TMC/DNA-EGF complexes; TMC lead to full-thickness of pig skin</td>
<td>(Guo et al., 2011)</td>
</tr>
<tr>
<td>CMC/gelatin hydrogel</td>
<td>Cell proliferation and neovascularization and almost complete wound healing in rats</td>
<td>(Huang et al., 2013)</td>
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<td>CMC-collagen matrix</td>
<td>Enhanced fibroblast migration and accelerated wound healing in rats</td>
<td>(Chen et al., 2006)</td>
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In vivo studies on rats were performed in order to enhance wound healing with CMC linked to collagen matrices containing chondroitin sulfate or an acellular dermal matrix. The acellular dermal matrix was derived from decellularized porcine full-thickness skin. Cell migration studies showed that fibroblasts migration was enhanced by adding CMC and increased with CMC concentration. In vivo, CMC-collagen matrices induced an enhanced wound healing with an increased cytokine secretion compared to pure collagen matrix (Chen et al., 2006). These findings suggest that CMC enhances wound healing and may be used for further clinical application.

A preclinical study was performed on a full-thickness wound with a crosslinked CMC/gelatin hydrogel in order to evaluate its biocompatibility and biodegradability as well as effects on wound healing in rats. CMC hydrogels promoted cell proliferation and neovascularization; after 15 days wounds were almost completely healed. This results suggested that CMC hydrogels are good candidates for inducing the formation of granulation tissue and to accelerate the healing process (Huang et al., 2013).

Examples of chitosan derivatives and their effects on wound healing are given in Table 2. Whereas TMC possesses intrinsic cytotoxicity mainly related to its degree of quaternization and thus surface charge, carboxylated or carboxymethylated chitosan derivatives are well tolerated independent of their substitution degree.

3.3. Chitosan and chitosan derivatives coupled to peptides

One attractive strategy for wound healing materials, besides the delivery of soluble factors or drugs, is to functionalize the scaffold materials with peptides that would interact with extracellular receptors of skin cells, helping cell differentiation, proliferation and migration to promote a faster or more complete healing. In this view signal peptides such as arginine-glycine-aspartic acid (RGD) were applied for promotion of cell adhesion to the extracellular matrix (ECM) (Chung & Park, 2007). The RGD sequence is known to induce cell adhesion by binding to integrin, which activates the Rho GTPase pathway (Hansson et al., 2012b; Johansson & Söderhäll, 1989). It was shown that RGD immobilization on chitosan 3-D scaffolds leads to an enhanced cell adhesion and
biocompatibility (Ho et al., 2005). Bound to the chitosan backbone, RGD is recognized by the adhesion receptors on the cell surface. The functionalized chitosan therefore takes on the role of an ECM substitute (Massia & Hubbell, 1990).

Karakecili et al. (Karakecili et al., 2007) studied cell behavior under the effect of chitosan membranes with immobilized RGD sequences. The *in vitro* analysis of L929 mouse fibroblasts showed that cell attachment increased with time and that the presence of RGD was critical for cell attachment, spreading and proliferation.

Jansma et al. (Jansma et al., 2003) conjugated tryptophan to 6-O-carboxymethyl-trimethyl chitosan oligomers (CM-TMC oligomers). TMC oligomers with 40% degree of trimethylation and conjugated to tryptophan were obtained. Results revealed that CM-TMC oligomers can be used for peptide conjugation, opening the possibility for a targeted drug and gene delivery platform.

Combining the advantages of TMC (drug complexation, nanoparticle formation) with those of CMC may be obtained by further derivatization of TMC to obtain carboxymethyl-trimethyl-chitosan (CMTMC). An optimized process has been developed to this end (Patrulea et al., 2015).

We recently synthesized polyelectrolyte nanoparticles based on the chitosan derivative CMTMC grafted with GRGDS peptide, (Figure 3) to induce cell adhesion and migration (Hansson et al., 2012b). Nanoparticles were obtained by an ionic complexation process with chondroitin sulfate, a biopolymer promoting wound-healing (Hansson et al., 2012a). For *in vitro* characterization of cell adhesion, human dermal fibroblasts were selected (Hansson et al., 2012b). Nanoparticles functionalized with the peptide were able, in vitro, to promote fibroblast adhesion and spreading of human dermal fibroblasts at a 3-fold increased area compared to an inactive scrambled peptide (Hansson et al., 2012b). This suggests a potential for nanoparticle-directed tissue engineering based on bio-activated chitosan derivatives.
4. Conclusions and future perspectives

Chitosan has been shown to be an efficient biomaterial to promote wound healing. Most of the studies revealed that chitosan is a non-toxic, slowly biodegradable and biocompatible material. It combines a unique set of useful properties for wound healing. However, as for many other biopolymers, care must be taken in the choice of the chitosan source as impurities such as animal proteins or other contaminants may affect the results. As for preclinical and clinical studies, many promising results have been reported. However, to rule out biopolymer variability, a more complete and standardized characterization of the materials would be highly beneficial.
We presented here some examples of chitosan derivatives used to accelerate wound healing and introduced some strategies for using them in different formulations: sponges, nanoparticles, scaffolds, gels, dressings or films.

Despite promising results of formulations based on chitosan and its derivatives for wound healing, many challenges remain, such as achieving an effective, controlled drug delivery adapted to the process of wound healing, the issue of sterilization and the development of controlled sourcing and manufacturing processes mandatory in view of medical applications.

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