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Abstract

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Chitooligosaccharides for wound healing biomaterials engineering

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ABSTRACT

Chitooligosaccharides (CHOS) are oligomers of β-(1-4) linked N-acetylglucosamine and D-glucosamine that are produced from chitin or chitosan using different enzymatic or chemical methods. CHOS is water-soluble and non-cytotoxic with diverse bioactivities such as antibacterial, anti-inflammation, anti-obesity, anti-tumour, and antioxidant. These biological features make CHOS promising compounds for several medical and food applications. In this review, we critically summarise the biological activities of CHOS toward biomaterials engineering with a particular focus on CHOS applications for skin tissue healing and regeneration. We also present an updated overview of CHOS fabrications into wound dressing biomaterials for several in vitro and in vivo studies. Besides, the prospect of CHOS applications for biomaterials engineering is discussed.

Keywords: Chitooligosaccharide; physicochemical properties; antibacterial and antifungal; antioxidant; anti-inflammatory; immunostimulatory; wound healing, in vitro and in vivo studies.

List of abbreviations

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

AgNPs: silver nanoparticles

BC: bacterial cellulose

CHOS: Chitooligosaccharide

CMCS: carboxymethyl chitosan

Cy: Cyclophosphamide

Đ: Dispersity

DA: Degree of acetylation

DC: Dendritic Cell

DD: Degree of deacetylation

DOPA: Dopamine

DP: Degree of polymerization

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DS: Degree of substitution

FN: Fibronectin

FA: Fraction of N-acetylated residues

G-CHOS: Quaternary ammonium CHOS

GTMAC: 2,3-epoxypropyltrimethylammonium chloride

HNTs: Halloysite Nano Tubes

HUVEC: Human umbilical vascular endothelial cells

IC_{50}: The half maximal inhibitory concentration

IBD: Inflammatory bowel disease

MW: Molecular weight

KGF: Keratinocyte growth factor

LMWC: Low Molecular Weight Chitosan

LPS: Lipopolysaccharide

MDM: Monocyte-derived macrophages

NHDF: Normal human dermal fibroblasts

NK: Natural killer

NO: Nitric oxide

OCHOS: Oxidized chitooligosaccharide

PA: Pattern of N-acetylation

pADM: Porcine acellular dermal matrix

PMA: Phorbol 12-myristate 13-acetate

PU: Polyurethane

PVA: Polyvinyl alcohol

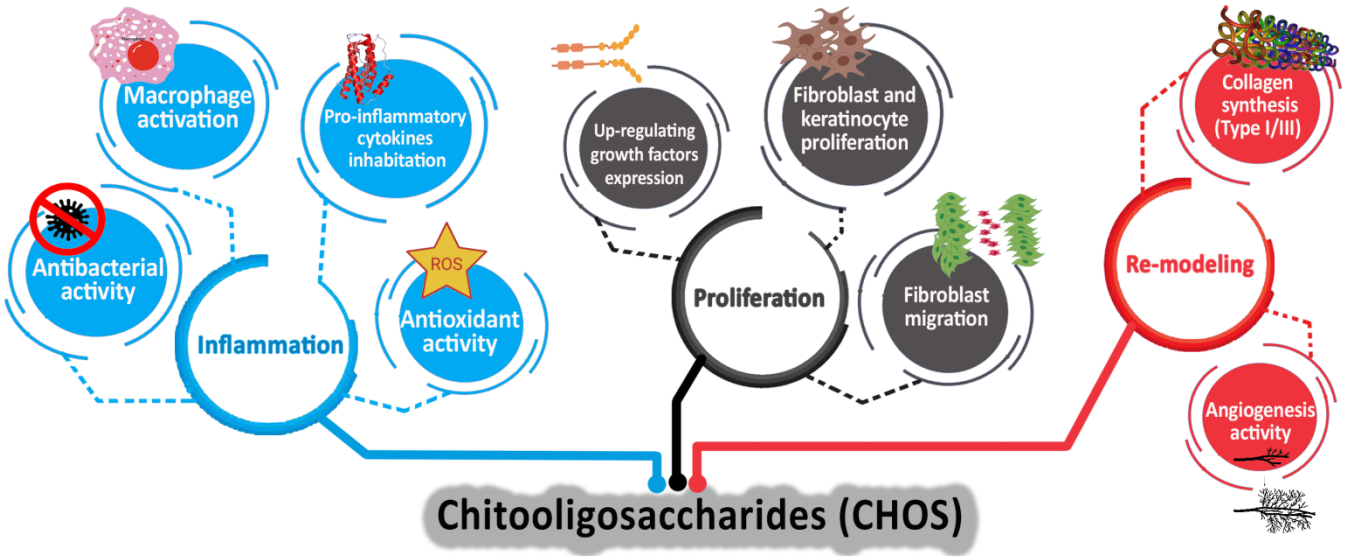
ROS: Reactive oxygen species

SC: Schwann cell

TGF- β: Transforming growth factor-β

VEGF: Vascular epidermal growth factor

Contents



Graphical abstract: Schematic illustration of wound healing potential of CHOS by its biological activities in the different phases of wound healing. CHOS with antioxidant, antibacterial, and anti-inflammatory activities can induce macrophages activation that leads to inhibition of pro-inflammatory cytokines expression (IL-1, IL-6, or TNF-α) result in the resolve of inflammation of wound healing. CHOS stimulates the production of growth factors involved in wound healing such as TGF-β, and VEGF followed by induction of fibroblast and keratinocyte proliferation and migration; besides, up-regulation of VEGF promotes angiogenesis, fibronectin (FN) and collagen protein production leads to the tissue granulation, and therefore the acceleration of re-modeling.

1 Introduction

Chitin (Fig. 1) is the most abundant natural polysaccharide after cellulose and is mainly found in hard-shelled crabs, shrimp, insect cuticles as well as in the cell wall of fungi [5]. Chitin is generally found in nature in complexes with other polysaccharides like cellulose, proteins, lipids, and phenols [6]. This natural polymer is structurally similar to mucopolysaccharides such as heparin and hyaluronic acid with high biological tolerance [7].

Chitin has three different crystalline forms, namely the α, β, and γ polymorphs in the solid phase [8]. The percentage of chitin-α in nature is higher than for the other two forms. Chitin-α is mostly found in the cell wall of fungi and in hard shells such as shrimp and crab, the β form could be extracted from diatoms and squid pen, and the γ form of chitin can be found in some insects such as larvae of the spider beetle, *Ptinus tectus*, and dragonfly silkworm larva (*Antheraea pernyi*) [10, 11].

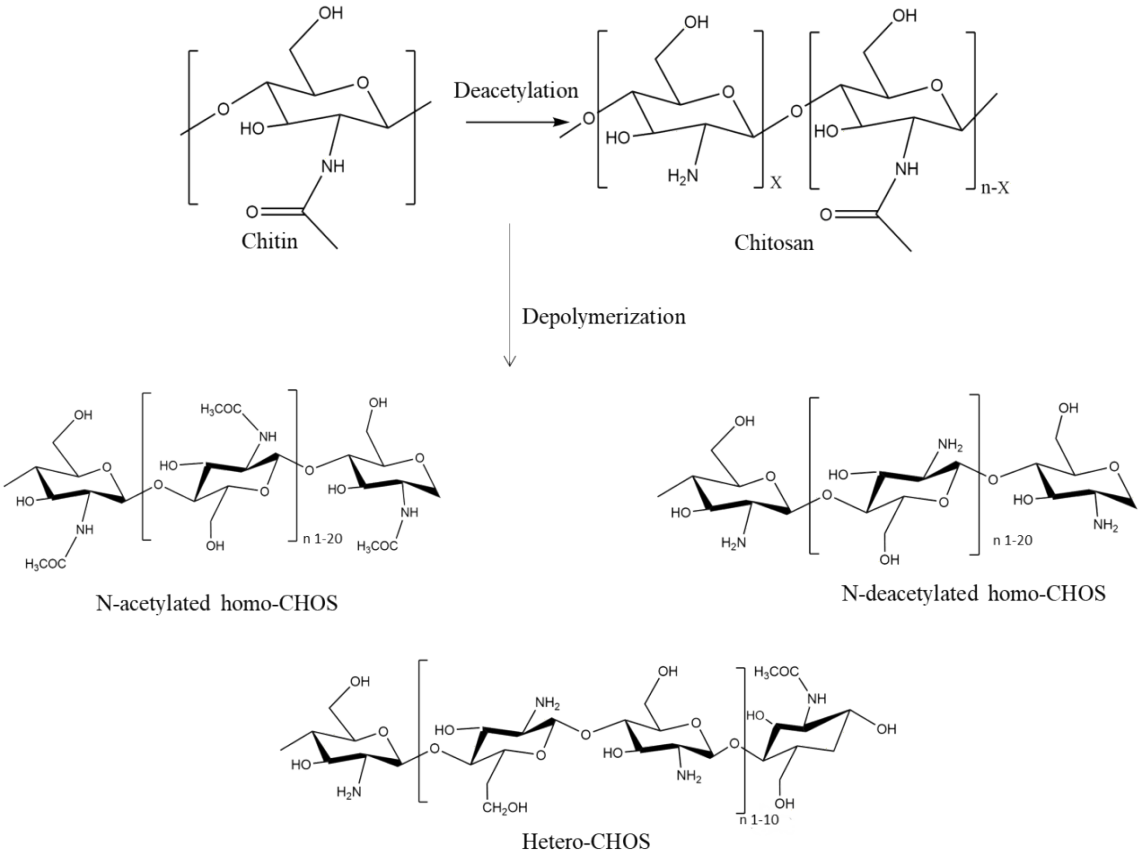


Figure 1. Chemical structure of chitin, chitosan, and homo- and hetero- CHOS.

The different orientation of the chitin polymeric chains causes such differences in the crystalline forms of chitin. The α and β chitin comprise of antiparallel and parallel polysaccharide layers, respectively; while the γ form, which only has low precedence in nature, is a combination of β and α chitin, having most similarity to α chitin [12]. α chitin has both intersheet hydrogen bonding between $–CH_2OH…O=C$ and intrasheet hydrogen bonding between N-H…O=C amide groups, while β chitin only has intrasheet hydrogen bonding indicating vital role of the acetyl group in such crystal formations.

Marine waste, which is generated in millions of tons annually, is the primary source of chitin [13]. Chitin based materials are biodegradable, non-toxic, and biocompatible, forming the basis of many biomedical, biotechnological and food applications [14, 15]. Chitosan (Fig. 1) is the most important derivative of chitin, which is partially deacetylated chitin and is composed of randomly and linearly distributed β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). The number of acetyl groups in the polymer chain indicates the difference between chitin and chitosan [16]. Chitin is a homopolymer of N-acetyl glucosamine with β-(1,4) linkages, while the degree of acetylation (DA) for chitosan is lower than 20%. Unlike the chitin, chitosan is not that common in nature and can be found in the cell wall of fungus, also in mycelia, stalks, and spores of basidiomycetes [17].

The chemical characteristics of chitosan such as the fraction of N-acetylated residues (FA) or degree of N-acetylation (DA), the degree of polymerization (DP) or the molecular weight (MW), the molecular weight distribution (Đ, for dispersity), and the pattern of N-acetylation (PA) or sequence have a significant impact on its biological and physicochemical properties [18]. The poor water solubility of chitin and chitosan restrict their biological activities and limit their pharmaceutical application in medical fields so that attention has been attracted to more soluble chitin and chitosan oligomers: chitooligosaccharides (CHOS) [19 20].

1.1 Chitooligosaccharide (CHOS)

CHOS (Fig. 1) is a chitosan oligomer obtained by hydrolytic depolymerization of chitin or chitosan. CHOS typically has a lower molecular weight (DP □ 20 and MW □ 3.9 kDa) and viscosity as well as superior solubility in aqueous solutions, which makes them attractive for use in biomedical applications [21, 22].

CHOS with different MW and DA can be produced using various methods such as microwave irradiation, acidic or enzymatic hydrolysis, oxidative degradation, ~~etc~~. The production of CHOS is beyond the scope of this review, and the readers are referred to the published reviews [23, 24]. The diverse potential biological activities of CHOS for plants and animals, including anti-tumor and immunostimulant activities, have also been widely reported and reviewed [25, 26]. The present review aims to critically evaluate and summarize recent studies on the application of CHOS for skin wound healing applications. In particular, the review focuses on antibacterial, antioxidant, and anti-inflammatory properties of CHOS as significant indicators of a bioactive compound for wound healing applications. As such, this review aims at providing an overall understanding of CHOS as a potential bioactive compound for the development of novel biomaterials for skin tissue healing and regeneration.

1.2 Physicochemical properties of CHOS

CHOS can be classified into homo-chitooligosaccharides, which contain N-Acetylglucosamine (GlcNAc) or glucosamine (GlcN) oligomers, and heterochitooligosaccharides, which comprise both GlcN and GlcNAc units (Fig. 1) [25]. CHOS consists of oligomers with different DP (2-20), DA, and PA. Physiochemical characteristics such as MW/DP, DA, and biological activities of CHOS are influenced by the CHOS production process. It is possible to produce CHOS with well-defined DP and DA; nevertheless, the pattern of acetylation in CHOS production is usually random [23]. Indeed, it is not possible to define a specific PA in CHOS production by chitosan hydrolysis. Still, it can be possible to define a specific pattern by using enzymes called chitin oligosaccharide deacetylases (CADs) [27].

The DP is the number of monomeric units, and therefore, an oligomer with two monomeric units is called di-saccharide (DP 2). The DP of CHOS can vary between 2 until 20. The DP of CHOS influences its solubility. CHOS with a DP lower than 4 is soluble in methanol, while CHOS with DP>5 is hardly soluble

[28]. Moreover, CHOS is not soluble in acetone, butanol, ethanol, ethyl acetate, propanol, and pyridine but fully soluble in water [28].

~~Moreover, it~~ has been reported that CHOS with a higher percentage of free amino groups than acetylated sequences shows higher antibacterial activity. Hence, DP/MW and PA play essential roles in the physicochemical and biological activities of CHOS. Therefore, to obtain a solid understanding of the relationship between biological activity and physicochemical structure of CHOS, it is necessary to have a well-controlled production and purification process. [29].

2 Wound healing characteristics of CHOS

The wound healing process depends on different healing phases such as hemostasis, inflammation, proliferation, and remodeling, which have been influenced by the immunostimulating activities of materials utilized for wound healing such as inflammatory cells, proinflammatory cytokines, and growth factors [30]. The demand for the utilization of biomaterials with superior biological activities such as antibacterial, antioxidant, anti-inflammatory, etc. has been growing fast. An ideal wound dressing material should provide a moist environment, prevent infection, and support the complete restoration of tissue layers. Additionally, an optimum wound dressing should decrease the production of the pro-inflammatory molecules in order to resolve the inflammation phase of wound healing. Furthermore, a wound matrix should upregulate the growth factors such as vascular epidermal growth factor (VEGF), keratinocyte growth factor (KGF), and transforming growth factor-β (TGF- β) that are involved in the re-epithelialization. Besides, a wound dressing should promote fibroblast and keratinocyte proliferation and migration as well as collagen synthesis. CHOS has exhibited a potential therapeutic effect in tissue injuries and wound healing due to its positive biological activities such as protecting injured tissues against infection, enhancing the permeability of air and moisture, supporting cell adhesion, and promoting cell proliferation [31, 32]. Moreover, different material types of CHOS with other biopolymers, such as fibers, sponges, hydrogels, and films, have been studied in wound healing applications. Indeed, CHOS can not be used directly as a wound dressing due to its low MW; however, it can be combined with other biopolymers

while taking advantage of the wound healing potential of CHOS. In addition to the biological activities, CHOS can improve wound healing by enhancing water absorption, flexibility, and mechanical strength of a wound dressing [30]. Along with the biological activities of CHOS, the physicochemical properties, such as MW and DA, also influence its wound healing potential. In this section, we discuss the specific biological features of CHOS that can accelerate the wound healing process.

2.1 Antibacterial and antifungal activity

The wound healing process is susceptible to infection, especially bacterial infection through different bacteria such as *Staphylococcus aureus*/MRSA, *Streptococcus pyogenes*, *Enterococci,* and *Pseudomonas aeruginosa*. The healing process takes more time in infected wounds due to the formation of unpleasant exudates and toxins, which lead to the concomitant killing of regenerating cells. Therefore, reducing the bacterial infection by the use of antibacterial compounds is highly necessary for the activation of the tissue repair process [33]. The antibacterial activity of CHOS plays a prominent role in wound healing acceleration, which leads to tissue granulation and collagenase activity during the wound healing process. As a result, it has been proposed that the wound healing potential of CHOS is derived from its antibacterial activity.

CHOS can be used as a potential antibacterial agent against a wide range of bacteria, such as S. *aureus,* E. *coli*, *Bacteroides/Prevotella, Prevotella melaninogenica,* which are the major infectious bacteria in the wound healing process. CHOS can be utilized in wound dressing materials to accelerate the wound healing through improving the antibacterial activity. Table 1 summarizes the recent studies that evaluated the antibacterial activity of CHOS.

Compared to chitosan, CHOS has been reported as a promising antibacterial agent due to its higher solubility, which is a prominent factor for penetrating the cell wall to inhibit the bacterial growth [34].

CHOS has different antibacterial activity against various bacteria. Indeed, CHOS with positively charged amine groups (C-2 position) can effectively inhibit growth of gram-negative bacteria such as E. *coli* with a negative cell surface charge. At the same time, CHOS is not similarly effective in inhibiting the growth of a gram-positive bacterial such as L. *monocytogenes.* The interplay between positively charged amine

groups from CHOS and negatively charges carboxylic groups from bacterial cell surface paves the way for creating polyelectrolyte complexes which can form an impermeable coating around the bacterial cell and repress its the metabolic activity [35]

The antibacterial activity of CHOS depends on its DP/MW, DA and PA, as well as the type of organism. Generally, the antibacterial activity of CHOS is increased with decreasing MW; however, a DP more than 5 is required to exhibit antibacterial property [36 37].

Although decreasing MW improves the antibacterial activity of CHOS, there is controversy on the impact of MW/DP and DA of CHOS on its antibacterial and antifungal activity in different studies. A recent study reported that CHOS with DP 9 and 14 at concentrations of 0.3–0.4% (w/v) were more effective in inhibitation the growth of *Leptographium procerum* and *Sphaeropsis sapinea* in comparison with a mixture containing lower-DP oligomers (DP 5). Moreover, the inactivity of N-acetylated CHOS with DP 14, as well as a higher antifungal activity at lower PH (4) indicate the importance of amino group protonation in the antifungal activity of CHOS [38].

Furthermore, it has been reported that increasing the DP of CHOS from 3 to 7 improved the antifungal activity against *Fusarium solani* with the maximum activity for the heptamer fraction at a concentration of 4 and 8 μg mL^{-1}, while the monomer and trimer of CHOS were not active [39]. Aparicio *et al*. studied the effect of different DA (8-47 %) and MW (2-28 kDa) of CHOS on human faecal microbiota during fermentation in batch cultures. Decreasing the DA from 28 to 2 % could reduce the populations of *Bifidobacterium spp*., E. *rectale*/C. *coccoides*, C.histolyticum, and Bacteroides/Prevotella. On the other hand, CHOS with more acetylated residues (DA 36-38 %) had not any probiotic effect. At the same time, it could maintain gut microbiota without any adverse effect and even increase the population of *Lactobacillus/Enterococcus*. Hence, low MW CHOS can be more effective for bacteria inhibition, but acetylation is needed to maintain the gut microbiota without any adverse effect. [40].

In another study, the presence of acetylated groups on CHOS was reported as one of the essential factors for its antibacterial activity. CHOS with a higher amount of acetylated sequences (73%) was more effective

in the inhabitation growth of *Escherichia coli* and *Listeria monocytogenes* than CHOS with 37 % fully acetylated sequences [29]. Sanchez *et al*. believed that the low presence of amino groups is an essential factor in CHOS to exert good antibacterial activity independent of the bacterial strain.

PU membranes modified by CHOS showed improved antibacterial activity E.*coli* and S.*aureus*, are more effective in preserving moisture content, and show wound healing acceleration in comparison to the non-modified PU membrane [41].

Recently, increasing the antibacterial activity of CHOS through surface modification has attracted a great deal of scientific consideration. Chen *et al*. reported that introducing quaternary ammonium groups such as 2,3-epoxypropyltrimethylammonium chloride (GTMAC) on the CHOS (DD = 85%, MW = 2000 Da) surface can increase CHOS antibacterial activity by increasing the CHOS solubility and its positive charges. Quaternary ammonium CHOS (G-CHOS) with positive charges can easily bind with negatively charged bacteria such as E. *coli*, and therefore causes an improvement in the antibacterial activity [35]. Moreover, incorporating the G-CHOS into a polyurethane (PU) membrane improved the antibacterial activity of PU [4], which could have potential applications as antibacterial wound dressing materials. In another study, quaternary ammonium CHOS (G-CHOS improved the antibacterial activity, moisture retention, and oxygen permeability of a PU membrane (Fig. 4 b). Moreover, NIH-3T3 cells exhibited higher proliferation and adhesion on the membrane surface in the presence of CHOS, possibly due to the relatively high surface roughness and active OH and NH_2 groups on CHOS layers [42].

It can be concluded that the quaternization of CHOS can improve the antibacterial activity by enhancing the water solubility and positive charge of CHOS and, as a result, a stronger bond to negatively charged bacteria. Therefore, PU/G-CHOS can be more effective in the wound healing acceleration than PU/CHOS due to the higher antibacterial activity, which leads to a more substantial reduction of bacterial infection in the wound healing phases. Hence, these two studies demonstrate the importance of the antibacterial activity of CHOS in the acceleration of wound healing.

Table 1. Summary of antibacterial activities of chitooligosaccharides (CHOS)

CHOS (MW/DP, DA/DD)	**Production method**	**Microorganism**	**Antibacterial activity MIC[1] (μg/ml) or inhabitation zone**	**Observations**	**Ref.**
CHOS	Chemical hydrolysis by HCl Chitin source (shrimp shell)	*S. aureus ATCC 25923* *S. aureus ATCC 43300* *Bacillus subtilis* *Bacillus cereus* *E. coli* *P. aeruginosa* *Salmonella typhimurium* *Shigella dysenteriae,* *Prevotella melaninogenica*	0.003 μg/ml (for all of the tested bacteria strains)		[43]
CHOS (DD = 87% CHOS-functionalized-quaternary ammonium (COS-GTMAC[2])	Commercial CHOS	S. *mutans* (ATCC 27351)	COS-GTMAC: 80% inhabitation growth	COS-GTMAC (DS[3] = 115%) higher antimicrobial activity	[35]
CHOS with different MW and DA (2-28 kDa**, 8-47 %)**	Enzymatic (chitosanase)	*Bifidobacterium* spp *Eubacterium rectale/Clostridium coccoides* *C.histolyticum* *Bacteroides/Prevotella*		Antibacterial (human Faecal microbiota)	[40]
CHOS (MW 1, 6, 17 kDa)	Enzymatic by chitosanase	*Escherichia Coli and* *Listeria monocytogenes*		CHOS with MW between 6-17 kDa showed higher antimicrobial than MW< 1 kDa.	[29]
CHOS (MW<5, <3 kDa, DD 80–85%)	Commercial CHOS [Nicechem (Shanghai, China)]	*Staphylococcus aureus* *Escherichia coli*	CHOS < 3 kDa E. *coli* (0.08% (w/v)) S. *aureus* (0.09% (w/v)) CHOS <5 kDa E. *coli* (0.5% (w/v)) S. *aureus* (0.5% (w/v))	Higher MW showed higher antibacterial activity in the case of gram-positive bacterium	[44]
CHOS (MW 2-5 kDa and DA 91.5 %)	Chemical hydrolyze of chitosan	*Actinobacillus actinomycetemcomitans* *Streptococcus mutan*	0.001%	CHOS markedly inhibited the growth of A. *actinomycetemcomitans*.	[45]
CHOS (1 kDa, 90% DA) grafted with **geraniol (CHOS-o-Ger)**	Commercial CHOS	*E. coli* *S. aureus*		The antibacterial activity of CHOS improved with increasing the degree of substitution of geraniol, and the CHOS-o-Ger had more antibacterial activity against S. *aureus*.	[46]
CHOS (DP 15-40)	Enzymatic hydrolysis of chitosan (DP 206 and FA[4] 0.15) by Chitosanase ScCsn46A	*Botrytis cinerea*		Antifungal activity reduced at < DP 1 Combination of CHOS and synthetic fungicides showed notable antifungal activity in plant-fungal infections	[47]
Three different mixtures **of CHOS** **Q_1 (DP2–DP10)** **Q_2 (DP2–DP12)** **Q_3 (DP5–DP8)**	Enzymatic degradation of chitosan	*Alternaria alternata* *Rhizopus stolonifera* *Botrytis cinereal* *Penicillium expansum*		Q2 and Q3 showed antifungal activity against *Alternaria alternata* and *Rhizopus stolonifer,* while Q1 stimulated the growth of these fungal. Q2, Q3 showedantifungal (*Botrytis cinerea*), and Q1 had no effect. Q2, Q3 slightly inhibited *Penicillium expansum,* while Q1 had no effect.	[48]

[1] Minimum inhibitory concentration
[2] 2,3-epoxypropyltrimethylammonium chloride (GTMAC)
[3] Degree of substitution
[4] Fraction of N-acetylated residues

CHOS-grafted-silk fibroin (CHOS-SF)	Commercial CHOS (MW< 2 kDa)	E. *coli.*	47.47% (antibacterial activity)	CHOS increased the antibacterial activity of silk from 15 to 47.47%.	[49]
CHOS 5 (MW of 5.1 kDa) **CHOS 14 (MW of 14.3 kDa)** **CHOS 41 (MW of 41.1 kDa)**	Enzymatic hydrolysis (lysozyme, papain, or cellulase)	*Escherichia coli* *Salmonella Typhimurium,* *Salmonella enteritidis*	CHOS 5 E. *coli.* (16 µg/ml)	The lowest MW CHOS exhibited the best antibacterial activity, and the samples were more effective against *Escherichia coli* than the other pathogens	[50]
CHOS with different MW (kDa) **CHOS I (MW>100)** **CHOS II (MW 100-10)** **CHOS III (MW 10-1)** **CHOS IV (MW<1)**	Enzymatic hydrolysis of chitosan (MW 300 kDa,) by immobilized pepper chitosanase	*Bacillus cereus* *Bacillus subtilis* *Staphylococcus aureus* *Escherichia coli* *Pseudomonas aeruginosa* *Candida albicans* *Saccharomyces chevalieri* *Macrophomina phaseolina* *Aspergillus niger*		groups (I, II, III), Antibacterial against *B. cereus,* groups (I, III, IV) Antibacterial against *B. subtilis* group (II, IV) against *Staphylococcus aureus* group (II) against Pseudomonas *aeruginosa* group (I, II, IV) against Candida *albicans* group (III) against Saccharomyces *chevalieri*.	[51]
CHOS-Naringin complex	Commercial CHOS	*Escherichia coli* *Staphylococcus aureus*		CHOS increased Naringin antibacterial activity.	[52]
CHOS with different MW (kDa) **CHOS III (10>MW>1)** **CHOS IV (MW<1)**	Enzymatic hydrolysis (immobilized pepper chitosanase)	Gram-positive bacteria (*Bacillus cereus*) Gram-negative fungi (*Pseudomonas aeruginosa*) Gram-positive yeast (*Candida albicans*)	0.11µg/ml (B. *c ereus*) 0.11 µg/ml (C. *albicans*)		[53]

2.2 Antioxidant activity

Reactive oxygen species (ROS) have a prominent role in different phases of the wound healing process, such as inflammation. However, excessive production of ROS can have adverse effects on the cutaneous wound healing by inducing processes such as inflammation and fibrotic scarring [54]. Hence, the utilization of small molecules with ROS scavenging activity in wound dressing biomaterials could be a promising strategy for accelerating the wound healing process. The antioxidant activity of chitosan and its derivatives has attracted a great deal of scientific consideration among researchers due to their biocompatibility, biodegradability, non-cytotoxicity, and their abundance [55].

Chitosan has antioxidant activity; however, its poor solubility and strong intermolecular and intramolecular hydrogen bonds limit this antioxidant potential [56]. CHOS, compared to chitosan, has a higher antioxidant activity. CHOS has short chains that causes a weaker intermolecular and intramolecular hydrogen bonding,

and therefore, the presence of more hydroxyl groups and activated free amine groups promote the antioxidant activity. Some recent antioxidant activities of CHOS are reported in Table 2.

The antioxidant activity of CHOS increases with the reduction of its MW. A study has been reported that CHOS with the MW of 963 Da showed the highest antioxidant activity among the tested CHOS with MW ranging from 963 Da to 13025 Da [57]. Moreover, the antioxidant activity of CHOS with different molecular weights of 2300, 3270, 6120, and 15,250 Da was investigated, and the CHOS with the lowest MW demonstrated the highest antioxidant activity (70 %) [58]. Indeed, the IC_{50}[5] of CHOS with MW of 2300, 3270, 6120, and 15,250 Da were 0.4, 0.76, and 1.54 mg/mL, respectively, and the maximal inhibiting efficacy of hydroxyl radicals were 70, 65, 51, and 7%, respectively.

Furthermore, decreasing in the DP from 10 to 3 enhances the antioxidant activity of CHOS from 34.97 to 48.19 % (hydroxyl radical scavenging activity); however, the presence of at least two saccharide units is essential for exhibiting antibacterial activity [59]. On the contrary, in the case of superoxide radical scavenging, the same samples with the highest DP (10) had a stronger ability to scavenge superoxide radicals (Ic_{50}: 0.68 mg/ml). Yang et al. reported that CHOS with MW 500 Da showed a lower capacity to scavenge superoxide radical compared to CHOS with MW 1100 Da [60]. Besides, Park *et al*. found that CHOS with a MW of 1000-3000 Da had more powerful superoxide radical scavenging activity in comparison with CHOS with a MW of 1000 Da [61]. Indeed, the superoxide radical scavenging activity of CHOS with a MW below 3000 Da has improved by increasing the MW/DP up to 3 kDa.

Another critical factor that has a noticeable effect on the CHOS antioxidant activity is the DA. The ratio of acetylated to deacetylated (A/D) units in the CHOS sequences revealed to play a significant role in the antioxidant activity of CHOS. When more than 70% of the N-acetyl-d-glucosamine/D-glucosamine (A/D) ratio is between 0 and 1, CHOS shows the best antioxidant activity. For example, CHOS with a 91% A/D

[5] The half maximal inhibitory concentration (IC_{50})

ratio below 1 exhibited the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (90%).

On the other hand, CHOS, with the presence of at least a 40 % A/D ratio above 2, can be predicted as non-antioxidant. Generally, the higher antioxidant activity in low MW CHOS lies in the easy reaction of amine and hydroxyl groups with different DPPH radicals [62].

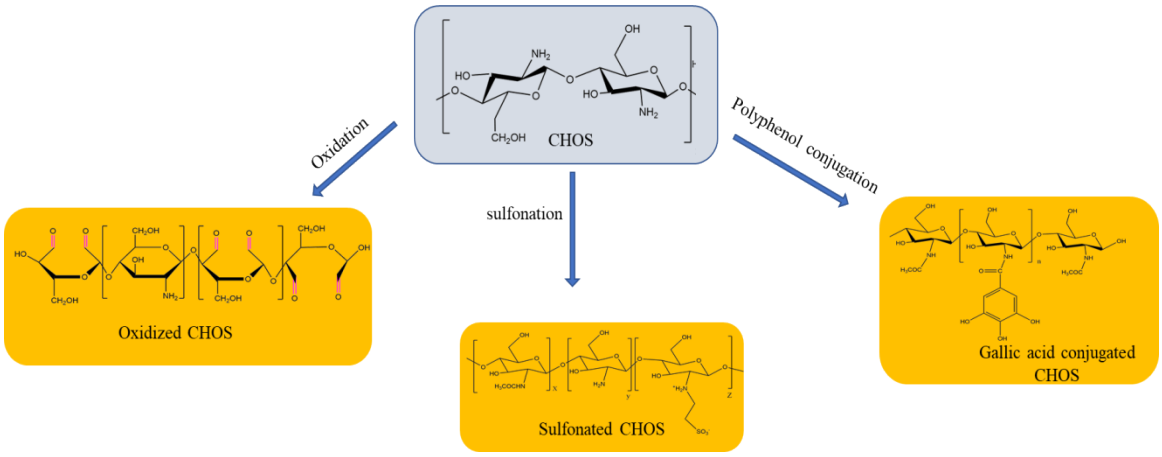


Figure 2. Chemical modifications of CHOS

A recent study revealed that a wound dressing with 54 % DPPH radical scavenging activity could improve wound closure (>90%) after 10 days, which indicates the importance of intracellular ROS production suppression in tissue regeneration [63]. It has been reported that CHOS has a hepatoprotective effect on Chang liver cells against tert-butyl hydroperoxide (t-BHP)-induced toxicity. Moreover, CHOS has the ability to mediate of production of ROS, lipid peroxidation, and glutathione (GSH) content [64].

Recently, chemical modification of CHOS has been widely investigated to enhance the biological activity of CHOS. Sulfonation [65], oxidation [66], quaternization, and carboxylation [67] are the main chemical modifications of chitosan derivatives (Fig. 2) made to improve its biological properties, particularly antioxidant activity. Conjugation of small antioxidant agents such as polyphenols and flavonoids to CHOS has also been reported to enhance the antioxidant activity. [68].

The antioxidant activity of gallic acid conjugated to CHOS has been evaluated against oxidative stress in human lung epithelial cells. Gallate-CHOS showed high DPPH radical scavenging activity and protective effect against H_2O_2-induced DNA damage. Besides, gallate-CHOS diminished the production of intracellular ROS in H_2O_2-stimulated A549 cells. The observed antioxidant activity related to the presence of hydroxyl groups in the phenolic structure of gallic acid has provided CHOS with the ability to destroy radicals and form stable phenoxy radicals [69].

Incorporation of CHOS (MW 1698 Da, DD 90%) into bacterial cellulose (BC) composite membranes by immersing the membrane in a CHOS solution (6 g/L) increased the antioxidant and antibacterial activity of the membrane against both Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*) in comparison with the pure cellulose membrane. BC-CHOS exhibited an inhibition ratio of 99.64 ± 0.18% (S. aureus) and 90.56 ± 0.06% (E.coli), and higher ABTS[6] radical scavenging activity (92.62 %) than the pure BC membrane (60 %), which demonstrates that BC-CHOS could be a promising candidate in wound healing applications [42]. However, attributing the wound healing potential of CHOS only to its antibacterial and antioxidant activity is an underestimation, and as a result, other biological activities of CHOS such as cell viability and proliferation, cell migration ability, collagen synthesis, and inflammatory activity should be considered to thoroughly investigate the determining parameters in the wound healing potential of CHOS.

[6] 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

Table 2. Summary of antioxidant activities reported for chitooligosaccharides

CHOS (MW/DP, DA/DD)	Production method/source	Antioxidant evaluation method(s)	IC_{50} [7](DPPH[8]) (mg/ml)/ scavenging effect	Observations	Ref.
Chitin oligosaccharides (NA-CHOS; MW 229.21–593.12 Da)	Chemical degradation of crab chitin (DA<10%)	Evaluation of the oxidative stress inhibitory activity of CHOS on Human myeloid cells (HL-60) and Raw 264.7.	NA	Antioxidant (RAW 264.7 cells and HL-60 cells)	[70]
CHOS (DD >90%) C1: 2.8 kDa, C2: 17.0 kDa,	Commercial CHOS[9]	DPPH and ABTS radical scavenging, and reducing power	C1: 0.87 mg/ml C2: 3.28 mg/ml	Antioxidant (human serum albumin (HSA))	[71]
N-acetyl chitooligosaccharides (NA-CHOSs) (NA-CHOS 1–3 kDa) (NA-CHOS < 1 kDa)	Chitin oligomers obtained by an ultrafiltration membrane system.	Determination of radical-mediated DNA damage, and cellular ROS determination by DCFH-DA[10]		Antioxidant (RAW 264.7 cells) Antioxidant activity of NA-COS 1–3 kDa was higher than that of NA-CHOS < 1 kDa.	[72]
CHOS I (5–10 kDa), CHOS II (1–5 kDa) CHOS III (below 1 kDa).	Enzymatic degradation of chitosan (DD 90%.)	Intracellular ROS determination (DCFH-DA), intracellular GSH[11] level determination, and Superoxide dismutase (SOD) activity		Antioxidant (Chang liver cell line (CCL-13)) against t-BHP[12]-induced toxicity. CHOS I exhibited superior ROS production inhibition.	[64]
Five low molecular weight Chitosans (CHOS1) 13025 Da (CHOS2) 7011 Da (CHOS3) 4169 Da (CHOS4) 2242 Da (CHOS5) 963 Da	Oxidative degradation from squid pens (β-chitin)	DPPH, Hydroxyl radical, Superoxide radical scavenging, and Reducing power	C1: 1.29 mg/ml C2: 1.09 mg/ml C3: 0.87 mg/ml C4: 0.31 mg/ml C5: 0.22 mg/ml	CHOS with the lowest MW showed the most effective antioxidant activities. (76.4 % at 0.4 mg/ml)	[57]
N-furoyl chitosan oligosaccharide (NF-CHOS) with MW 5000 Da	Oxidative degradation (H_2O_2) from commercial chitosan (120 kDa, 97% deacetylation)	DPPH, Hydroxyl radical, and Reducing power	CHOS: 0.62 mg/ml NF-CHOS: 0.76 mg/ml		[73]
Five fractions of CHOS DP (2-12) F2 (GlcN)3 F3 (GlcN)4 F4 (GlcN)5 F5 (GlcN)6 F6 (GlcN)7 (GlcN)8 (GlcNAc) (GlcN)9 GlcNAc**	Chemical degradation of Commercial chitosan (DD 82% and MW 658 kDa)	Hydroxyl and superoxide radical scavenging, and reducing power	F2: 1.30 mg/ml F3: 1.30 mg/ml F4: 1.29 mg/ml F5: 1.11 mg/ml F6: 0.89 mg/ml	$(GlcN)_3$ had the highest antioxidant activity	[59]
F30 (30 × 10^3 g/mol) F10 (10 × 10^3 g/mol) F5 (5 × 10^3 g/mol)	Enzymatic hydrolysis by chitosanase	DPPH radical scavenging activity, and ferric reducing antioxidant power assay (FRAP)	F30: 36% F10: 46% F5: 83%	CHOS with DA higher than 70% can exhibit better antioxidant activity	[62]

[7] The half maximal inhibitory concentration (IC_{50}).
[8] 2,2-diphenyl-1-picrylhydrazyl
[9] Dainichiseika Color & Chemicals Mfg. Co., Ltd (Tokyo, Japan).
[10] Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay
[11] Glutathione
[12] Tert-butylhydroperoxide

Gallic acid-conjugated chitooligosaccharides (gallate-CHOS) **CHOS (3–5 kDa)**	CHOS produced from chitosan (DD 89%) via using an ultrafiltration membrane reactor. Gallate-CHOS synthesized by solution mixing of CHOS and gallic acid.	DPPH radical scavenging activity, ROS[13] production measurement .	CHOS: 70% G-CHOS: 36 % (at 200 µg/ml)	Antioxidant (human lung epithelial A549 cells)	69
$CHOS_5$ **(MW of 5.1 kDa)** **$CHOS_{14}$** **(MW of 14.3 kDa)** **$CHOS_{41}$** **(MW of 41.1 kDa)**	Enzymatic hydrolysis of chitosan (DD 90%) by papain.	DPPH radical scavenging activity, Reducing power, and Metal chelating activity	IC50 $CHOS_5$: 2.36 mg/ml $CHOS_{14}$: 2.64 mg/ml $CHOS_{41}$: 2.71 mg/ml		50
CHOS (DD ≥95%; MW, <1,000 Da	Commercial CHOS (Dalian, China)	Measurement of ROS production (DCFH-DA), and determination of lipid peroxidation and reduced GSH		Antioxidant (Human L02 normal liver cells) against ethanol-induced oxidative stress. CHOS was effective in ROS production and lipid peroxidation inhibition.	74
CHOS-grafted-Silk fibroin (CHOS-SF)	Commercial CHOS (MW< 2 kDa).	ABTS radical scavenging	SF (8.58%) CHOS-SF (15.42%)		49
CHOS with different hydrolysis time (min) **CHOS (60 min)** **CHOS (72 min)** **CHOS (84 min)** **CHOS (120 min)**	Enzymatic hydrolysis by chitinolytic enzymes	Hydroxyl and superoxide radical scavenging activity, and reducing power	CHOS (60): 2.8 mg/ml CHOS (72): 1.9 mg/ml CHOS (84): 3.2 mg/ml CHOS (120): 3.6 mg/ml	CHOS (72) exhibited the highest antioxidant activity among the samples (80 % at 4 mg/ml)	75
β-carotene (CAR)-CHOS complex by 2 methods: • **Freeze-drying** • **Sonication**	acid hydrolysis of chitosan (MW 500000 Da, DD 75–85%),	DPPH radical scavenging activity	Freeze-drying method 0.23 mg/ml 94.72 % Sonication method 0.26 mg/ml 89.45%		76
Hesperidin-conjugated CHOS complex **CHOS (98% purity, DD 97% and MW 980 Da)**	Commercial CHOS Complex (Hesp-CHOS) has been prepared by the spray-drying method	ABTS and DPPH radical scavenging activity	Hesperidin : 187.2 µmol L^{-1} TE g^{-1} CHOS : 439.8 µmol L^{-1}TE g^{-1} Hesp/CHOS (1 :10) 446 µmol L^{-1}TE g^{-1}	The higher antioxidant activity can result from the improvement of the solubility by CHOS and addition of hydroxyl groups on CHOS by hesperidin	77

* This assay evaluated Cu^{+} levels derived by the reduction of Cu^{2+} by the action of antioxidants present in the sample.

** The main component of F1 was the unwanted glucosamine monomer.

[13] Reactive oxygen species

2.3 Anti-inflammatory activity

The primitive action of host protection against any infection in the human body is known as an inflammatory response, and several diseases result from a prolonged or severe inflammatory event. For inhibiting the inflammatory event, different cell types, and inflammatory mediators in the human body, such as cytokines, are involved in the resolution of acute inflammation [78].

CHOS has been used for regulating the inflammatory activity in macrophages, as a potential therapy of inflammatory bowel disease [79] and allergic asthma [80]. Some recent studies dedicated to the anti-inflammatory activities of CHOS are reported in Table 3.

Ma et al. studied the inflammatory effect and mechanism of chitosan oligomers with a DP of 2-6 on the lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells. The chitosan oligomers had an inhibitory effect on the LPS-induced IL-6 and TNF-α expression in macrophages. The inhibitory mechanism is based on the down-regulating the phosphorylated levels of MAPK and PI3K/Akt signaling pathways and subsequent NF-κB and AP-1 activation [81]. The physicochemical properties of CHOS affect its anti-inflammatory activity. Although it has been reported that CHOS with the lower MW has higher biological activity such as antioxidant and antibacterial activities [25], there is a controversy about the effect of MW on the inflammatory activity of CHOS.

On the one hand, some researchers revealed that chitosan and its derivatives with a MW of 50–190 kDa [82], 50 kDa [83], or 3 kDa [84] were not efficient in the inhibition of NO production, and the cytokines expression of IL-1, IL-6 or TNF-α in the RAW 264.7 macrophage. On the other hand, some opposite results were reported that CHOS with a MW of 10–20 kDa [85], or 5–10 kDa [85] remarkably exhibited NO production inhibition in LPS induced macrophages. This controversy can be related not only to the variation in the MW but also other physiochemical properties such as DA or PA and even the CHOS production method. The inflammatory phase of the wound healing process is mainly involved in the expression and induction of the proinflammatory cytokines (IL-1, IL-6, or TNF-α). Wound healing acceleration and scar formation reduction are highly dependent on modulating the production of the inflammatory mediators [86]. In a study

from 2016, a wound dressing with the ability to down-regulate cytokine production (IL-1, IL-6 or TNF-α) had a powerful effect in promoting the healing of burned skin induced by midrange ultraviolet radiation (UVB) [87].

In one study, the anti-allergic and anti-inflammatory effects of CHOS against calcium ionophore A23187-stimulated RBL-2H3 cells were investigated in a MW-dependent manner. The authors investigated the inhibitory effects of different MW of CHOS (1–3 kDa, 3–5 kDa, and 5–10 kDa) on degranulation and cytokine generation of mast cells. The CHOS with a MW of 1-3 kDa at a concentration of 1000 μg/ml presented the highest inhibitory effect, and the anti-allergic effect of CHOS reported to be due to the regulation of the MAPK pathway [88]. Although the author could not clarify the reason for the higher anti-inflammatory activity of CHOS (1–3 kDa), we assumed that the different activity is probably due to the distinctive absorption influenced by different MW, which leads to diverse inhibitory effects in mast cells.

On the contrary, another recent study investigated the impact of five different MW of chitosan from 3.3 to 300 kDa and a CHOS mixture on the NO production and cytokine expression (TNF-α and IL-6) production in LPS-induced RAW264.7 macrophages. Higher MW chitosan (300, 156, 72 kDa) significantly inhibited NO production and cytokine expression (TNF-α and IL-6), while a mixture of lower MW chitosan (7.1 and 3.3 kDa) and CHOS induced their production. Indeed, high MW chitosan down-regulated the phosphorylation of MAPK signaling proteins such as ERK, JNK, and p38 by binding to CR3 receptor (156 kDa chitosan), and TLR4 and CR3 receptors (72 kDa chitosan) which led to inhabitation of the LPS induced NF-κB activation, decrease in the expression of TNF-α and IL-6, and iNOS expression, and as a result, diminishing the NO production. On the contrary, the CHOS mixture acted oppositely via binding to CD14, TLR4, and CR3 receptors in macrophages. (Fig. 3) [1].

NO is a highly reactive free radical formed through the conversion of $_{L}$-arginine to $_{L}$-citrulline via NO synthase enzyme. NO can be secreted in activated macrophages by interferon-gamma (IFNγ) and bacterial LPS, and excessive NO (>10 M) leads to inflammatory cellular cytokines and cytokine-induced cell death. Therefore, reduction of NO production is essential in the inflammatory phase of wound healing to inhibit

cell death and inflammation [89]. It is crucial to prepare well-defined and highly purified CHOS preparations for a more robust understanding of the biological activity. A recent study investigated the anti-inflammatory activity of CHOS prepared by single-step enzymatic hydrolysis by chitosanase, and a two-step chemical-enzymatic hydrolysis against LPS induced RAW264.7 macrophages. It was reported that CHOS prepared by single-step hydrolysis exhibited higher anti-inflammatory activity against LPS-induced RAW264.7 macrophages than the two-step chemical-enzymatic method. The CHOS prepared by two-step chemical-enzymatic hydrolysis promoted the level of MAP kinases such as ERK and p38α. The higher activity of CHOS from single-step preparation might be due to the presence of a higher proportion of fully deacetylated and monoacetylated oligomers (42% and 54%, respectively) than the second one, which consisted of 50% of fully deacetylated and 27% of monoacetylated oligomers. Consequently, the higher proportion of fully deacetylated and monoacetylated oligomers could establish a stable complex with the LPS [90].

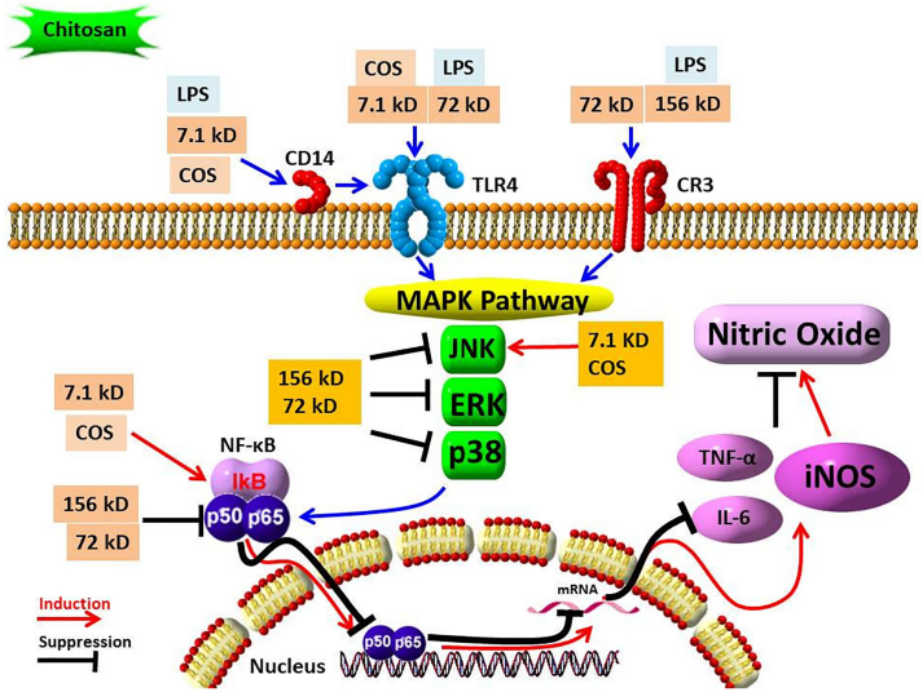


Figure 3. Schematic illustration of NO regulation mechanisms for chitosan with various molecular weights. High molecular weight chitosan (156 kDa and 72 kDa) inhibits the production of TNF-α and IL-6, iNOS expression, and NO production by binding to TLR4 and CR3 receptor in macrophages and as a result down-regulating the phosphorylation of MAPK signaling proteins ERK, JNK, and p38 which leads to inhibition of the LPS-induced NF-κBactivation; however, CHOS increased the production of NO through binding to CD14, TLR4, and CR3 receptors [1]. The figure is reproduced from [1] with permission from Elsevier (License no: 4796561511310)

Table 3. Anti-inflammatory activities of chitooligosaccharides

CHOS (MW/DP, DA/DD)	CHOS production methods/ source	Anti-inflammatory activity evaluation	Observations	Ref.
CHOS-A (MW 10-20 kDa) CHOS-C (MW 1-3 kDa)	Enzymatic degradation of chitosan by chitosanase	Nitric oxide measurement, prostaglandin E_2 production measurement, and immunoblotting	**Anti-inflammatory (LPS-stimulated RAW264.7 macrophage cells)** Both CHOS exhibited inhibition of LPS-induced NO production and suppression of LPS-induced PGE2 production without any severe reaction on the subject skin. .	85
CHOS (DD 95%) (DP 2–6)		cell viability and proliferation (MTT), determination of IL-6 and TNF-α production (ELISA)	**Anti-inflammatory (LPS-stimulated RAW264.7 macrophage cells)** CHOS was effective in the inhibition of the expression of both inflammatory cytokines and phosphorylated levels of p38 MAPK, ERK1/2, and JNK.	81
CHOS (DP 2-5, DD 100%, and MW< 1 kDa)	Enzymatic degradation by chitosanase	Cell viability (MTT), β-Hexosaminidase release assay, analysis of cytokine mRNA production, and Enzyme-linked immunosorbent assay (ELISA)	**Anti-asthmatic and anti-inflammatory (allergic airway inflammation in RBL-2H3 cells) (in vitro, in vivo)** CHOS inhibited β-hexosaminidase release from the RBL-2H3 cells stimulated with IgE–antigen complexes. LM-COS was found to inhibit the asthma-related cytokine expression (IL-4, IL-13, and TNF-α) in IgE–antigen complex-stimulated rat basophilic leukemia RBL-2H3 cells.	80
CHOS (different MW) CHOSI (1–3 kDa) CHOSII (3–5 kDa), CHOSIII (5–10 kDa)	Enzymatic degradation of chitosan by chitosanase in an ultra-filtration (UF) membrane reaction system	Histamine and β-hexosaminidase release assay, and intracellular Ca^{2+} levels and cytokine production measurement	**Anti-allergic anti-inflammatory (against calcium ionophore A23187-stimulated RBL-2H3 cells)** Inhibition in degranulation via attenuating histamine (32%, 48%) and β -hexosaminidase (34%, 42%,) releases (CHOS I, II) Inhibition of the expression of IL-1 β, IL-4, IL-6, and TNF-α induced by A23187 plus PMA[14] (CHOS I) Inhibition of the activation of MEK, ERK, and	88

[14] Phorbol 12-myristate 13-acetate

			p38 kinase but not of JNK. (CHOS I)	
CHOS (DP 2-8)	Commercial CHOS (Koyo Chemical Co. Ltd. (Tokyo, Japan))	Survival study, histopathologic evaluation, nuclear factor-kappa B (NF-κB) detection, immunohistochemical analysis, and measurement of serum levels of cytokines	**Anti-inflammatory activity (in vivo (mice), bowel disease)** Inhabitation of colonic inflammation, suppression pro-inflammatory cytokines, and reduction in tissue injury in the IBD[15] model by CHOS.	[91]
Chitosan (MWs of 156, 72, 7.1, and 3.3 kDa) (DD 81.63–84.4%) CHOS mixture DP of 1–5	Cellulose degradation of chitosan (300 kDa)	Nitrite assay, cytokine measurements, western blotting, and measurement of NF-κB activation	**Anti-inflammatory (LPS-stimulated RAW264.7 macrophage cells)** Larger MW (>29.2 kDa) chitosan exhibited anti-inflammatory activity, while smaller MW (<29.2 kDa) had pro-inflammatory activity	[1]
GC mixture (glucosamine (80%) and chitooligosaccharides (9.80%))	Single-step enzymatic degradation of chitosan (MW 20–30 KDa, DD 85–95%) by recombinant chitosanase (McChoA) and Exo-β-D-glucosaminidase (AorCsxA).	Immune organ indices. Measurement of levels of four cytokines. Measurement of serum immunoglobulin levels (IgG, IgA, IgM) Biochemical assays. Histopathology analysis	**Anti-inflammatory (in vivo) against osteoarthritis (OA)** higher Spleen and Thymus index in GC group than the model group inhabitation of the level of cytokines production (IL-1β, IL-6, TNF-α) in a dose-dependent manner (GC group) promoting serum immunoglobulin levels (IgG, IgA, IgM) .	[92]
P1CHOS (MW8 kDa) (enzymatically by chitosanase) (PA was 0.8) **P2CHOS (MW 5 kDa) (two-step chemical-enzymatic) (PA was 0.9)**	Single-step enzymatic hydrolysis (chitosanase), and two-step chemical-enzymatic hydrolysis of chitosan (89 kDa; DA 17%)	Cell viability (MTT), western blot analysis, and measurement of the level of MAP kinases (ERK, JNK, and p38α)	**Anti-inflammatory (LPS-stimulated RAW264.7 macrophage cells)** Cell viability decreased to 60% by 2 μg/ml P2, while P1 had no effect. P1 was more effective in inflammatory activity than P2 in terms of suppression of the level of ERK and p38α.	[90]

2.4 Immunostimulatory activities

Immunomodulation is known to be an alternative for the prevention and healing of neoplastic disorders [93]. The immune system is accountable for recognizing and obliterating foreign pathogenic substances from the body, and macrophages play a significant role in the host defense mechanism. Macrophage activation prompts a generation of adaptive immunity through an expression of accessory and costimulatory-molecules, and interaction with T cells. Indeed, macrophages are considered a critical factor in immunomodulation with a prominent role in wound healing, angiogenesis, cell proliferation, and transition

[15] Inflammatory bowel disease

out of the inflammatory phase [94, 95]. Many studies reported that chitosan and its derivatives have immunostimulatory activities such as activation of macrophage migration, in vitro or in vivo cytokine secretion, and NO release (Table 4) [96, 97]. It is considered that chitosan and its derivatives seem to activate macrophage secreting cytokines such as interferon-γ (IFN-γ), interleukins [98]; moreover, CHOS in cultured human monocytes could provoke tumor necrosis factor-alpha (TNF-α), which is a pro-inflammatory implicated as an essential mediator of cardiovascular complications [99]. Like other biological features such as antibacterial and antioxidant activity, immunostimulatory activity of CHOS depends on the physicochemical structure of the CHOS, such as MW/DP, and DA. CHOS with lower MW can activate RAW264.7 macrophages by up-regulating mRNA expression [84].

Furthermore, the immunomodulatory effects of low MW chitosan (MW, 21–92 kDa) on Der f-stimulated human monocyte-derived macrophages (MDM) have been notified. Indeed, CHOS brought about a shift in Th2 cytokine polarization and lessened the production of the inflammatory cytokines IL-6 and TNF-α [100].

On the other hand, in non-injured skin, there are few resident macrophages which are essential for the wound healing process. These inflammatory amplification molecules like cytokines (IL-1β, IL-6, IL-12, IL23, and TNF-α) play an indispensable role in the inflammation phase of wound healing by inducing differentiation of intravascular monocytes into macrophages [101]. However, the low rate of transition from a pro- to an anti-inflammatory macrophage phenotype, or excess amount of pro-inflammatory cytokines maintain the inflammatory phase and a chronic wound results [102, 103].

In a mouse model study, the wound healing process was impaired due to the excess expression of NO synthase (iNOS), IL12, and TNF-α, which led to the persistence of macrophages in a pro-inflammatory state [104]. Besides, it has been reported that CHOS with a MW of 3 kDa had higher immunomodulatory through the expression of TNF-α, IFN-γ, and IL-6 from RAW264.7 cells in comparison with the CHOS MW 50 kDa [100]. Furthermore, the stimulatory impacts of CHOS on macrophages have been reported through the fusion of interferon-γ as indicated by the improvement of NO synthesis and enhanced cytotoxicity towards murine fibrosarcoma Meth A cells [96, 105].

A recent study has compared the immunomodulatory activities (RAW 264.7 macrophages) of CHOS prepared from two different chitosans (α and β-chitosan) by a microwave-assisted degradation method. According to the results, α-CHOS exhibited higher activity than β-CHOS in inducing NO production; besides, α-CHOS was more active in promoting cytokines, such as tumor necrosis factor- α (TNF-α) and interleukin 6 (IL-6) [106].

All these discoveries prove that CHOS can be employed to intensify the innate and adaptive immune responses. However, the immunomodulatory activities of CHOS require further investigations such as the exact mechanism of immunomodulation, the impact of the DA and PA on immunomodulation, and identifying the most active molecules in the immunomodulatory activity of CHOS with a single DP.

Table 4. Reported immunostimulatory activities of the chitooligosacchairdes (CHOS).

CHOS (MW/DP, DA/DD)	Production method	Evaluation for immunostimulatory activity	Main results	Ref
CHOS (MW 3.5 kDa)	Commercial CHOS (Kitto Life Co. (Seoul, Republic of Korea))	• Measurement of serum cytokine production • **Subject**: Elderly (age range, 74–86 years) volunteer donors without immune diseases • Oral administration of CHOS (5.1 g/day) for 8 weeks	**Immunostimulation and anti-inflammation** (tested by serum cytokine levels in elderly adults after oral intake) • Increase in IL-12 and IFN- □ levels in the CHOS group but a slight decrease in the levels of IL-1β and TNF-α in the CHOS group. • Oral administrated CHOS caused activation and proliferation of Th 1 response	107
CHOS (DP 4-11)	Enzymatic degradation of chitosan (DD 90%) with chitosanase	Immune organ indices (thymus and spleen), mononuclear phagocytic system function assay by carbon, and biochemical assay	**Immunostimulation in mice against cyclophosphamide (Cy)-induced immunosuppression** • Higher organ indices, strengthen the T cell-mediated immune response, and improvement of macrophage phagocytic activity in CHOS groups than the group treated with Cy[16] alone	108

[16] Cyclophosphamide: it works by slowing or stopping the growth of cancer cells

ZCOS (MW 1.36 kDa) **WCOS (MW 1.46 kDa)** **YCOS (MW 1110 Da)**	CHOS produced from chitosan (DD 82% and MW 658 kDa) by three different methods: ZCOS: Traditional method WCOS: Microwave irradiation YCOS: Enzymatic hydrolysis	Macrophage phagocytosis capacity assay, lymphocyte proliferation assay, natural killer (NK) cells activity assay.	**Immunostimulation (RAW 264.7 macrophages)** • WCOS exhibited the best immunomodulation activity • Unlike in vitro results, degraded chitosan demonstrated higher in vivo immunomodulation activities YCOS, ZCOS (10 μg/ml) and WCOS (1 μg/ml) increased the activity of chitosan on macrophage phagocytosis • YCOS had a significant increase in the proliferation of splenocytes, but ZCOS and WCOS had no effect. • ZCOS was the most efficient for inducing NK-cell activation, and WCOS was the second. • WCOS could stimulate the macrophage activity at a low concentration (1 mg/mL; $P < 0.01$). • The high MW chitosan exhibited the best splenic lymphocyte proliferation activity in vitro. However, the in vivo immunomodulation activities of chitosan was lower than that of degraded chitosan. • Only WCOS increased delayed-type hypersensitivity (stimulatory effect on T lymphocytes and lymphokines)	109
CHOS (DP 3-8)	Enzymatic hydrolysis of chitosan by chitosanase	Determination of mitogenic activity and neutral red phagocytosis of macrophages, measurement level of nitric oxide (NO) and TNF-α in macrophages, and determination of organ weights and serum IgG, IgM contents.	**Immunostimulatory (RAW 264.7 macrophages)** • Significant increase in cell proliferation, neutral red phagocytosis, stimulated NO, and TNF-α[17] secretion through RAW264.7 macrophage cells. • Increase in spleen index and serum IgG[18] contents after oral administration of CHOS to mice	110
LMWCs[19] (3 kDa and 50 kDa)	Commercial LMWC	Pinocytic Activity Assay, measurement of Cytokine levels in RAW264.7 macrophage, measurement of nitric oxide (NO) release and intracellular contents of iNOS, and measurement of the mRNA expression	**Immunostimulation (RAW264.7 macrophages)** • LMWC (3 kDa) enhanced the pinocytic activity of macrophages and secretion levels of NO and *iNOS* more than LMWC (50 kDa) • Both LMWCs induce the TNF-α secretion levels, the mRNA expression levels of TNF-α and iNOS, but LMWC (3 kDa) could induce a significant increase in INF-γ and IL-6. .	84
LMWCs (3 kDa and 50 kDa)	Commercial LMWC	Determination of mRNA expression levels of COX-2, IL-10, IKKβ, key molecules (TRAF6, JNK1) and MCP-1 in RAW264.7 macrophages by RT-PCR[20]	**Immunostimulatory (RAW264.7 macrophages)** • LMWCs (3 kDa) significantly increased the mRNA expression level of IL-10 and levels of IKKβ compared with the LMWCs (50 kDa) at the same dosage, but no difference between two samples in the mRNA expression levels of key molecules. • The primary mechanism of macrophage activation was based on the NF-κB and AP-1 signaling pathways.	111
Chitosan-hydrolysate **LMWC (MW 20 kDa)** **CHOS (DP 1–6)**	Cellulose degradation of chitosan (shrimp)	Superoxide assay, phagocytic activity, cell cycle assay, and DC[21]-associated surface maker assay	**Immunostimulation (RAW264.7 macrophages)** • All three samples markedly inhibited superoxide production. • CHOS and chitosan hydrolysate increased the phagocyte activity to 65.4 ± 11.1 % and 41.1 ± 8.2%, respectively. • CHOS induced expression of dendritic cell surface markers (B7.1, B7.2, and CD40) • CHOS can induce differentiation of RAW264.7 macrophages into dendritic-like cells	112

[17] Tumor necrosis factor alpha (TNF-α)
[18] Serum immunoglobulin G
[19] Low Molecular Weight Chitosan
[20] real-time fluorescent quantitative reverse transcription-polymerase chain reaction
[21] Dendritic Cell

α-CHOS (1874 Da) **β-CHOS (2186 Da)**	Microwave-assisted degradation of two different chitosans (α (1856 kDa) and β-(4574 kDa) chitosan)	NO, and cytokines quantitation, the mRNA expression levels of cytokines, and immunoblotting	**Immunostimulation (RAW264.7 macrophages)** • The NO-promoting activity of α -CHOS was better than that of β-CHOS. • Promotion of the expression of TNF-α, IL-6, COS-2, improvement of $I_{\kappa}B\alpha$ degradation and activation of the translocation of NF-κB	[106]

3 CHOS for skin wound healing: in vitro studies

The proliferation and remodeling phase of wound healing is highly involved in the proliferation and migration of keratinocytes from the wound edge and epithelial stem cells from the basal layer of the epidermis [113]. Moreover, tissue granulation is involved in fibroblasts proliferation and migration and the formation of new blood vessels [114]. The in vitro studies of CHOS with the emphasis on the proliferation and migration of different cell lines presented in the skin has been listed in Table 5. One of the most critical properties of CHOS in wound healing application is its ability to proliferate different cell lines such as fibroblasts, and keratinocyte epidermal stem cells, which exist in different layers of the skin. Moreover, the cell migration ability of CHOS improves the tissue remodelling phase of the wound healing process.

The effect of chitin and chitosan monomers/oligomers on the migration of fibroblast (3T6) and vascular endothelial cells (human umbilical vascular endothelial cells: HUVEC) via a direct migratory assay using the blind well chamber method have been investigated. Migratory activity of HUVECs was increased by chitin monomer (GlcNAc) but reduced by chitosan oligomer (DP 2-6) at a concentration of 10 mg/mL. Both chitin and chitosan had an insignificant effect on the proliferation of HUVECs [32]. In another study, CHOS has been utilized in combination with halloysite as a wound dressing. CHOS incorporation (4 µg/mL) into the halloysite composites, improved cell viability, and proliferation of normal human dermal fibroblasts (NHDF). Moreover, enhanced cell migration is obtained after the incorporation of CHOS, which acts as a wound-healing accelerator. Furthermore, CHOS enhanced wound healing properties of fish collagen/alginate scaffolds by the improvement of normal human dermal fibroblasts-neonatal (NHDF-neo).

Among the CHOS with different MW (1–3 kDa, 3–5 kDa, 5–10 kDa, >10 kDa), CHOS (1-3 kDa) increased the cell viability and proliferation significantly indicating that like other biological activities, decrease in MW can enhance the wound healing potential of CHOS. The positive change in the cell morphology has been attributed to the interaction of positively charged CHOS and negatively charged cell surfaces [31].

Human skin fibroblasts exhibited good cell viability on polyvinyl alcohol (PVA) electrospun nanofibers incorporated with CHOS (3000 Da). Hence, these studies demonstrated that CHOS with a MW of 1-3 kDa could be a promising candidate for the preparation of wound dressing materials due to its effects on skin cell fibroblasts proliferation and migration [115].

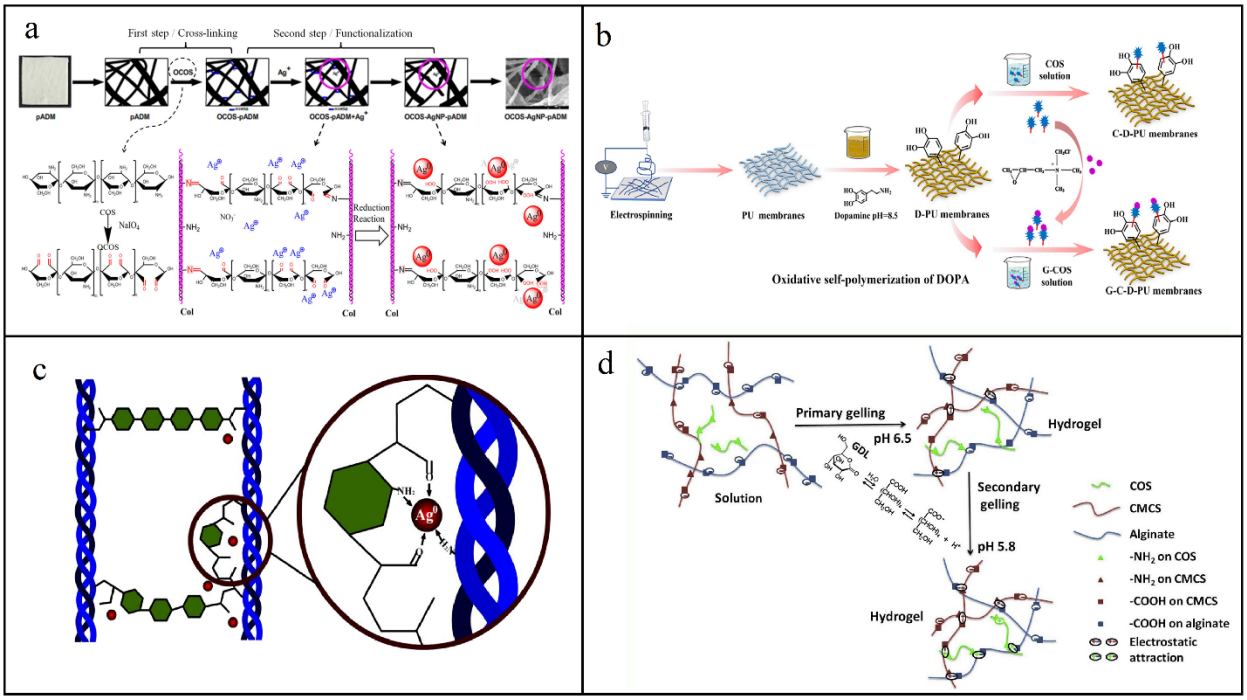


Figure 4. Schematic illustration of CHOS incorporation in wound dressings. a) Schematic outline of the process of in situ synthesis of silver nanoparticles (AgNPs) on oxidized chitooligosaccharide (OCHOS) crosslinked porcine acellular dermal matrix (pADM) scaffold. Oxidized chitooligosaccharide (OCHOS) acted as a crosslinker of pADM by the Schiff base reaction between aldehyde groups in OCHOS and amine groups in pADM, and at the same time stabilized AgNPs without any additional chemicals [2]. Reproduced from [2] with permission (Elsevier, License no: 4796560645121) , b) Schematic diagram of the reaction mechanism of the oxidative self-polymerization of dopamine (DOPA) and subsequent quaternary ammonium chitooligosaccharide (G-CHOS) immobilization on polyurethane (PU) fibrous membrane [4]. Reproduced from [2] with permission (Elsevier, License no: 4813700724893) c) illustration of reaction between OCHOS and AgNPs [2]. Reproduced from [2] with permission (Elsevier, License no: 4796560645121) d) Schematic illustration of the carboxymethyl chitosan (CMCS)/alginate/CHOS gelling system. The addition of protonated CHOS caused a secondary crosslinking due to the increase of the electrostatic interaction with the negatively charged alginate [9]. Reproduced from [9] with permission (Elsevier, License no: 4796551320138)

Moreover, the wound-healing effect of CHOS has been attributed to its angiogenesis ability, which leads to the generation of new tissue. A recent study reported that CHOS with a suitable DP of 5 (Chitopentaose) could modulate osteogenesis/angiogenesis processes (proliferation, migration, and cytokine production) for tissue regeneration without using any inductive agent. Indeed, chitopentaose was effective in the secretion of angiogenic factors such as EGF[22], VEGF-D, basic fibroblast growth factor (bFGF), VEGF-B[23], TGFb1[24] which are the main initiators of the re-epithelialization phase of wound healing [116] [117]. Certainly, one of the essential aspects of chitosan-based biomaterials in tissue regeneration is the activation of macrophages influencing osteogenesis and angiogenesis.

Not only CHOS can act as a bioactive agent, but also it can play a role as a green crosslinker in the development of wound dressing materials without using any additional chemicals. CHOS can act as both physical and chemical crosslinkers due to its high positive charge and functional groups such as amino/acetamido groups, and primary/secondary hydroxyl groups. CHOS can crosslink alginate in the presence of a proton donor. Protonated CHOS with a highly positive charge improves the electrostatic interaction with negatively charged alginate, which leads to the formation of a polyelectrolyte complex (PEC) (Fig. 4 d) [118]. Hence, CHOS can be used as a substitute for ionic crosslinking in alginate; alternatively, it can be combined with ionic crosslinking in alginate.

Moreover, another study investigated the crosslinking role of CHOS in wound healing applications. The addition of CHOS to chitosan/alginate scaffolds prompts a secondary crosslinking through the enhancement of electrostatic interactions with negatively charged alginate. The storage moduli of the hydrogels increased 10^4 times (about 1 MPa) after adding 1% CHOS due to the higher crosslinking density induced by CHOS. The addition of CHOS to the hydrogel from 0 to 0.5 % increased cell proliferation of human umbilical cord mesenchymal stem cells (HUMSCs) from 80% to 160% [9]. However, the higher concentration of CHOS (1

[22] Epidermal growth factor (EGF)
[23] Vascular Endothelial Growth Factor B
[24] Transforming Growth Factor Beta 1

%) reduced the cell viability and proliferation to 12 %, which is probably due to the oxidative stress induction on cells [119]. Moreover, CHOS can act as a chemical crosslinker by forming a Schiff-based bond in wound dressing materials (Fig. 4 a). Indeed, oxidized CHOS with aldehyde groups has the ability to build a 3-dimensional crosslinked network with the free amine groups in a wide range of biopolymers such as chitosan, collagen, and gelatin [66]. Even though the excess of aldehyde groups on CHOS may reduce the cytocompatibility of a wound dressing, the unconsumed aldehyde groups can provide the opportunity to act as reducers in a redox reaction such as in the preparation of AgNPs (Fig. 4 c).

Incorporation of CHOS-Ag NPs into a porcine acellular dermal matrix (pADM) did not have an adverse effect on the cell viability of L929 fibroblasts. Increasing the concentration of Ag NPs from 0.4% to 1.2% decreased the cell viability of L929 fibroblasts, and Human Umbilical Vein Endothelial Cells (HUVECs) from 90.8 ± 3%, to 86.3 ± 3.3%, and 91.6 ± 4.1% to 82.1 ± 5.1%, respectively [2]. It is noteworthy to mention that the use of CHOS as a green method for stabilizing Ag NPs can reduce the risk of toxicity in comparison with the common methods, which mainly impart the addition of chemical reducing agents.

Table 5. In vitro studies on chitooligosaccharides (CHOS) in wound healing applications

CHOS (DP/MW, DD/DA)	Fabrication/ CHOS production	Cells	CHOS role (wound healing)	Ref.
COSs (1–3 kDa, 3–5 kDa, 5–10 kDa, >10 kDa)	Fish collagen/alginate scaffold	Normal human dermal fibroblasts-neonatal (NHDF-neo)	Improve mechanical strength, cell viability, and proliferation. Reduce biodegradation	[31]
quaternary ammonium chitooligosaccharide (G-COS) CHOS (DD = 85%, MW = 2000 Da)	electrospinning polyurethane (PU) fibrous membrane	NIH-3T3 fibroblast	Improve hydrophilicity, antibacterial activity, cytocompatibility, moisture retention, and oxygen permeability	[4]
CHOS (MW > 3 kDa)	PVA/AgNPs electrospun nanofibers	Human skin fibroblasts	PVA/CHOS (0.8 mg/ml) showed the highest cell viability	[115]
Chitooligosaccharide (CHOS) (1000 Da, > 90%)	Chitosan/alginate hydrogel	Human umbilical cord mesenchymal stem cells (HUMSCs)	The hydrogel with 0.1, 0.25 and 0.5% COS exhibited the cell viability of 160%, 140%, 150%, respectively.	[9]
Oxidized chitooligosaccharide (OCHOS)	Porcine acellular dermal matrix (pADM) sca□old and AgNPs	L929 fibroblasts and Human Umbilical Vein Endothelial Cells (HUVECs)	OCHOS has not an adverse effect on cell viability and migration; however, increasing AgNPS dosage decreased cell viability	[2]
CHOS with different polymerization degrees (3, 4, 5, and 6)	Commercial CHOS	Human umbilical vein endothelial cells (HUVECs)	CHOS (DP 5) with the concentration of 4μg/ml is the optimum CHOS for osteogenesis/angiogenesis processes induction	[117]

4 Preclinical studies on skin wound healing

Although several studies demonstrated the active role of CHOS in the wound healing process, few animal reports have been performed to investigate the role of CHOS as a wound-healing accelerator. The animal studies on the wound-healing effect of CHOS have been listed in Table 6. A significant difference has been reported on the wound closure ability of a PVA sponge containing CHOS after eight days in comparison with the sponge without CHOS. The average wound area decreased from 90.8% to 27.7 % by increasing CHOS content from 10 to 50 % in the PVA sponge. The impact of CHOS on wound closure could be due to its homeostatic effect and antibacterial activity at the early stage of wound healing [120].

Moreover, it has been reported that the incorporation of CHOS as a bioactive compound in PVA/AgNPs electrospun nanofibers improved the wound healing process in the early stage of wound healing after 14 days. However, the authors did not clarify if the wound healing acceleration of the nanofiber is due to the presence of CHOS or AgNPS. Nevertheless, it could be found that CHOS with the homeostatic activity improved the wound healing in comparison with the commercial wound dressing. Moreover, the antibacterial activity derived from silver nanoparticles had a positive impact on wound healing [115].

In another study, CHOS has been used for stabilizing silver chloride nanoparticles to prepare wound dressing ointment. The histological analysis of the CHOS/AgCl NPs ointment exhibited little infiltrated inflammation in comparison to the untreated and vaseline ointment. The proliferation of fibroblasts in the granulation tissue was significantly higher in the CHOS/AgCl NPs from day 14. Moreover, collagenase activity in the wound healing process increased for the CHOS/AgCl NPs compared to the untreated group. Furthermore, the wound closure ability was higher in the hydrogel containing CHOS than the hydrogel without CHOS. The authors reported that the wound healing effects of CHOS/AgCl NPs was due to the dual antibacterial activity, which was obtained by the stabilization of AgCl NPs with CHOS [121].

The incorporation of CHOS into carboxymethyl chitosan (CMCS)/alginate hydrogel increased the wound closure on mice models after 11 days. The hydrogel without and with CHOS exhibited 77% and 84% wound closure after 11 days, respectively (Fig. 5 a). The hydrogel with CHOS was more effective in generating new blood vessels through upregulating the expression of vascular endothelial growth factors compared with the control group due to the higher angiogenic activity of CHOS (1000 Da, > 90%) compared to its parents. Moreover, CHOS improved the thickness and integrity of epidermal tissue formation of collagen fibers [9].

Besides, halloysite nanotubes (HNTs) nanocomposites containing CHOS (MW 1000 Da, 75.4% DD) exhibited enhanced wound healing properties. Incorporation of CHOS (4 µg/mL) into HNTs (300 µg/mL) improved the wound closure after 15 days; moreover, nanocomposites containing CHOS exhibited an early re-epithelialization phase after 7 days limited to the lesion borders [122]. (Fig. 5 b).

Furthermore, CHOS with angiogenesis activity can accelerate the wound healing process by stimulating the formation of granulation tissue and matrix and promoting epithelization by up-regulating the expression of growth factors. A cellular porcine dermal matrix (pADM) crosslinked with oxidized chitosan oligosaccharide containing silver nanoparticles (AgNPs) promoted the expression of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) which are the important growth factors indicating the ability of biomaterials to promote healing. The wound closure in the scaffold containing CHOS (OCOS-AgNPs-pADM group) was more significant (90%) than in pADM crosslinked by glutaraldehyde (GA-pADM group) (72%) (Fig. 5 c). Not only CHOS improved the thermal and mechanical stability of the scaffold as a crosslinker, but it also accelerated the wound healing process due to its biological properties such as antibacterial and angiogenesis activity in the different stages of the wound healing [2].

Like other biological activities, the wound healing potential of chitosan and its derivatives depends on the MW. A study investigated the effect of MW on the wound healing potential of chitin and chitosan. It has been reported that chitin and chitosan oligomers were more effective in the wound break strength compared to high MW chitin and chitosan. Hence, chitin and chitosan with low MW can be absorbed quickly in the wound due to their lower viscosity and higher biodegradation, which leads to wound healing acceleration.

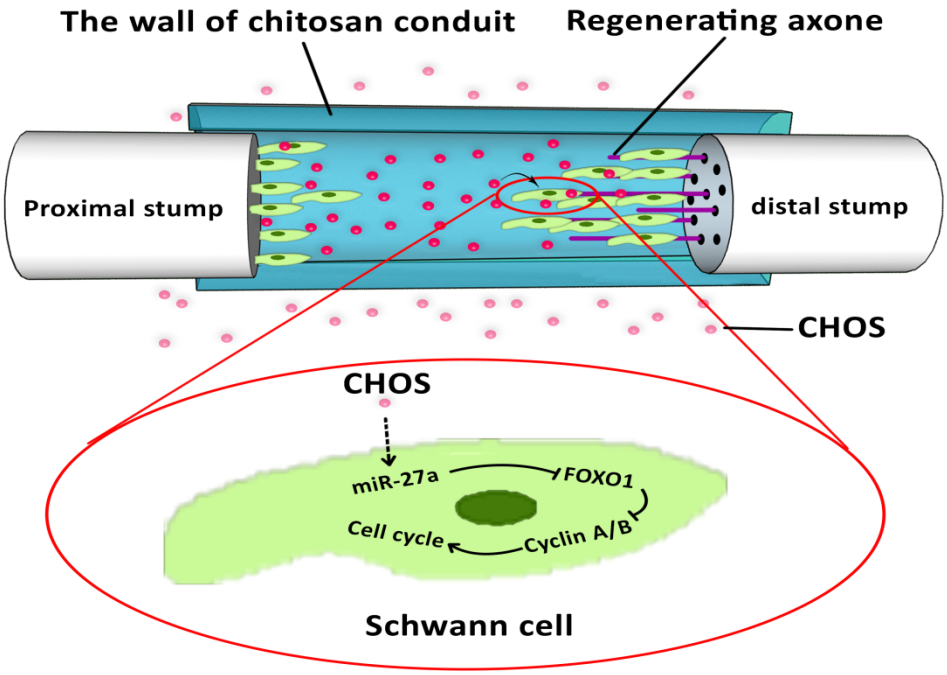


Figure 5. Schematic illustration of the tissue regeneration mechanism of CHOS in peripheral nerve regeneration in rats and Schwann cell (SC) proliferation during nerve regeneration. CHOS induced the SC proliferation by the miR-27a/FOXO1 axis as the primary signaling pathway. CHOS improved the miR-27a expression level, which leads to a reduction of FOXO1 resulting in the acceleration of the cell cycle and stimulation of SC proliferation and nerve regeneration. Redrawn from [3].

Moreover, in terms of the DA, a higher DA is more effective in stronger break strength, more collagenase activity, and activation of fibroblast possibly due to the importance of amine residues in wound healing acceleration (Fig. 5 d) [123]. Moreover, it has been reported that chitin with a DDA of more than 70% is more effective in macrophage activation, which can accelerate the wound healing process by decreasing the inflammation phase of wound healing [124].

Two main mechanisms for wound healing and tissue regeneration activities of CHOS have been reported; namely, the up-regulation of miR-27a and the activation of the transforming growth factor-beta (TGF-ß)-1-Smad2/3 pathway, that are further discussed. Wang et al. reported that CHOS induced the proliferation of Schwann cells (SCs) during nerve regeneration. The underlying tissue regenerative mechanism of CHOS on the Schwann cells involves the up-regulation of miR-27a, which leads to the down-regulating of FOXO1 (Fig. 6) [3]. FOXO1 as the regulator of cyclin A/B is involved in a wide range of critical cellular processes, namely being metabolism, cellular differentiation, apoptosis, and cell cycle progression [3].

Alternatively, Li et al. reported that the wound healing mechanism of CHOS with incorporated PVA/ AgNPs nanofibers is based on the activation of the transforming growth factor-beta (TGF-β)-1-Smad2/3 pathway (Fig. 7). The wound healing acceleration resulted from the significant increase in transforming growth factor-beta (TGF-β1) and its receptors (TGFβRI, TGFβRII), collagen I, collagen III during the early stage of wound healing. TGF-β1 acts as a chemoattractant for monocytes and fibroblasts and stimulates fibronectin (FN) and collagen proteins when acting on fibroblasts in the wound [125]. Indeed, the porous structure of nanofiber and CHOS biological activities such as anti-inflammatory activity brings about the macrophages activation, which leads to the stimulation of growth factors production such as TGFβ1.

In other words, a transition of macrophages from pro-inflammatory (M1) to anti-inflammatory macrophages (M2) is essential in diminishing the inflammation phase and moving to proliferation, which leads to the preparation of the wound for an effective repair. On the other hand, the excessive production of pro-inflammatory molecules (NO synthase (iNOS), IL12, IL6, and TNF-α) decreases the transition rate of macrophages from pro- to anti-inflammatory resulting in a chronic wound. CHOS with high anti-inflammatory activity paves the way for the transition of macrophages (macrophages activation) by inhibiting the production of pro-inflammatory cytokines. In the proliferation phase, CHOS effectively promotes the restoration of the vascular network known as angiogenesis by inducing VEGF from pro-repair macrophages [2, 104, 126]. CHOS increases the production of TGF-β that is significantly involved in collagen synthesis and fibroblast proliferation as the primary cell tangled in granulation tissue formation. This event

is followed by the proliferation and migration of fibroblasts and keratinocytes, collagen synthesis, and angiogenesis results in the acceleration of re-epithelization and therefore enhances the wound healing process. [127]

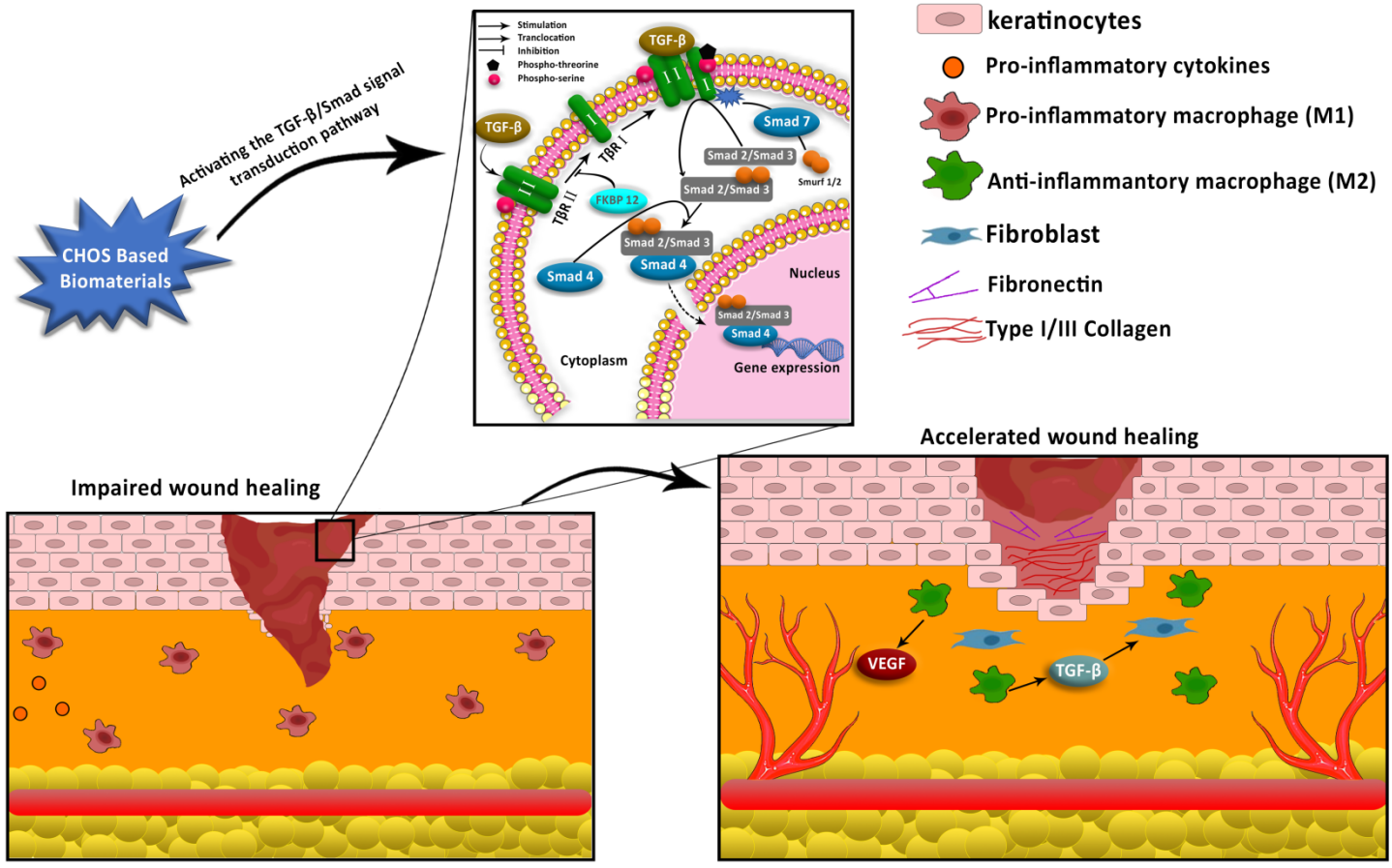


Figure 6. Schematic illustration of the wound healing mechanism of CHOS-based biomaterials. During the inflammation phase of wound healing, excessive presence of pro-inflammatory macrophages (M1) induced by danger signals and pro-inflammatory cytokines (IL12, IL6, and TNF-α) leads to the chronic wound and non-repaired wound. CHOS with biological activities can influence the expression level of transforming growth factor-beta (TGF-β) by activating the TGF-β/Smad signal transduction pathway. Increasing the TGF-β result from the activation of macrophages by CHOS; in other words, transformation from pro-inflammatory macrophages (M1) to anti-inflammatory macrophages (M2) results in the stimulation of TGF-β and subsequent fibroblast and keratinocyte proliferation and migration. Besides, CHOS increases the production of vascular endothelial growth factor (VEGF) involved in the new blood vessel formation, referred to as angiogenesis.

Table 6. Preclinical studies on CHOS on wound healing applications

CHOS (MW/DP, DA/DD)	Fabrication	Animal	Main result	Ref.
DP2-9, 87% DD	PVA sponge	Rat	The average wound area decreased from 90.8% to 27.7 % by increasing CHOS content from 0 to 50 % in the PVA sponge.	120
Chitin oligomer (NACHOS) DP 1-5 Chitosan oligomer (CHOS) DP 1-6	Pure chitin and chitosan oligomer	Wistar female rats	More wound healing acceleration in chitin and chitosan oligomers compared to chitin and chitosan	123
CHOS (MW > 3 kDa),100% water soluble	PVA/AgNPs electrospun nanofibers	New Zealand White rabbits (weighing 2–2.5 kg)	PVA/CHOS/AgNPs sponge exhibited a significant difference in wound closure after 14 days compared to commercial dressing.	115
CHOS (DD = 87%)	CHOS/AgCl NPs ointment	Sprague-Dawley rats (4 weeks)	Stabilization AgCl NPS by CHOS increases the antibacterial activity, which led to an increase in the tissue granulation, and collagenase activity during the wound healing process.	121
CHOS (DP ∼7, MW 900 Da)	Chemical hydrolysis of chitosan (DD 92.3 %, MW 250 kDa) via acetic acid	Male Sprague Dawley rats	CHOS induces the proliferation of Schwann cells (SCs) during nerve regeneration.	3
CHOS (MW > 3 kDa), 100% water soluble	PVA/CHOS-AgNPs nanofiber	Sprague–Dawley rats	PVA/CHOS-AgNPs improved wound healing through activation of the TGFβ1/Smad signaling pathway.	3
chitooligosaccharide (CHOS) (1000 Da, > 90%)	carboxymethyl chitosan/alginate hydrogel	Adult male SD mice	Hydrogel without and with CHOS exhibited 77%, and 84% wound closure after 11 days, respectively.	9
Oxidized chitooligosaccharide (OCHOS)	Porcine acellular dermal matrix (pADM) sca□old	Sprague-Dawley rats	CHOS, as a green crosslinker, improved the thermal, mechanical, and wound healing properties of the scaffold.	2

5 Conclusion and perspectives

The wound healing potential of CHOS has been thoroughly discussed. CHOS can accelerate the wound healing process by its enhanced biological features such as cytocompatibility, antibacterial, antioxidant, anti-inflammatory, and immunostimulatory activities. The remarkable antibacterial activity of CHOS is due to its lower molecular weight and solubility compared to chitin and chitosan, as well as its positive charge, which can form polyelectrolyte complexes with negatively charged bacteria. Besides, the role of CHOS as a green crosslinker for stabilizing nanoparticles and for hydrogel formation has been reported. CHOS can resolve the inflammation phase of wound healing through down-regulating of pro-inflammatory molecules such as IL-1, IL-6, and TNF-α. Moreover, CHOS can accelerate the proliferation and remodeling phase of wound healing by improving the proliferation and migration of fibroblasts contemporaneously with angiogenesis and new blood formation. Two possible mechanisms of CHOS in wound healing and tissue regeneration acceleration are the up-regulation of miR-27a and, alternatively, the activation of the transforming growth factor-beta (TGF-β)-1-Smad2/3 pathway. Although mechanisms for tissue regeneration and wound healing-acceleration effects of CHOS have been reported, the potential of CHOS in the context of developing new biomaterials for wound healing acceleration has not been exploited. Moreover, to our knowledge, the impact of the physiochemical properties (MW, DA, DP, PA) of CHOS in the context of biomaterials for wound healing potential has not been reported. Further investigation is required to fully discover the cellular and molecular mechanism of CHOS in wound healing applications; besides, the development of well-defined CHOS with the enhanced therapeutic potential in wound healing applications can be an interesting topic. Furthermore, different types of modifications of CHOS, such as sulfation, oxidation, conjugating by polyphenol, etc. can be further investigated to obtained enhanced properties in wound healing applications.

Conflicts of interest

There are no conflicts to declare.

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