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Chitosan-based nanosystems and their exploited antimicrobial activity

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Abstract

Chitosan is a biodegradable and biocompatible natural polysaccharide that has a wide range of applications in the field of pharmaceutics, biomedical, chemical, cosmetics, textile and food industry. One of the interesting characteristics of chitosan is its antibacterial and antifungal activity, and together with its excellent safety profile in human, it has attracted considerable attention in various research disciplines. The antimicrobial activity of chitosan is dependent on a number of factors, including its molecular weight, degree of deacetylation, degree of substitution, physical form, as well as the structural properties of the cell walls of the target microorganisms. While the sole use of chitosan may not be sufficient to produce an adequate antimicrobial effect to fulfil different purposes, the incorporation of this biopolymer with other active substances such as drugs, metals and natural compounds in nanosystems is a commonly employed strategy to enhance its antimicrobial potential. In this review, we aim to provide an overview on the different approaches that exploit the antimicrobial activity of chitosan-based nanosystems and their applications, and highlights the latest advances in this field.

Keywords: polysaccharide; polycation; nanoparticles; nanocomposites; wound healing; food packaging.

1. Introduction

Chitosans are a family of polysaccharides characterized by a randomly distributed β-(1→4)-linked D-glucosamine and *N*-acetyl-D-glucosamine monomers, with a wide range of molecular weights (MW), degree (DA) and pattern (PA) of *N*-acetylation. Although the most common source of chitosan is the exoskeleton of marine organisms (e.g. crustacean shells and squids) (Younes and Rinaudo, 2015), chitosans produced by fungi or insects become available on the market in recent years (Ghormade et al., 2017). Due to its favourable properties such as low toxicity, high biodegradability and biocompatibility, the technological applications of chitosan span the food, cosmetic, biomedical and agricultural fields (Dash et al., 2011) (Bellich et al., 2016) (Aider, 2010). Moreover, chitosan has achieved the Generally Recognized as Safe (GRAS) status from the Food and Drug Administration (FDA) and its chloride salt form has been described in a monograph of the European Pharmacopeia (EP), making it an attractive biopolymer for pharmaceutical applications (*European Pharmacopoeia 6.0, 1774 Chitosan hydrochlode monograph, Council of Europe*, 2008). Thanks to its large versatility, many companies worldwide have started to commercialize chitosan-based products (Khor, 2014).

As widely recognized, the unicity of this class of polysaccharides mainly depends on the presence of the amino groups on their backbone, configuring chitosan as a natural polycation in acidic pH (Ruel-Gariépy and Leroux, 2006). The naturally occurring positive charge has ascribed chitosan to peculiar characteristics such as mucoadhesiveness, permeability enhancing effect and antimicrobial activity, which have further extended its possible utilization in different fields (Vllasaliu et al., 2012)(Vllasaliu et al., 2010)(Sogias et al., 2008). Spanning from biomedical to cosmetic and from food to agriculture applications, one of the most investigated properties of chitosan is its antimicrobial effect. Several studies have been conducted with the intent to exploit the antimicrobial activity of chitosan together with its peculiar features in order to produce self-preserving materials with improved characteristics. This has led to the design of a large range of products containing chitosan as beads, films, fibers, membranes, hydrogels that are intended for various uses. Although the first studies regarding the antimicrobial activity of chitosan have been published almost three decades ago (Allan and Hadwiger, 1979) (Kendra and Hadwiger, 1984), the scientific interest on this property it has never been declined, as attested by the thousands of publications produced only in the last few years. Besides, the interest on the antibacterial properties of chitosan has been riding the crest of the nanotechnology, leading to the formulation of several mixed nanosystems (e.g nanoparticles, nanocomposites) in which chitosan is formulated with metals or acts as carriers for natural or synthetic compounds with an intrinsic antibacterial activity. Particularly, special attention has been devoted to enhance the antimicrobial

effect of these nanoparticulate formulations. Despite the considerable body of literature produced, it lacks a critical review, which would be auspicable to provide insights for the future directions in this field.

Based on the current scenario, this review is intended to summarize and discuss the recent studies which evaluated the antimicrobial activity of nanoparticulate systems (e.g. nanoparticles, nanofibers or nanocomposite) containing chitosan alone or, in the most of the cases, in association with other materials (e.g. metals, natural compounds, small molecules). In the second part of the review, a general overview of the potential applications of these systems (e.g. wound healing, textiles and food packaging) is provided.

2. Physico-chemical characteristics of chitosans influencing their antimicrobial activity

Several properties of chitosan are recognised to influence its antimicrobial activity and they have been already comprehensively reviewed (Kong et al., 2010) (Goy et al., 2009). Among the different studies, several mechanisms have been proposed to explain this antimicrobial effect. It is generally accepted that the main antimicrobial effect of chitosan is related to the ability of its positively charged amine groups to bound onto the negatively charge surface of bacterial wall or plasma membrane. This causes a modification of the cell permeability, leading to an osmotic damage with the efflux of ions and proteins from the cytoplasm to the extracellular space (Liu et al., 2004) (Figure 1). Therefore, all intrinsic factors of chitosan influencing its positive charge density are of critical importance. In fact, higher the positive charge density of chitosan, stronger the electrostatic interactions with the bacterial cell surface. Intrinsic factors related to the chemical structure of chitosan that affect the positive charge density include the degree of deacetylation (DD) and degree of substitution (DS) on the amino groups. All studies in the literature generally agree that chitosans with higher DD shows more pronounced antimicrobial activity (Kong et al., 2008) (Takahashi et al., 2008) (Mellegård et al., 2011) due to the higher number of free amino groups on the polymer backbone. Despite this, some controversial data were reported in the literature because both structural characteristics of chitosan (e.g. molecular weight) and other extrinsic factors (e.g. pH or solvent used for the preparation of chitosan solutions) were not, in all cases, accurately controlled.

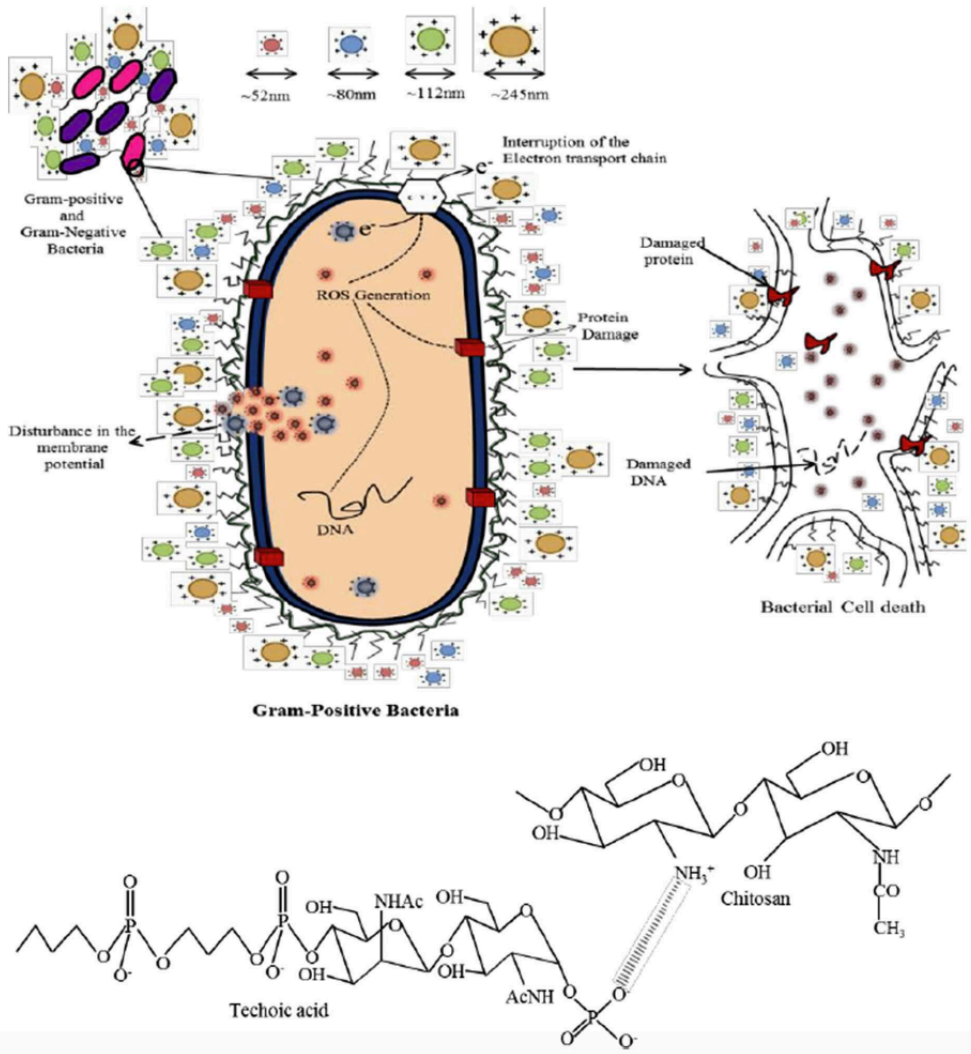

Fig. 1. Proposed mechanism of the antibacterial effect of chitosan nanoparticles on *S. aureus* according to the work of Tyagi et al. Reprinted from (Tyagi et al., 2014).

Recently, Younes et al. published a study aimed to investigate the antibacterial and antifungal effect of 15 different chitosans with well-defined characteristics in term of DD and MW, and they were prepared under homogeneous conditions (Younes et al., 2014). The antimicrobial effect was evaluated by the agar diffusion method, the viable cell counting and the minimum inhibition concentration (MIC), performed at different pH values. It was found that Gram-negative bacteria have a higher susceptibility to chitosan with the respect to Gram-positive bacteria. In particular, the antibacterial activity of chitosan increases with decreasing of the degree of acetylation (DA), MW and pH. These factors showed a more pronounced effect on Gram-negative than Gram-positive bacteria (Campana et al., 2017). Moreover, for some Gram-positive bacteria, an improved activity was obtained for higher MW chitosans. The antifungal activity was also depended on DA and MW, and the effect was mainly correlated to the species of the fungus studied. This study gives a structured overview about the experimental factors influencing the antimicrobial effect of chitosans, by examining the relationship between the characteristics of the polymer (i.e. DA and MW) and the nature of the microorganisms being investigated. It also verified the results previously obtained by

Zeng and Zhu regarding the opposite effect of MW on Gram-positive and Gram-negative bacteria (Zheng and Zhu, 2003).

The DS, the other structural factor affecting the positive charge density, is directly linked to the hydrophilic/hydrophobic character of chitosan. In fact, *N*-modification on chitosan through alkylation, acylation, saccharization, quaternarization, and metalization is a common strategy to manipulate the hydrophilicity / hydrophobicity of the polymer, depending of the nature of the derivatization itself. Generally, by preparing ammonium quaternary derivatives, the hydrophilicity increases to obtain water-soluble chitosans. According to the literature, quaternarized chitosans display higher antibacterial activity and a broad spectrum of action against bacteria and fungi at neutral pH (Tan et al., 2013) (Martins et al., 2014). This can be explained by the introduction of a permanent positive charge in the backbone of the polymers, enable the polymers to strongly interact with bacterial cell membrane, independently of the pH.

Quaternarization with short alkyl chains showed the best antimicrobial activity (Sajomsang et al., 2010). Recently, Sahariah et al. investigated the antimicrobial activity of a series of quaternarized water-soluble cationic chitosan derivatives prepared by chemoselective functionalization at the amino group of five different chitosans varying in DD (from 66% to 94%) and MW (from 180 to 308 kDa) (Sahariah et al., 2014). All the derivatives displayed various degrees of acetylation and MW, and were *N*-modified by introducing alkyl quaternary ammoniumyl or pyridiniumyl group distanced from the polymer backbone by different alkyl spacer lengths. Higher MIC values for *Staphylococcus aureus* as compared to *Escherichia coli* was found, suggesting the greater inhibitory effect on Gram-positive bacteria. Moreover, trimethylammonium derivatives showed a higher antibacterial activity than the pyridinium derivatives, with a decreasing effect by increasing the length of the alkyl spacer from C-2 to C-6. This study confirmed that in addition to the positive charge density, an optimal hydrophilic/hydrophobic ratio is crucial in determining the antimicrobial activity of ammonium derivatives of chitosan.

3. Factors affecting the antimicrobial activity of empty chitosan nanoparticles

Since the first work of Qi et al. on antibacterial activity of chitosan nanoparticles in 2004 (Qi et al., 2004), there was a flourishing number of papers reporting the antibacterial effect of chitosan nanoparticles (Anitha et al., 2009) (Xing et al., 2009) (Chávez de Paz et al., 2011) or the enhanced effect exerted by chitosan nanoparticles when they were loaded with metals, antimicrobial drugs or other active compounds (Wei et al., 2009) (Sanpui et al., 2008) (Keawchaoon and Yoksan, 2011).

According to the literature, chitosan nanoparticles have a greater antibacterial effect than chitosan solutions, despite the exact mechanism of action has not been completely elucidated. It has been proposed that chitosan nanoparticles have similar antimicrobial mechanisms to those reported for chitosan solutions. Consequently, the increased antimicrobial activity has been ascribed to the higher density of the positively charged amino groups when chitosan is packed into nanoparticles. The higher positive charge density of chitosan nanoparticles promotes a stronger binding to the negatively charged surface of bacteria (Chung et al., 2004). This may explain the higher activity reported for chitosan nanoparticles against Gram-negative (*Escherichia coli)* than Gram-positive bacteria (*Staphilococcus aureus*) (Qi et al., 2004) (Katas et al., 2011), although some authors reported otherwise, indicating a more pronounced effect on Gram-positive bacteria (Sadeghi et al., 2008) (Sarwar et al., 2014).

Moreover, the enhanced activity of nanoparticles can be also related to the increase of surface area to volume ratio and the associated quantum size effect. Generally, a higher activity is associated with the smaller particles size, which is dependent on the concentration and the MW of the chitosan used (O'Callaghan and Kerry, 2016) (Sarwar et al., 2014) (Mohammadi et al., 2016b). In fact, smaller the particles size, larger the area of the particles in contact with the bacterial surface. Sarwar et al. provided evidence of the ability of chitosan nanoparticles to destabilize and disrupt the bacterial membrane, confirming their bactericidal effect. In this work, not only the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined for *Escherichia coli* and *Staphilococcus aureus*, the cell membrane integrity and outer/inner membrane permeability were also evaluated. The study demonstrated that the inner membrane permeability of *Escherichia. coli* treated with chitosan nanoparticles was compromised, causing a cytoplasmic β-galactosidase release into the medium (Sarwar et al., 2014).

Recently, the interest in antimicrobial effect of chitosan nanoparticles was extended to bacteria not generally employed for routine screening, but selected according to the specific application of nanoparticles that are intended for. Mohammadi et al. investigated the use of chitosan or chitosan nanoparticles as natural additive for the preservation of vegetables from plant pathogenic bacteria (Mohammadi et al., 2015a) (Mohammadi et al., 2016b) (Mohammadi et al., 2016a) (Mohammadi et al., 2015b). In a recent paper, they evaluated the antibacterial activities of low and medium MW chitosan through MIC and MBC assays. These tests were performed against *Pseudomonas fluorescens* which was responsible for causing head rot in broccoli, *Erwinia carotovora* which was responsible of fungal infections in different vegetables, and the common Gram-negative *Escherichia coli.* Recently, low and medium MW chitosan nanoparticles were also examined for their

antimicrobial effect against cheese-derived cultures, such as *Bacillus cereus* and *Pseudomonas fluorescens* in addition to *Escherichia coli* and *Pseudomonas aeruginosa* as model for Gram-positive and Gram-negative bacteria (O'Callaghan and Kerry, 2016).

Many papers have also shown the antifungal activity of chitosan and chitosan nanoparticles against different pathogenic species for humans such as *Candida, Aspergillus* and *Fusarium,* demonstrating their disruption effect on fungal lipid membrane (Seyfarth et al., 2008) (Pena et al., 2013) (Park et al., 2008) (Ing et al., 2012). More recently, chitosan nanoparticles have been proposed for controlling the growth of pathogenic fungi in agriculture (Saharan et al., 2015)(Saharan et al., 2013). A review was also published in 2015 dealing with the applications of chitosan nanoparticles in terms of sustainable agricultures, highlighting their role in crop protection against phytopathogenic fungi. The antifungal activity of oleoyl-chitosan nanoparticles was investigated against different pathogenic fungi for plants. It was found that the different susceptibility is related to the composition of fungal membrane in terms of fatty acids. In fact, chitosan sensitive-fungi have lower levels of non-saturated fatty acid than those chitosan-resistant (Xing et al., 2016).

4. Antimicrobial activity of chitosan nanoparticles loaded with antibiotics or other microbiocidal substances

Chitosan was employed as nanocarrier for the delivery of both synthetic and natural substances in order to potentiate or modulate their antimicrobial activity. These substances include antibiotics, antimicrobial peptides (AMP), natural compounds and proteins (Figure 2). Different classes of antibiotics were encapsulated in chitosan nanoparticles such as tetracycline (Maya et al., 2012), penicillins (Anal et al., 2006) (Ngan et al., 2014) (Ngyuen et al., 2017), cephalosporins (Zaki and Hafez, 2012) (Chifiriuc et al., 2012) (Jamil et al., 2016) (Brito et al., 2011), aminoglycosides (Lu et al., 2009), vancomycin (Cevher et al., 2006) (Chakraborty et al., 2012) (Esmaeili and Ghobadianpour, 2016) with the aim to increase the effectiveness of the drug against bacterial growth. In particular, chitosan nanoparticles were employed to improve the internalization of the antibiotics into cells infected by intracellular bacteria or to increase their efficacy against multi-resistant microorganism. Zaki et al. reported the enhanced antibacterial activity against *Salmonella typhimurium* of ceftriaxone sodium encapsulated in chitosan nanoparticles. They demonstrated the cellular uptake of drug-loaded chitosan nanoparticles in Caco-2 and J774.2 (macrophages) cells and the intracellular antibacterial effect of the drug-loaded nanoparticle was higher than the drug in solution (Zaki and Hafez, 2012).

A similar study was also performed using tetracycline-loaded O-carboxymethyl chitosan nanoparticles. In this case, the drug-loaded chitosan nanoparticles were found to enhance the efficacy of the antibiotics against intracellular infections caused by *Staphylococcus aureus* (Maya et al., 2012). Jamil et al. evaluated the efficacy of cefazolin-loaded chitosan nanoparticles against multi-resistant Gram-negative bacteria such as *E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The drug-loaded chitosan nanoparticles showed antibacterial activity against the three microorganisms, as determined by agar well diffusion method and microdilution broth assay, superior to cefazolin in solution which was inactive (Jamil et al., 2016). The efficacy of drug-loaded chitosan nanoparticles against antibiotic-resistant bacterial strains was also demonstrated for vancomycin against drug-resistant *Staphylococcus aureus* (Chakraborty et al., 2012).

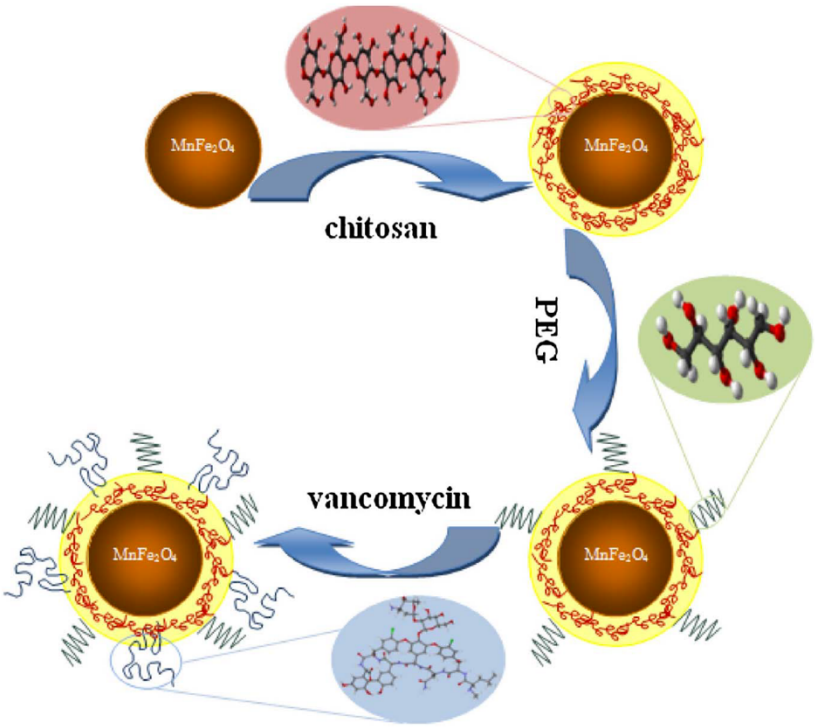

Fig. 2. Architecture of mixed chitosan/metals/antibiotic nanoparticles effective against Gram-negative bacteria. Reprinted from (Esmaeili and Ghobadianpour, 2016).

Recently, peptides and proteins with antimicrobial activity were encapsulated in chitosan nanoparticles. Among these molecules, lysozyme has received attention for its application as preservative in food products and pharmaceuticals (Wu et al., 2017), while the amphiphilic peptide temporin B has shown a strong and fast killing ability, especially against Gram-positive, multi-drug-resistant nosocomial bacterial species (Mangoni et al., 2008). The temporin B loaded chitosan nanoparticles showed a prolonged and strong bactericidal activity *in vitro* (up to 4 log) against clinically-relevant isolate of *Staphylococcus epidermidis* (Piras et al., 2015). The same research group also characterized the chemical-physical and antimicrobial properties of lysozyme-loaded chitosan nanoparticles against *Staphylococcus epidermidis.* Similarly, a prolonged antibacterial

activity of lysozyme was observed up to five days as a consequence of the protein release slowly from nanoparticles (Piras et al., 2014). Daptomycin, a natural lipopeptide active against most Gram-positive, was encapsulated in chitosan nanoparticles. This nanoparticulate system, due to the mucoadhesive properties of chitosan, was proposed for the topical ocular administration of daptomycin for the treatment of bacterial endophthalmitis (Silva et al., 2015).

The co-adjuvant or additive effect of chitosan as antimicrobial agent was explored in association with a large variety of synthetic or natural compounds (Martínez-Hernández et al., 2017) (Hu et al., 2017). For instance, the therapeutic effect of the tretinoin on skin diseases including bacterial infections as acne, can be potentially ameliorated when it is encapsulated in chitosan-solid lipid nanoparticles. This can be attributed to the antibacterial effect of chitosan in addition to the comedolytic activity of tretinoin (Ridolfi et al., 2012). In another study, curcumin-chitosan nanoparticles showed an *in vitro* growth inhibition of methicillin-resistant *Staphilococcus aureus* and *Pseudomonas aeruginosa* and an improved wound repair in a murine model (Krausz et al., 2015). On the contrary, another work showed that when chitosan was used for the encapsulatation of coriander oil, the antimicrobial activity of the microcapsules was lower than that of pure chitosan. The authors explained that there was no activity observed for coriander oil alone, the presence of the essential oil onto the surface of loaded chitosan microcapsules could override the intrinsic antimicrobial effect of chitosan (Duman and Kaya, 2016). An example of the antimicrobial effect of an essential oil (thyme essential oil) loaded chitosan nanoparticles was reported by Sotelo-Boyas et al. The authors have clearly displayed the antimicrobial activity of the oil-loaded nanoparticles against six foodborne bacteria without testing chitosan nanoparticles and the essential oil alone as a control (Sotelo-Boyás et al., 2017).

5 Antimicrobial activity of chitosan/metals nanocomposites

The combined antimicrobial effect of chitosan and metals was explored to prepare novel nanocomposite materials with improved microbicidal properties (Pelgrift and Friedman, 2013) (Palza, 2015) (Figure 3). In particular, a broad spectrum of activities against both Gram-positive and Gram-negative bacteria were demonstrated for gold-, silver- or copper-loaded chitosan nanoparticles. Such nanoparticles were prepared by adding metal ion solutions into chitosan nanosuspension or by reducing a soluble salt of the metal in the presence of chitosan solutions (Rhim et al., 2006). Their antibacterial activities were evaluated in nanoparticulate colloidal dispersion or in the form of a thin film, in which the metal nanoparticles remained embedded inside the chitosan polymeric matrix. In

all cases, a higher antimicrobial effect was observed against Gram-negative bacteria, which can be attributed to the more negatively charged surface and the thinner cell wall.

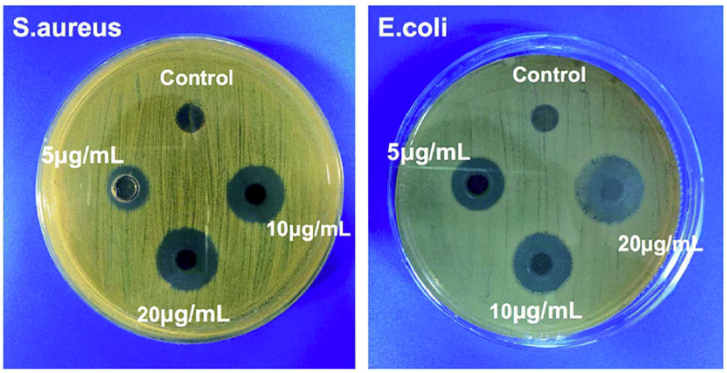

Fig. 3. Inhibition bacterial growth zone (according to the disk diffusion test) produced by different concentrations of chitosan/silver mixed nanoparticles against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria. Chitosan solution was used as control. Reprinted from (Huang et al., 2016).

Silver-based nanocomposites are the most frequently investigated metal-chitosan complexes (Sanpui et al., 2008) (Rhim et al., 2006) (Hernández-Sierra et al., 2008). Du et.al., demonstrated that Ag^{+}/chitosan complex nanoparticles exhibited higher antimicrobial activity against Gram-positive and Gram-negative bacteria than chitosan solution, chitosan nanoparticles and chitosan nanoparticles containing other metals including Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+} (Du et al., 2009). The antibacterial effect of Ag^{+}/chitosan composite materials was found to increase with the amount of silver (Akmaz et al., 2013). In order to investigate if the greater antibacterial effect of the silver-based chitosan composite material was exerted by the presence of silver as metallic nanoparticles or ions, Kumar-Krishnan et al. prepared chitosan films containing Ag nanoparticles or Ag^{+} ions at different concentrations and tested for their activity against *Staphilococcus. aureus* and *Escherichia coli* (Kumar-Krishnan et al., 2015). They found that the maximum bactericidal effect of the chitosan films was obtained with those containing 1% w/w of silver nanoparticles or 2% w/w of silver ions, concluding that Ag/chitosan nanoparticles have higher antibacterial effect than Ag^{+} ions. The maximum antibacterial effect was observed at a concentration close of that of the electrical percolation threshold. It was also proposed that nanoparticles size could affect the antimicrobial effect. Smaller silver nanoparticles have a higher specific surface area and release Ag^{+} ions at a faster rate (Sotiriou and Pratsinis, 2010). Apart from silver, the antimicrobial effect of copper and gold loaded or absorbed chitosan nanoparticles was also reported (Manikandan and Sathiyabama, 2015) (Mallick et al., 2012) (Regiel-Futyra et al., 2015) (Gu et al., 2007), despite, especially for copper nanoparticles, concerns about their toxicity was raised (Arora et al., 2016) (Qi et al., 2005). Non-cytotoxic nanocomposite films containing gold and chitosan were prepared by the solvent evaporation method. Among these formulations, films composed of chitosan with average-MW and high degree of DD (MW 1278 Da; DD 89%) embedding gold

nanoparticles, showed the highest antibacterial activity against resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains. Moreover, the nanocomposites were not cytotoxic on human cell lines, HaCaT cells (immortal human keratinocyte) and A549 lines (human lung adenocarcinoma epithelial cells) (Regiel-Futyra et al., 2015).

6 Antimicrobial activity of chitosan nanoparticles on bacterial biofilm

Biofilms are microbial communities embedded in a matrix of slimy extra-cellular polymers. Microorganisms in biofilms are significantly more resistant to antimicrobial agents. Natural biological molecules are currently being evaluated for their anti-biofilm activity in order to develop alternative preventive or therapeutic rationale. In this context, chitosan-streptomycin conjugates/gold nanoparticles were evaluated in terms of their anti-biofilm properties against Gram-negative *Pseudomonas aeruginosa* and *Streptococcus typhimurium*, and Gram-positive *Listeria monocytogenes* and *Staphilococcus aureus*. In particular, the nanoparticles showed a disruption effect on biofilms formed by Gram-negative or Gram-positive bacteria, and an inhibition effect on the formation of biofilm of Gram-negative bacteria. The conjugation of streptomycin to chitosan and gold nanoparticles facilitated its penetration into the biofilm matrix and improved the contact with the bacterial surface, thereby enhancing its bactericidal effect (Mu et al., 2016). Another application of chitosan nanoparticles is represented by photodynamic activation. Darabpour et al. investigated the effect of chitosan nanoparticles on the efficiency of methylene blue (MB)-mediated antimicrobial photodynamic inactivation (APDI) of *Staphilococcus. aureus* and *Pseudomonas aeruginosa* biofilms. The authors observed that chitosan nanoparticles enhanced the efficacy of MB-APDI, causing the disruption of biofilm structure and subsequently a deeper and effective penetration of MB into *Staphilococcus. aureus* and *Pseudomonas aeruginosa* biofilms (Darabpour et al., 2016).

The use of chitosan nanoparticles has also been reported to combat biofilm formed by oral pathogens on tooth surfaces, which are associated with human caries, gingivitis and periodontitis (Costa et al., 2014). Chavez de Paz et al. investigated the antimicrobial activity of chitosan nanoparticles of different DA and MW on *Streptococcus mutans* biofilm. The low MW formulations disturbed the cell membrane integrity of *Streptococcus mutans* in a homogenous manner across the entire biofilm and the chitosan particles directly interacted with bacterial cell (Chávez de Paz et al., 2011). In the study reported by Aliasghari et al., the antimicrobial activity of chitosan and chitosan nanoparticles on four different strains of cariogenic streptococci (*S. mutans*, *S. sobrinus*, *S. sanguis* and *S. salivarius)* was evaluated. The results showed that these substances have bacteriostatic or bactericidal and anti-

adhesion effects, and they can reduce biofilm/plaque formation *in vitro*, indicating the high potential of chitosan and chitosan nanoparticles as anticariogenic agents (Aliasghari et al., 2016). Moreover, the activity of chitosan nanoparticles was also assessed on *Candida albicans* biofilm. Panwar et al. observed the inhibition of *Candida albicans* biofilm and the disruption of cellular morphology in the presence of ferulic acid encapsulated chitosan nanoparticles. It was suggested that the positive surface charge of the nanoparticles played a crucial role in facilitating their interaction with the negatively charged plasma membrane of fungal, thereby inhibiting *Candida albicans* biofilm formation or destroy its structural integrity (Panwar et al., 2016). These data were in line with the study performed by Ing et al., who reported that chitosan nanoparticles at concentration of 1 mg/ml were established as an effective antifungal agent against *Candida albicans* (Ing et al., 2012).

A summary of the most relevant *in vitro* experimental studies reporting the antibacterial activity of chitosan dispersion or unloaded and loaded chitosan nanoparticles can be found in table 1.

Table 1
Summary of the *in vitro* studies evaluating the antimicrobial activity of chitosan-based nanosystems.

	Antimicrobial assay	Microorganisms	References
Chitosans dispersions	Agar diffusion method	*E. coli, P. aeruginosa, K. pneumoniae, S. thypi*	(Younes et al., 2014)
	Viable count estimation	*S. aureus, Micrococcus luteus, Bacillus cereus, E. faecalis*	(Younes et al., 2014)
	Minimum Inhibitory Concentration (MIC)	*Aspergillus niger, Fusarium oxysporum, Alternaria solani*	(Younes et al., 2014)
	Minimum Inhibitory Concentration (MIC)	*B. cereus, E. coli, Salmonella typhimurium*	(Mellegård et al., 2011)
	Minimum Bactericidal Concentration (MBC)		
	Growth inhibition assay	*E. coli, S. aureus*	(Zheng and Zhu, 2003)
	Cell viability	*Candida albicans*	(Pena et al., 2013)
	Chitinase assay		
	Fermentation		
	Binding of chitosan to the cells		
Chitosan nanoparticles (CSNP)	Turbidimetric method at 620 nm	*E. coli*	(Kong et al., 2008)
	Minimum Inhibitory Concentration (MIC)	*E. coli, Salmonella choleraesuis, S. typhimurium, S. aureus*	(Qi et al., 2004)
	Minimum Bactericidal Concentration (MBC)		
	Minimum Inhibitory Concentration (MIC)	*C. albicans, Fusarium solani, Aspergillus niger*	(Ing et al., 2012)
	Anti-biofilm activity	*Streptococcus mutans*	(Chávez de Paz et al., 2011b)
	Agar well diffusion method	*S. mutans, S. sobrinus, S. Sanguis, S. salivarius*	(Aliasghari et al., 2016)
	Minimum Inhibitory Concentration (MIC)		
	Minimum Bactericidal Concentration (MBC)		
	Anti-adhesion effect		
	Antimicrobial photodynamic inactivation in biofilm	*S. aureus, P. aeruginosa*	(Darabpour et al., 2016)
CSNP-antibiotics	Minimum Inhibitory Concentration (MIC)	*S. aureus*	(Maya et al., 2012)
	Turbidimetric method at 610 nm	*S. aureus, Streptococcus pneumoniae*	(Ngan et al., 2014)
	Invasion assay on Caco-2 and macrophages	*S. typhimurium*	(Zaki and Hafez, 2012)
	Minimum Inhibitory Concentrations (MIC)	*S. aureus* and *E. coli*	(Chifiriuc et al., 2012)
	Microdilution broth assays and agar well diffusion assay	*K. pneumoniae* and *P. aeruginosa*	(Jamil et al., 2016)
	Growth inhibition assay	*Mycobacterium tuberculosis*	(Lu et al., 2009)
	Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Killing kinetic studies	*S. aureus*	(Chakraborty et al., 2012)
	Minimum Inhibitory Concentration (MIC)	*S. aureus, S. epidermidis, Bacillus subtilis, E. coli, P. aeruginosa, MRSA*	(Esmaeili and Ghobadianpour, 2016)
	Test tube serial dilution method	*S. aureus*	(Anal et al., 2006)
CSNP-essential oil	Killing kinetic studies	MRSA, *P. aeruginosa*	(Krausz et al., 2015)
CSNP-metal ions	Inhibition of mycelia growth	*Alternaria solani, Fusarium oxysporum*	(Saharan et al., 2015)
	Spore germination		
	Inhibition of mycelia growth	*Alternaria alternata, Macrophomina phaseolina, Rhizoctonia solani*	(Saharan et al., 2013)
	Spore germination		
	Minimum Inhibitory Concentration (MIC)	*E. coli*	(Sanpui et al., 2008)
	Minimum Bactericidal Concentration (MBC)		
	Minimum Inhibitory Concentration (MIC)	*Streptococcus mutans*	(Hernández-Sierra et al., 2008)
	Minimum Bactericidal Concentration (MBC)		
	Minimum Inhibitory Concentration (MIC)	*E. coli, S. choleraesuis, S. aureus*	(Du et al., 2009)
	Minimum Bactericidal Concentration (MBC)		
	Minimum Bactericidal Concentration (MBC)	*E. coli, Acinetobacter baumannii, S. aureus, E. faecalis, P. aeruginosa, S. pneumoniae*	(Akmaz et al., 2013)
	Disk diffusion method	*E. coli, Salmonella paratyphi, Bacillus spp.*	(Manikandan and Sathiyabama, 2015)
	Turbidimetric method at 595 nm	*E. coli, B. cereus*	(Mallick et al., 2012)
	"Disinfection technique standard"	*S. aureus*	(Gu et al., 2007)
	Minimum Inhibitory Concentration (MIC)	*E. coli, S. aureus*	(Tran et al., 2010)
	Minimum Bactericidal Concentration (MBC)		
	Agar well diffusion method	*Aspergillus flavus, Rhizoctonia solani, Alternaria alternata*	(Kaur et al., 2012)
	Mycelium growth inhibition		
	Bacterial growth kinetics	*E. coli, S. aureus*	(Chen et al., 2008)
	Minimum Inhibitory Concentration (MIC)		
	Minimum Bactericidal Concentration (MBC)		
	Agar diffusion method	*S. aureus, P. aeruginosa, B. cereus, E. coli*	(An et al., 2010)
CSNP-others molecules			
CSNP-tretinoin	Minimum Inhibitory Concentration (MIC)	*Propionibacterium acnes, S. aureus*	(Ridolfi et al., 2012)
CSNP-AMP	Killing kinetic studies	*S. epidermidis*	(Piras et al., 2015)
CSNP-lysozyme	Standard liquid microdilution susceptibility	*S. epidermidis*	(Piras et al., 2014)
	Killing kinetic studies		
CSNP-daptomycin	Minimum Inhibitory Concentration (MIC)	MRSA, *S. epidermidis, Staphylococcus lugdunensis, Staphylococcus haemolyticus, Staphylococcus hominis Staphylococcus warneri, E. faecalis*	(Silva et al., 2015)
CSNP-ferulic acid	Anti-biofilm activity	*C. albicans*	(Panwar et al., 2016)
CSNP-polyphenols	Minimum Inhibitory Concentration (MIC)	*B. cereus, E. coli, S. aureus, S. typhimurium, Listeria innocua, Y. enterocolitica*	(Madureira et al., 2015)
	Minimum Bactericidal Concentration (MBC)		

7 Applications of the antimicrobial activity of chitosan-based nanosystems

Due to versatility, biocompatibility and biodegradability of chitosan, chitosan-based nanosystems have attracted a large interest in the last years especially for the formulation of mixed systems with improved properties. The antimicrobial activity of chitosan has been exploited for a wide range of applications, ranging from agriculture to biomedical area. The following sections introduce the advances of chitosan-based nanomaterials in wound healing, textiles and food packaging fields.

7.1 Wound healing

Skin wound treatment is an important research area. Poor wound management could lead to severe complications and loss of function. Wound healing is a complex process where numerous steps take place in order to re-establish the normal functionality of the skin. It includes an inflammatory, a proliferation, and finally a remodelling phase. Many factors have to be considered when designing a wound material to provide an adequately moist environment and allow gas exchange in order to avoid dehydration and exudates accumulation. Moreover, wound related infections represent a serious problem since bacteria can easily invade the tissues and proliferate, hampering the regeneration process. Hence, agents that are able to prevent infection and promote wound healing have been extensively explored. Chitosan has been widely applied as wound dressing due to its properties that include biocompatibility, biodegradability, haemostatic and antibacterial activities (Siafaka et al., 2016). As such, chitosan have been approved in commercial medical devices for topical applications in wound healing (e.g. HemCon bandages) . Moreover, the gradual depolymerisation of chitosan to *N*-acetyl glucosamine promotes fibroblast proliferation, thereby accelerating wound closure. As this review aims to highlight the main advances of chitosan in the nanotechnology field, a brief overview in wound healing treatment is given, focusing on the application of chitosan as a nano-dressing material.

Tremendous efforts have been devoted to the development of innovative nanofibrous wound dressing materials that are able to assist and accelerate the healing process (Figure 4). Chen et al. were the first to fabricate an electrospun collagen/chitosan nanofibrous membrane, exploiting the recognized properties of chitosan and collagen as wound dressing materials. Their studies demonstrated that the new membrane was able to promote and accelerate wound healing compared to the conventional gauzes or collagen sponges (Chen et al., 2008). In another study, a nanofibrous membrane made of

chitosan and silk-fibroin was fabricated. Its antibacterial activity against Gram-negative *Escherichia coli* was demonstrated to be directly dependent on the chitosan concentration in the composite nano-dressing, with a higher effect with increasing chitosan proportion (Zulianello et al., 2006). A slightly different result was obtained by Sarhan et al. who studied the combined antibacterial activity of honey, chitosan and polyvinyl alcohol electrospun nanofibrous wound dressing. The nanofibers showed pronounced antibacterial effect against the Gram-positive *Staphylococcus aureus*, and the effect increased with increasing chitosan concentration. However, a weak antibacterial activity was observed against the Gram-negative *E.coli* (Sarhan and Azzazy, 2015). Recent studies reported the loading of different compounds such as antibiotics, antimicrobial agents and metal nanoparticles within the chitosan nanofibers with the attempt to increase chitosan antibacterial activity and accelerate the wound healing process. Fazli et al. studied the antimicrobial activity of a nanofibrous mat made of chitosan-polyethylene oxide and loaded with hydrocortisone and imipenem/cilastatin zinc oxide nanoparticles. The chitosan-polyethylene oxide nanofibrous mat showed antimicrobial activity on its own against both *Staphylococcus aureus* and *Escherichia coli* but, as expected, a higher effect was observed when the mat was loaded with the drugs. Moreover, when comparing the antimicrobial activity, a greater antibacterial activity was observed against the Gram-positive *Staphylococcus aureus* than the Gram-negative *Escherichia coli* (Fazli et al., 2016).

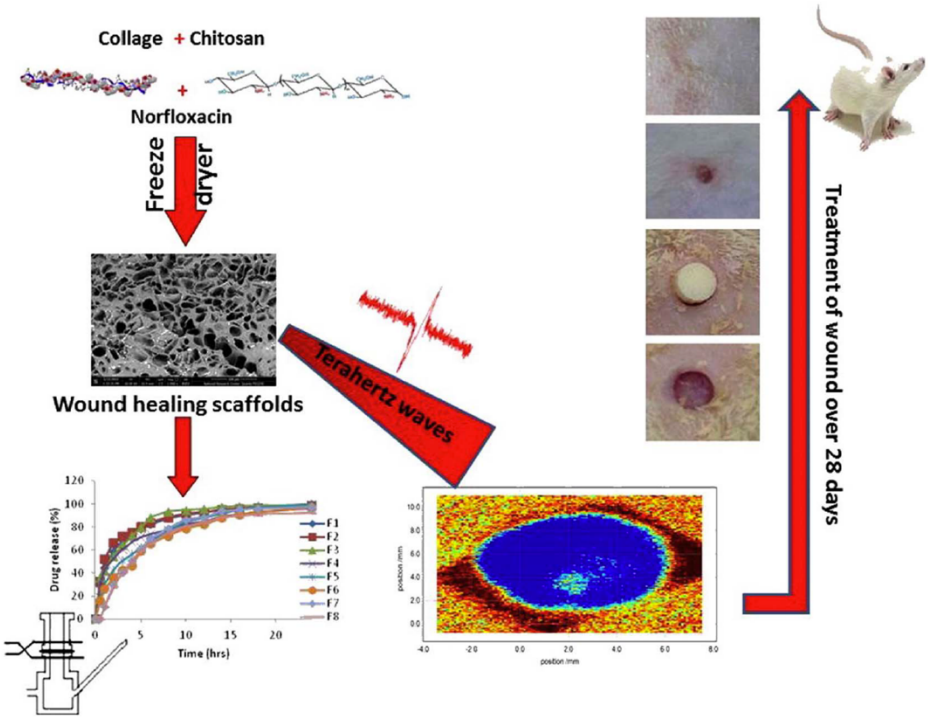

Fig. 4. Preparation, characterization and *in vivo* evaluation of the skin regeneration ability of a mixed chitosan/collagen wound dressing loaded with norfloxacin. Reprinted from (Mahmoud and Salama, 2016).

Many studies have been focused in the past few years on developing alternative drug delivery systems to tackle the problems associated with conventional antibiotic treatments of open wounds which are

highly exposed to bacteria proliferation and infections. The over consumption of antibiotics has led to the development of drug-resistance, rendering the conventional therapies ineffective. In this regard, metal nanoparticles have emerged as a promising strategy to address drug-resistance, due to their antimicrobial and haemostatic properties. The combination of metals or metal oxide nanoparticles with chitosan has recently attracted a growing interest as an encouraging alternative to the conventional antibiotics (Lu et al., 2017) (El-Feky et al., 2017) and it has recently reviewed (Bui et al., 2017). Chitosan is combined with metal nanoparticles for different purposes. A recent study reported the crucial role of chitosan oligomers in the stabilization of silver chloride nanoparticles, exploiting the combined effect of chitosan and AgCl nanoparticles on the antibacterial activity in burn wounds (Kang et al., 2016). Wounds treated with chitosan-stabilised AgCl nanoparticles recovered faster and showed a lower number of white blood cells, demonstrating that these nanoparticles were able to prevent the infection at the wound site. In another recent study, a low MW chitosan was used as coating for silver nanoparticles in order to reduce the toxicity which is often associated with Ag. The nanoparticles were tested on a methicillin-resistant *Staphilococcus aureus* (MRSA) wound infection mouse model. It was demonstrated that the chitosan-coated silver nanoparticles exhibited a higher biocompatibility and lower body absorption characteristics when compared to polyvinylpirrolidone (PVP)-coated or uncoated silver nanoparticles, together with a good anti-MRSA effect (Peng et al., 2017).

Chitosan has also been used in the preparation of wound dressing materials, in which metal nanoparticles are introduced to the nanocomposite with the attempt to obtain a synergistic antibacterial and wound healing effect. Archana et al. prepared a wound dressing using chitosan, polyvinylpyrrolidone and silver oxide nanoparticles, and studied the additive antibacterial effect of chitosan and silver oxide against *Escherichia Coli* and *Staphilococcus aureus*. The ternary system demonstrated a stronger inhibitory effect against the Gram-positive *Staphilococcus aureus* than the Gram-negative *Escherichia coli* (Archana et al., 2015). In another recent study from the same group, titanium dioxide nanoparticles were incorporated into the chitosan-polyvinylpyrrolidone nanocomposite and the antimicrobial activity was evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. Results from this study were in agreement with the previous one, showing a higher efficacy of the nanocomposite against Gram-positive bacteria (Archana et al., 2013). Moreover, a faster wound closure rate was achieved with the ternary nanocomposite compared to chitosan alone. A similar antibacterial effect was observed with a castor oil polymeric film reinforced with chitosan-modified zinc oxide nanoparticles. The developed film was found to have a more efficient antibacterial activity against Gram-positive *Staphylococcus aureus*

435 and *Micrococcus luteus* bacteria. Furthermore, the antibacterial effect was directly proportional to the
436 concentration of chitosan-zinc oxide (Díez-Pascual and Díez-Vicente, 2015a).
437
438 Most of the studies conducted so far on wound dressing investigated the combination of chitosan with
439 metal nanoparticles, while few studies have been performed on chitosan as a nano-carrier system for
440 local drug release. Active compounds with well-known wound healing properties have been loaded
441 in chitosan nanoparticles and these systems have been assessed for their efficacy. In a recent research
442 paper, melatonin has been loaded into lecithin/chitosan nanoparticles, demonstrating the enhanced
443 effect of the nano-carrier system and melatonin in improving wound epithelialisation. Different MW
444 and DA chitosans have been tested, since biological properties of chitosan are known to be highly
445 dependent on these two parameters. The lecithin/chitosan nanoparticles showed better wound healing
446 ability and biocompatibility when compared to the chitosan solutions. The melatonin-loaded chitosan
447 provided an increased wound re-epithelisation and wound closure ability, demonstrating the central
448 role of melatonin in the wound healing process (Blazevic et al., 2016). Other studies have investigated
449 the antimicrobial and wound healing properties of curcumin-loaded chitosan nanoparticles, either
450 alone or incorporated in a nanocomposite matrix. Curcumin has been intensively studied as wound
451 healing agent due to its antimicrobial, anti-oxidant and anti-inflammatory properties. The
452 combination of curcumin with chitosan could potentially increase the wound healing process through
453 a synergistic action. Jahromi et al. reported the potential antibacterial activity of curcumin-loaded
454 chitosan tripolyphosphate (TPP) nanoparticles against *Staphylococcus aureus* and *Pseudomonas*
455 *aeruginosa* bacteria on mouse skin. The curcumin control had a lower antimicrobial activity when
456 compared to curcumin-loaded chitosan TPP nanoparticles. On the other hand, the curcumin-free
457 chitosan-TPP nanoparticles could not inhibit bacterial infection. These results suggest an enhanced
458 antimicrobial effect of chitosan nano-carrier and curcumin against both Gram-positive and Gram-
459 negative bacteria on the examined wounds (Mofazzal Jahromi et al., 2014). Curcumin-loaded
460 chitosan nanoparticles have also been incorporated into different wound dressing systems. Lin et al.
461 used a bacterial cellulose-chitosan membrane designed to prevent bacterial infection at the wound
462 site and to accelerate tissue regeneration (Lin et al., 2013), while in another study, an innovative
463 nanohybrid scaffold was reported by dispersing curcumin-loaded chitosan nanoparticles into a
464 collagen scaffold. In the *in vivo* wound studies using diabetic rats, the nanohybrid scaffold treated
465 group showed a significant wound contraction of the treated wound, faster than the control group
466 (sterile gauze) and the reference group (collagen scaffold without curcumin). Chitosan nanoparticles
467 were found to play a central role in the healing process, being involved in fibroblast proliferation and
468 collagen deposition as well as in the sustainable release of curcumin (Karri et al., 2016).

7.2 Textile and fabrics

Chitosan has been proposed to act as an antimicrobial agent in fabrics or textile in order to prevent microbial growth. In fact, textiles, especially those made of natural fibers such as proteins (silk) or cellulose (cotton), represent a favourable environment for the proliferation of different microorganisms including bacteria or fungi, due to extensive surface area, high porosity and ability to retain humidity. The research in the field of antimicrobial textiles has a great impact on many technological applications such as clothing, furnishing, filtering, medical devices, healthcare and hygienic products. With the increasing interest in the use of silk, collagen or cellulose for the production of membranes and supports for biomedical applications (as regenerative medicine), there is an enhanced demand for safe and biodegradable antimicrobial agents.

Chitosan is a good candidate, with attractive characteristics in comparison to other commonly used organic antimicrobial agents (eg. phenolic and formaldeyde derivatives), especially in terms of toxicological profile. The major drawbacks regarding the use of chitosan include its poor solubility in most of the solvents except acidic aqueous solution, the high viscosity of concentrated high MW chitosan solution for coating application, and the thermal stability of chitosan. Nevertheless, some commercial textile products based on chitosan are available on market. For example, Craybon® (SWICOFIL AG) is a material produced by a blend of chitosan with viscose, which find application in clothing (as underwear, pyjamas, sport apparel) thanks to its strong antibacterial activity, biodegradability, anallergic characteristics and high moisture absorption (Morais et al., 2016). Another exemplum is Chitopoly® (Fuji-Spinning) which is made of polinosic fiber and chitosan (Morais et al., 2016). Although the mentioned commercial products have reached the market over than 10 years ago, the research on the use of chitosan in textiles has been flourishing yet. Different strategies have been applied in textiles and fabrics to improve the antimicrobial activity of chitosan. Microencapsulation is one of the most explored approaches (Ibrahim et al., 2017). Recently, cellulose fibers were functionalized with chitosan or its water-soluble derivative, *N, N, N* trimethyl chitosan, as nanoparticles dispersed at different pH values (4 and 7). The derivatized fibers were used to prepare vaginal tampon with enhanced antimicrobial and drug delivery properties, acting as a preventive medical device in gynaecological diseases (Ristić et al., 2017). The ability of chitosan to be desorbed by the tampon, its antimicrobial activity and its drug encapsulating properties, demonstrated the effectiveness of the product as medical device. As reported by the same authors, fibres functionalized with chitosan or chitosan nanoparticles exhibit a comparable antimicrobial activity (Ristić et al.,

503 2015). The incorporation in chitosan nanoparticles of substances with intrinsic antibacterial activity
504 have also been proposed for textile applications. Similar to other cases, it is not clear if the
505 antibacterial effect should be attributed to chitosan, the encapsulated substances or to their interaction.
506 An example is from the work of Revathi and Thambidurai, in which chitosan–neem seed composites
507 were prepared, and were coated or crosslinked with the cotton fabrics (Revathi and Thambidurai,
508 2017). The antimicrobial activity of these composites against Gram-negative and Gram-positive
509 bacteria was evaluated in comparison to the unmodified cotton fabrics. The composite with
510 crosslinked coated cotton fabric exhibited better antibacterial activity than those without crosslinking,
511 and as expected, the unmodified cotton fabrics did not display any activity. However, no data
512 regarding the activity of chitosan alone (without neem seed extract) were reported.
513
514 ***7.3 Food packaging***
515
516 Food industry is facing the problem of microbial contamination. Foodborne bacteria are responsible
517 for many serious human infections and they can also accelerate food spoilage with enormous
518 economic losses. In this regard, food packaging represents a solution to prevent and retard bacterial
519 invasion and proliferation. Different biopolymers, characterized by a good environmental profile,
520 biodegradability and biocompatibility, have been screened in order to find alternatives to the
521 conventional petroleum derived materials for food packaging.
522
523 Many researches focused on the development of chitosan-based systems, as its intrinsic properties
524 could enhance the antimicrobial efficacy of the packaging. The film forming properties of chitosan
525 has led to the development of film packaging materials in combination with various natural
526 polysaccharides such as starch, pectin and hydroxypropyl methylcellulose (HPMC). An example was
527 given by Möller et al., who studied the antimicrobial activity of a chitosan-HPMC film against
528 *Listeria monocytogenes,* demonstrating a complete growth inhibition. When chitosan-HPMC films
529 were cross-linked with citric acid by the amino groups of chitosan, the antibacterial activity decreased
530 drastically, thus demonstrating the critical role of the protonated amino groups of chitosan for the
531 antimicrobial activity (Möller et al., 2004). Chitosan nanofibers have also been exploited as a
532 packaging material by many research groups due to their numerous advantages such as
533 biocompatibility, large surface area, good functional and antimicrobial properties. Arkhoun et al.
534 studied the antibacterial activity of chitosan/polyethylene oxide electrospun nanofibers against
535 *Escherichia coli, Staphilococcus aureus, Listeria innocua and Streptococcus typhimurium*, verifying
536 the importance of the protonated amino groups of chitosan for the antibacterial activity. The authors

demonstrated that activity of the nanofibers is strain-dependent rather than Gram-dependent, with the highest effect observed against *Escherichia coli* and the lowest against *Streptococcus typhimurium* (Arkoun et al., 2017). In another study, chitosan nanofibers have been incorporated into a poly(butylene adipate-co-terephthalate) (PBAT). Their antimicrobial activity was evaluated against four common foodborne bacteria and the efficacy was found to be dependent on the chitosan nanofibers concentration. Moreover a stronger antibacterial activity was observed against Gram-negative *Escherichia coli* and *Salmonella enteritidis* than Gram-positive *Bacillus subtilis* and *Staphilococcus aureus* (Díez-Pascual and Díez-Vicente, 2015b).

Nano-carrier systems have attracted an increasing attention in food packaging, being able to load different active compounds, including those with low solubility and stability. Chitosan nanoparticles have been intensively exploited for this purpose, and with their well-known antimicrobial properties, a potential combined effect with the loaded active compound could be achieved. As proof of concept study, Feyzioglu and Tornuk examined the antimicrobial activity of summer savory essential oil-loaded chitosan nanoparticles against three different foodborne bacteria (*Escherichia coli*, *Listeria monocytogenes* and *Staphilococcus aureus*) showing the promising application in food packaging materials. Both chitosan nanoparticles and summer savory essential oil-loaded chitosan nanoparticles displayed antibacterial activity, with the loaded nanoparticles demonstrating a higher effect (Feyzioglu and Tornuk, 2016). In another study from Zhang et al., catechin and catechin-Zn complex were loaded into β-chitosan nanoparticles with the aim of increasing cathechin availability and antimicrobial effect. The antibacterial activity was evaluated against *Escherichia coli* and *Listeria innocua*, and the catechin-Zn complex-loaded-chitosan nanoparticles exhibited a higher effect than the cetachin-loaded chitosan nanoparticles. Moreover a stronger antibacterial activity against *Listeria innocua* was observed for all samples including the unloaded chitosan nanoparticles (Zhang et al., 2016).

Chitosan nanoparticles were also incorporated into different food packaging materials and their antimicrobial activity was assessed using different foodborne bacteria. An example was given by Priya et al. that chitosan nanoparticles were incorporated into a HPMC film and evaluated for its antimicrobial activity against four food pathogenic bacteria, representative of *Salmonella, Klebsiella, Bacillus and Pseudomonas* species. A higher effect against Gram-positive bacteria was observed (Shanmuga Priya et al., 2014). In another study, Cui et al. demonstrated a good antibacterial activity of nisin-loaded poly-γ-glutamic acid/chitosan nanoparticles electrospun with poly-ethylene oxide against *Listeria monocytogenes* (Cui et al., 2017) (Figure 5). Hosseini et al. developed a bio-

nanocomposite system by combining gelatin and chitosan nanoparticles. Chitosan nanoparticles were used to improve the mechanical properties of gelatin film and the composite system was evaluated for its antimicrobial activity. Moreover, an antimicrobial active packaging was proposed, by loading oregano essential oil into chitosan nanoparticles which were added to a fish gelatin-based film. The antibacterial activity of the nanocomposite film against *S. aureus*, *L. monocytogenes*, *S.enteritidis* and *E.coli* was studied. Fish gelatin/chitosan nanoparticles used as control did not show any antimicrobial activity, while all the oregano essential oil containing nanocomposite films demonstrated a clear concentration dependent antimicrobial activity, showing the efficacy of the active packaging system (Hosseini et al., 2016).

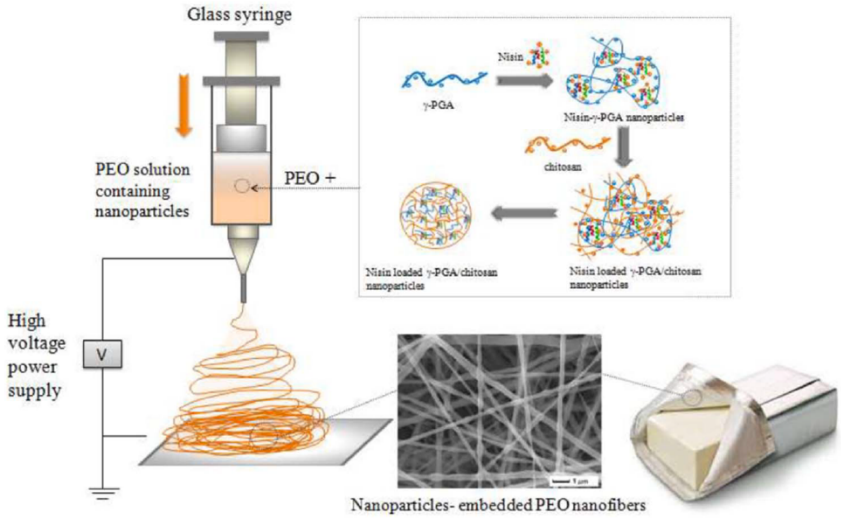

Fig. 5. Nisin-loaded poly-γ-glutamic acid (γ-PGA)/chitosan nanoparticles (NGC)-embedded in PEO nanofibers for food packaging application. Reprinted from (Cui et al., 2017).

Another intensively explored strategy in food packaging is the combination of chitosan and metal nanoparticles to obtain an enhanced antimicrobial effect. Al-Naamani et al. studied the potential antibacterial activity of chitosan and chitosan-zinc oxide nanocomposite as a coating on polyethylene against the pathogenic bacteria *Eschericha coli*, *Salmonella enterica* and *Staphilococcus aureus*. While the chitosan zinc oxide nanocomposite-coated films completely inhibited the bacterial growth, the chitosan-coated films showed only a mild reduction of 10-fold decline of cell counts in the tested bacteria after 24h compared to the control (Al-Naamani et al., 2016). Tripathi et al. described the antibacterial activity of a chitosan-silver oxide nanocomposite films against *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa and Staphilococcus aureus* by agar diffusion method. An enhanced effect was observed when silver oxide nanoparticles were incorporated into the chitosan films (Tripathi et al., 2011). In another recent study, a nanocomposite based on chitosan and titanium dioxide nanoparticles was used for coating a commercial paper. The antimicrobial activity was evaluated against *Escherichia coli*, and a combined effect of chitosan and titanium dioxide nanoparticles was observed. Chitosan coating without nanoparticles showed an antibacterial activity

compared to the blank paper, with an obvious increase when the nanocomposite was used as coating (Tang et al., 2016).

Conclusion

Many publications have reported the antimicrobial effect of chitosan alone or incorporated in different formulations for a wide range of applications. However, the reported data on the antimicrobial effect of chitosan are sometimes controversial and a clear conclusion on it is still far to be drawn up. Several mechanisms have been proposed for the antibacterial and antifungal activity of chitosan, with the focus on understanding the effect of physico-chemical characteristics of chitosan (e.g. MW, DA etc.) and the different structural features between Gram-positive, Gram-negative and fungal cellular wall. Despite most of the proposed mechanisms, appear to be plausible, none of them has been demonstrated successfully at a molecular level. Moreover, the use of non-standardized protocols and assays as well as the lack of controlled experimental conditions in different laboratories contributed to the mixed and inconclusive results, which has generated some confusion on this subject. There are sufficient evidences to support the antimicrobial and antifungal activities of both chitosan as a free polymer and as nanoparticles. However, it is not certain whether there is an intrinsic susceptibility of different microorganisms to chitosan, or such differences (highlighted by the different studies) were resulted from the effect of different experimental conditions. It is also arguable the mode of antimicrobial action ascribed to chitosan nanoparticles in order to explain the supposed enhanced effect (e.g. charged surface area, particle diffusion, quantum-size effect). Also, in this case, controversial results are reported in terms of comparison between the antimicrobial activity of chitosan and chitosan nanoparticles. Overall, current studies generally suggest that chitosan alone is not sufficient to exert a satisfactory antimicrobial activity, but it can be employed together with other active compounds such as small molecules, natural products or metals, to induce an enhanced antimicrobial effect. In this way, chitosan configures as an adjuvant polymer in the design and fabrication of self-antimicrobial materials for a large range of applications in the pharmaceutical, biomedical, food and textile fields.

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Figure 1 Proposed mechanism of the antibacterial effect of chitosan nanoparticles on *Staphilococcus aureus* according to the work of Tyagi et al. Reprinted from (Tyagi et al., 2014).

Fig. 2 Architecture of mixed chitosan/metals/antibiotic nanoparticles effective against Gram-negative bacteria. Reprinted from (Esmaeili and Ghobadianpour, 2016).

Figure 3 Inhibition bacterial growth zone (according to the disk diffusion test) produced by different concentrations of chitosan/silver mixed nanoparticles against *Staphilococcus Aureus* (Gram-positive) and *Escherichia Coli* (Gram-negative) bacteria. Chitosan solution was used as control. Reprinted from (Huang et al., 2016).

Figure 4 Preparation, characterization and *in vivo* evaluation of the skin regeneration ability of a mixed chitosan/collagen wound dressing loaded with norfloxacin. Reprinted from (Mahmoud and Salama, 2016).

Figure 5 Nisin-loaded poly-γ-glutamic acid (*γ*-PGA)/chitosan nanoparticles (NGC)-embedded in PEO nanofibers for food packaging application. Reprinted from (Cui et al., 2017).

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