STUDIA UBB CHEMIA, LXVII, 1, 2022 (p. 209-226)
(RECOMMENDED CITATION)
DOI:10.24193/subbchem.2022.1.14

PREPARATION, PHYSICO-CHEMICAL CHARACTERIZATION AND ANTIBACTERIAL PROPERTIES OF CHITOSAN AND CHITOSAN–NISIN MEMBRANES

MARIANA ADINA MATICA[a], FINN LILLELUND AACHMANN[b], ANNE TØNDERVIK[c], HÅVARD SLETTA[c], VASILE OSTAFE[a]*

ABSTRACT. Different types of chitosan were used to prepare membranes with enhanced antibacterial properties, via the solvent casting method. Nisin, an antimicrobial peptide, already use in food preservation, was incorporated in chitosan membranes to enhance the bactericidal effect, to obtain a starting material intended for use as wound dressings. The physico-chemical properties of the membranes were monitored and the results showed a good swelling capacity and water vapor transmission rate of the membranes. Optical characterization data showed that chitosan-based membranes could provide ultraviolet light protection while *in vitro* biodegradability assay demonstrated good stability of the films under enzymatic degradation. Nisin improved significantly the antibacterial effect of the membranes, while the nisin-chitosan membrane-forming solutions had a bactericidal effect against both Gram-positive and Gram-negative bacteria.

Keywords: *chitosan, membrane, nisin, antimicrobial peptide, biodegradable polymer*

INTRODUCTION

The new approach in developing antibacterial materials for wound treatment implies the use of bioactive molecules, with innate properties that are safe to the human body as well as safe to the environment. Chitosan is

[a] *West University of Timișoara, Advanced Environmental Research Laboratories, 4 Oituz str., RO-300086, Timișoara, Romania*
[b] *Norwegian Biopolymer Laboratory (NOBIPOL), Department of Biotechnology and Food Sciences, NTNU Norwegian University of Science and Technology, Sem Sælands vei 6/8, 7491 Trondheim, Norway*
[c] *SINTEF Industry, Department of Biotechnology and Nanomedicine, Richard Birkelands veg 3 B, 7034 Trondheim, Norway*
** Corresponding author: vasile.ostafe@e-uvt.ro*

one of the most studied non-toxic biomaterials with ideal characteristics such as antimicrobial properties [1,2], biodegradability [3], and biocompatibility [4]. Currently, on the market, there are many wound dressings based on chitosan, mainly hemostatic dressings and the interest in chitosan as wound dressing starting material will only increase in the future [5,6] as it has become more and more difficult to treat and heal infected wounds.

While pristine chitosan possesses an antibacterial effect, as many studies have proven this over the years, its antibacterial properties are dependent on several physico-chemical characteristics, mainly molecular weight (MW) and degree of deacetylation (DDA%) [5]. The use of different polymers in combination with antibacterial agents has become a common practice, but due to the increase of antibiotic resistance, other molecules, such as antimicrobial peptides, are replacing conventional antibiotics. Antimicrobial peptides (AMPs), both synthetic or from natural sources, are small molecules, from 6 up to 100 amino acids, usually of cationic nature, with activity against a wide range of microorganisms, from bacteria, yeast, fungi, to viruses and even tumor cells [7].

Nisin, a polycyclic cationic peptide produced by *Lactococcus sp*., is composed of 34 amino acids and is classified as a Type A (I) lantibiotic molecule. Mainly used in food preservation [8], nisin is active on both Gram-positive and Gram-negative bacteria, by interfering in the cytoplasmic membrane permeability and cell wall disruption. While nisin is FDA (Food and Drug Administration) approved and has a GRAS (Generally Regarded as Safe) status, it`s application has extended to biomedical fields [9], especially due to the proven activity against drug-resistant bacterial strains [10].

In this study, several types of chitosan were used to prepare chitosan and chitosan-nisin membranes. The physico-chemical characteristics of the above-mentioned membranes were investigated, as well as their antibacterial effects against Gram-positive and Gram-negative bacteria.

RESULTS AND DISCUSSION

Preparation of chitosan membranes

Different types of chitosan were used (Table 1) to prepare chitosan and chitosan-nisin membranes (Figure 1), via the solvent casting method. Membranes were dried at 37°C and peeled using 1 M NaOH. Chitosan membranes were transparent and flexible, while the addition of nisin made the membranes less flexible and brittle. The main characteristics of chitosan (Cs) and chitosan-nisin (Cs-N) membranes, based on visual observations, are described in Table 2.

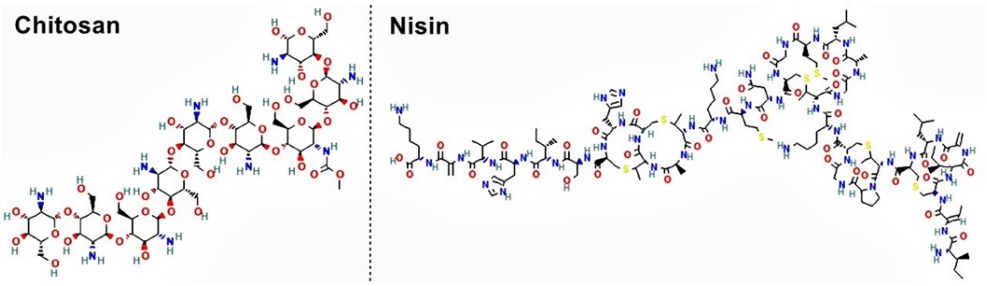

Figure 1. Chemical structure of chitosan and nisin

All chitosan membranes were pealed off easily, after 10 to 15 minutes after NaOH addition, except Cs-50 membrane, which needed more time to be removed from the Petri plate. Also, this type of membrane was more flexible, presented higher elasticity upon removal from Petri plate for neutralization, low resistance to mechanical stress, and required longer time to dry, compared to the other chitosan membranes.

Table 1. Characteristics of chitosan types used for membrane preparation

Chitosan type	Chitosan code	Molecular weight (kDa)	Fraction of acetylation (FA)/Degree of deacetylation (DDA%)
Chitopharm S	Cs-S	198	FA 0.19
Chitopharm L	Cs-L	604	FA 0.17
Chitopharm 50	Cs-50	190	FA 0.52
ChitoClear	CC	270	DDA 95%
Chitosan Low Molecular Weight	Cs-SA	50-190	DDA 74 – 85%

Chitosan-nisin membranes peeled off in a matter of seconds after adding 1 M NaOH, but were less flexible and brittle, especially CCN membrane, which shattered easily upon handling, after drying. After complete drying, Cs membranes turned out less wrinkled than Cs-N membranes (Figure 2). Cs membranes presented homogeneity and absence of insoluble particles, while the presence of nisin, in some cases, lead to a lower homogeneity of membranes, except Cs-50N membrane, which kept its homogenous appearance, and that might depend on the chitin ability to form

protein retention membranes [11]. As seen in Table 1, this type of membrane is based on chitosan with an acetylation fraction of 0.52, meaning there is at least an equal number of acetylated and deacetylated glucosamine units in the polymer backbone.

Table 2. Observed morphological characteristics of membranes

Membrane code	Observations regarding membrane properties
Cs-S	A transparent, flexible, smooth surface membrane
Cs-SN	A slightly transparent, brownish membrane, moderately flexible, smooth surface
Cs-L	A transparent, flexible, smooth surface membrane
Cs-LN	A slightly transparent, brownish membrane, moderately flexible, smooth surface
Cs-50	A transparent, very flexible, smooth surface membrane,
Cs-50N	A transparent, very flexible, smooth surface, membrane
CC	A transparent, slightly yellowish, moderately flexible, smooth surface membrane
CCN	A slightly opaque, brownish, very brittle, slightly rough surface membrane
Cs-SA	A transparent, yellowish, flexible, smooth surface membrane
Cs-SAN	A slightly transparent, brownish, moderately flexible, slightly smooth surface membrane

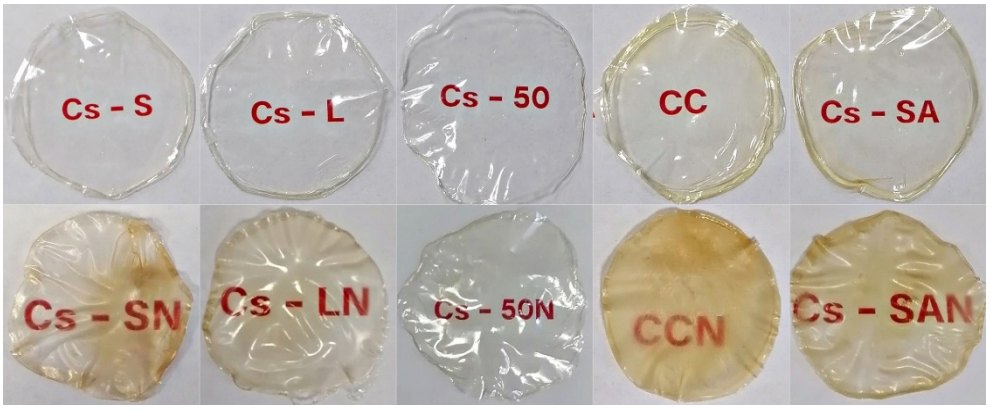

Figure 2. Digital images of the membranes

Moisture content and total soluble matter

The moisture content (right side) and total soluble matter of the membranes are shown in Figure 3 (left side). The presence of nisin in Cs membranes slightly increased the membrane moisture content. This could be explained by the fact that besides the strong hydrogen bonds, that occur between the functional groups in the chitosan chain (–OH, $–NH_2$) and water molecules [12], the presence of nisin, which is an amphipathic molecule (the C-terminal hydrophilic region and the N-terminal hydrophobic region) increases the hydrophilicity of membrane [13].

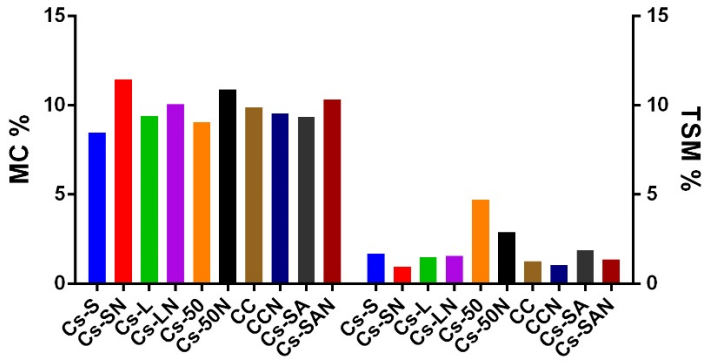

Figure 3. Moisture content (MC % - left) and total soluble matter (TSM % - right) of membranes

The values obtained from measuring total soluble matter give information for the resistance of the membranes against water, and the results obtained (Figure 3 – right side) showed that solubility of membranes decreased with the addition of nisin, which may also be due to the hydrophobic nature of the antimicrobial peptide.

The Cs-50 and Cs-50N membranes are based on a type of chitosan having FA 0.52, meaning that the chitosan backbone contains at least, an equal number of glucosamine and N-acetylglucosamine units. It is known that chitosan has a better film forming ability than chitin [14], and we observed that during the membrane preparation step, when Cs-50 membrane was difficult to handle and less resistant to mechanical stress. Therefore, the higher soluble matter in this membrane type may be caused by the less structured membrane, compared to the other Cs membranes, based on chitosan with a higher deacetylation degree.

Swelling measurements

The swelling of Cs and Cs-N membranes involves the diffusion of water molecules into the polymer matrix followed by the expansion of the polymer matrix into the surroundings [15]. As shown in Figure 4, Cs-50 and Cs-50N membranes had the highest swelling ratio (4.7 and 5.9 gram of solvent sorbed per gram of dry membrane), twice compared to the other membranes (Figure 4). This high swelling capacity of Cs-50 and Cs-50N membranes may be due to the chitin sorbent ability [16].

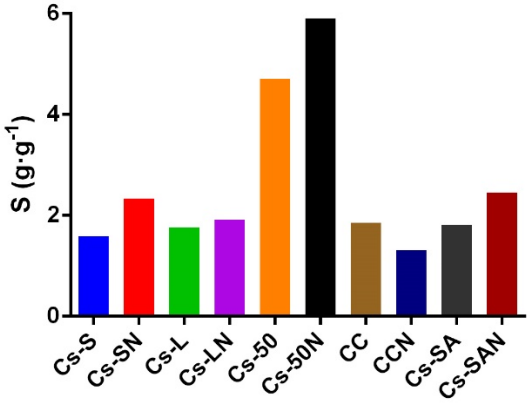

Figure 4. Swelling measurements of membranes

Swelling capacity may depend also on the deacetylation degree and molecular weight [17], as the CC and Cs-SA and Cs-L containing membranes had the highest swelling values (Figure 4), compared to the other chitosan types, which had smaller deacetylation degrees and lower molecular weight (Table 1).

Table 3. Thickness and opacity of the films

Membrane code	Thickness (μm)	O_m value
Cs-S	37.14	3.925
Cs-SN	40.05	5.405
Cs-L	44.29	2.996
Cs-LN	51.43	5.264
Cs-50	25.71	3.385
Cs-50N	52.86	5.324
CC	54.43	2.942
CCN	67.14	6.529
Cs-SA	60.01	2.340
Cs-SAN	71.02	7.266

The Cs-N membranes had high swelling capacity compared to Cs, except CC type (Figure 4). There are two ways for water to be absorbed by the membrane, by binding to the membrane itself or by being retained in the pores formed in the membrane structure [18]. On the other side, Cs-N membranes were thicker than Cs membranes, and the chitosan-nisin blend would form a denser structure, therefore they would retain more water molecules in their structure (Table 3).

Water vapor permeability

The water vapor permeability transmission rate (WVTR) showed different results based on the type of chitosan (Figure 5). Although, the swelling capacity increased slightly with the molecular weight of chitosan, it seems that the WVTR will decrease based on the same criteria [19]. Moreover, the addition of nisin decreases, even more, the WVTR, probably due to the formation of a denser structure within the membrane. If considering that the vapor rate for injured skin, the value can reach up to 5138 $g \cdot m^{-2} \cdot d^{-1}$ for a granulating wound, therefore, the results we obtained for both Cs and Cs-N membranes, between 2000 and 3000 $g \cdot m^{-2} \cdot d^{-1}$, the membranes would meet the requirements for a wound dressing material [18]. Moreover, the values we obtained are comparable to commercial wound dressings products available on the markets [20].

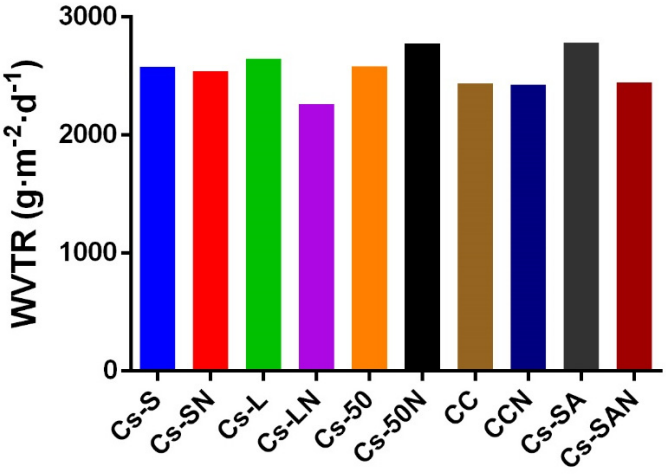

Figure 5. Water vapor permeability rate of membranes

Optical characteristics and thickness of membranes

The measurements regarding the thickness and opacity of membranes showed that the addition of nisin lead to an increase of both parameters. The opacity of the membranes doubled when nisin was added, and CCN and Cs-SAN presented the highest O_m values, while all Cs membranes showed similar opacity (Table 4).

The addition of nisin affected the transmittance rate of membranes, as shown in Table 5, where T_{800nm} is starting from a range of 66 to 76% for all membranes, and gradually decreases until zero at T_{200nm} (Figure 6). Four of the membranes exhibit lower transmittance rate compared to the other membranes, Cs-LN, CC, CCN, and Cs-SAN. The lowest transmittance rate is observed for CCN membrane, which starts from 12% at T_{800nm} and reaches 0 at T_{200nm}. Given the results obtained, we can suggest that chitosan-based membranes would provide protection against UV light to some wounds. Although the effectiveness of UV radiation in wound care, for bactericidal effect was proven [21], there are some limitations and further analysis must be employed, as UVC and UVB can damage the genetic material in the host cell [22].

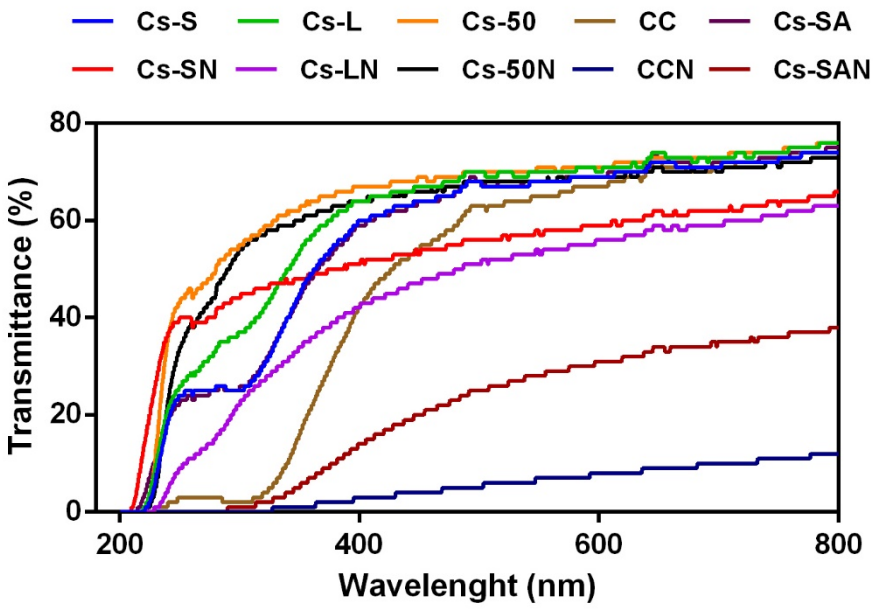

Figure 6. The transmittance of the membranes

Table 4. Light transmittance of membranes

Membrane code	Transmittance (%) at different wavelengths (nm)												
	200	250	300	350	400	450	500	550	600	650	700	750	800
Cs-S	0	24	25	45	60	64	68	68	69	72	71	73	74
Cs-SN	0	40	44	48	51	53	56	57	59	62	62	63	66
Cs-L	0	26	37	54	64	67	70	70	70	74	73	74	76
Cs-LN	0	9	22	34	42	47	51	53	56	59	59	61	63
Cs-50	0	43	55	62	67	69	70	71	71	73	73	74	76
Cs-50N	0	34	54	60	64	66	68	68	69	71	70	71	73
CC	0	3	2	16	42	54	63	65	67	72	71	72	74
CCN	0	0	0	1	3	4	5	7	8	9	10	11	12
Cs-SA	0	23	26	44	59	64	68	68	69	73	72	73	75
Cs-SAN	0	0	1	5	14	20	25	28	31	34	35	36	38

In vitro degradation

Biodegradable polymers, as chitosan, desired to be used for wound treatment, are susceptible to *in vivo* oxidative and enzymatic degradation, when such molecules are secreted during the inflammatory phase [23]. Therefore, the biodegradation of membranes was determined by monitoring the weight loss, in phosphate buffered saline (PBS – used as control), lysozyme, and H_2O_2, after 24 hours at 37°C. The degradation assay revealed that there is no significant difference between PBS degradation and lysozyme degradation, for the membranes, except Cs-50 and Cs-50N membranes, that lost almost 50% of their weight, after 24 hours of incubation in lysozyme. Given the fraction of acetylation of this type of chitosan (FA 0.52), and the results obtained during the swelling measurements (Figure 4), thickness (Table 3) and in vitro degradation (Figure 7), confirm the weak film-forming ability of low deacetylation degree of chitosan, that can be easily degraded.

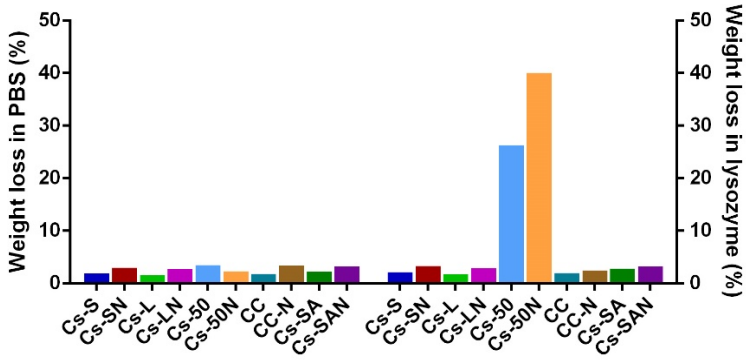

Figure 7. In vitro degradation of membranes (PBS – left and lysozyme – right)

As for the H_2O_2 degradation assay, we were not able to measure the weight loss, because, after 24 hours, all membranes were almost entirely degraded, resulting in very small pieces of membranes, and for Cs-50 and Cs-50N, remaining only a gel-like solution. Therefore, membrane resistance in oxidative environments was very low. On the other side, the long-term stability of membranes under enzymatic degradation indicates that the obtained membranes could be appropriate for external use as wound dressing materials. A controlled degradation process of polymeric matrix can be advantageous for drug release, especially in biomedical application, such as wound healing [24].

Antibacterial testing

The antibacterial effect of Cs and Cs-N membranes was tested using the disk diffusion method, which is a qualitative test that indicates the inhibitory effect of a sample. The inhibition zone diameter in susceptibility testing of conventional antibiotics indicates the sensitivity or resistance of bacteria to antimicrobial agents. Here, the measure of inhibition zone diameter is mainly a qualitative result, as there are no standards available for these new emerging antimicrobial molecules. Is possible that the zone diameter depends on the diffusion rate of the chitosan-based on its molecular weight. However, the results showed a significant difference between the chitosan-nisin membrane inhibitory effect and chitosan solutions.

As seen in Figure 8, all membrane-forming solutions showed an inhibitory effect against both bacterial strains tested (Table 5), while Cs membranes instead, showed no inhibition zone (Figure 10). It is known that the antibacterial effect of chitosan is based on its polycationic nature in an acidic environment. Therefore, in an aqueous acidic environment, the positive charge of chitosan increases, because the $–NH_2$ are converted to soluble protonated form $–NH_3^+$. The antibacterial effect of chitosan is based on its protonated form, due to electrostatic interaction between protonated amino groups and anionic structures from the bacterial cell surface [5]. This would explain the lack of inhibition zone when testing the antibacterial effect of dried and neutralized Cs membranes. One drawback observed when adapting the disk diffusion method to chitosan-based membranes was the poor adherence of membranes to agar media. Once placed on top of the agar surface and incubated at 37°C, the edges of the membranes would curl up. Therefore, to overcome this drawback, before placing them into the incubator, the Petri plates were kept at 4°C for 2 hours to ensure better adherence to the agar surface. The same step was applied to membrane-forming solutions, to prevent the drying of the solution on the filter paper disk and to allow a better diffusion of viscous chitosan to agar media.

The addition of nisin increased highly the inhibitory effect on both bacterial strains, while seemingly, the inhibitory effect against *S. aureus* MRSA was higher than against *P. aeruginosa*. Nisin is known to have a bactericidal effect against a broad spectrum of Gram-positive bacteria [25], but its inhibitory effect is lower against Gram-negative species [26]. However, our results are similar to other studies [27] and show that the nisin-chitosan blend exhibit a high inhibitory effect on both Gram-positive and Gram-negative strains, hence the synergistic effect of both antimicrobials (Figure 9).

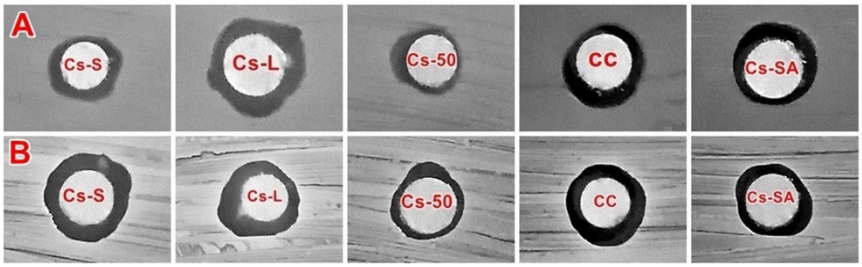

Figure 8. Disk diffusion method: Inhibition zone of Cs solutions against A) Pseudomonas aeruginosa (G-), B) Staphylococcus aureus MRSA (G+)

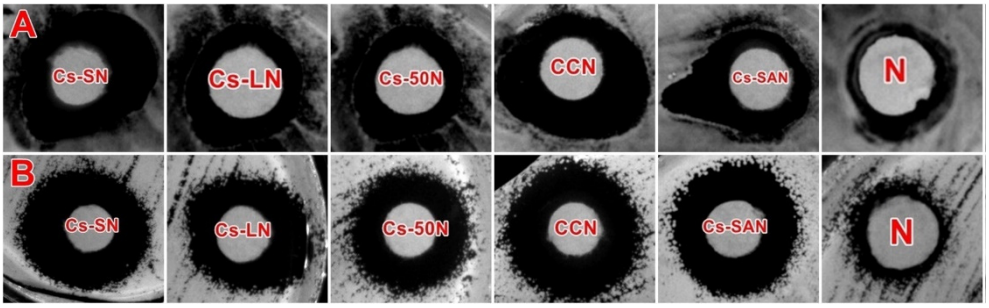

Figure 9. Disk diffusion method: Inhibition zone of Cs-N solutions against A) Pseudomonas aeruginosa (G-), B) Staphylococcus aureus MRSA (G+)

The inhibitory effect of Cs and Cs-N membranes was lower, compared to membrane-forming solutions, as explained above. As seen in Figure 10, a clear inhibitory effect, based on inhibition zone, was observed against S. aureus MRSA (methicillin-resistant Staphylococcus aureus) strain. Four types of membranes inhibited the growth of this strain, Cs-SN, Cs-LN, Cs-50N, and Cs-SAN. The other membranes (CC and CCN) did not inhibit bacterial

growth, and some of them did not adhere completely to the agar surface. This drawback could influence the inhibitory effect, due to the poor diffusion of the antimicrobials to agar media. While none of the membranes tested showed any inhibition zone against P. aeruginosa growth, it was observed that the bacterial cells did not grow on the surface of the membranes, compared to S. aureus MRSA growth, where we observed bacterial growth on the surface of Cs membranes. Our results are comparable to other studies, which show that the antibacterial effect of chitosan and nisin as well, depends on the bacterial strains [10,28,29].

Table 5. Inhibition zone diameter of membranes-forming solutions

Membrane code	Inhibition zone (mm)	
	P. aeruginosa	*S. aureus MRSA*
Cs-S	10	11
Cs-SN	24	24
Cs-L	11	11
Cs-LN	20	22
Cs-50	8	9
Cs-50N	20	22
CC	10	11
CCN	25	25
Cs-SA	10	10
Cs-SAN	19	24
N	11	10

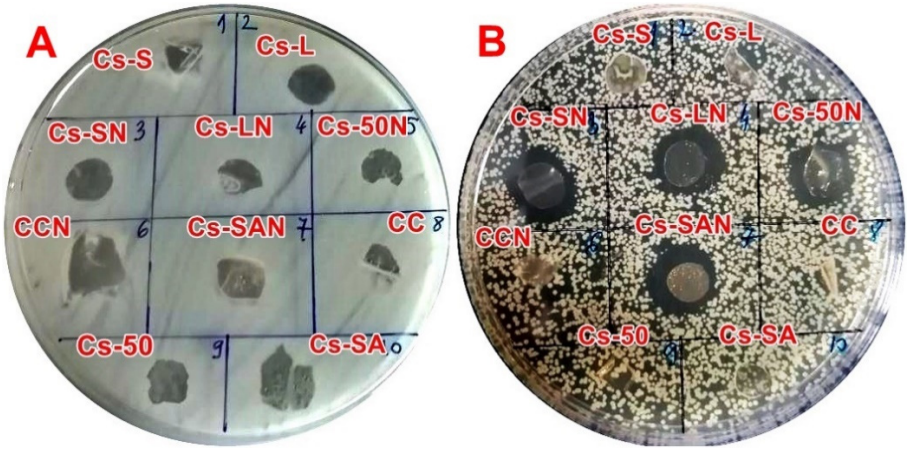

Figure 10. Disk diffusion method: Inhibition zone of Cs and Cs-N membranes against A) Pseudomonas aeruginosa (G-), B) Staphylococcus aureus MRSA (G+)

CONCLUSIONS

The use of antimicrobial peptides incorporated in the biopolymer matrix could provide better protection against bacterial colonization and infection of different wound types [30,31]. In this study, membranes were successfully prepared, via solvent casting method, from chitosan powder of different molecular weight and deacetylation degrees. The antibacterial effect of pristine chitosan was enhanced by blending nisin, an antimicrobial peptide already used in food preservation.

The physico-chemical characteristics showed that the membranes developed had a good swelling capacity and water vapor transmission rate, which could provide a suitable environment for wound healing application. The optical characteristics showed that the membranes could provide UV protection, as genetic material from host cells can be affected by ultraviolet light exposure [22]. The antibacterial effect of the membranes, tested against two bacterial strains, common to wound infections, was confirmed by both membrane-forming solutions and membrane as well. By using the chitosan-nisin blend, the inhibitory effect was higher, the two components working synergistically.

These findings are comparable to previous studies [9,27,29,32], which show the effectiveness of nisin to human pathogenic bacterial strains and the use of AMPs in combination with chitosan could become a promising derivative with intense use in biomedical and pharmaceutical applications. Moreover, based on our results, the membranes based on low molecular weight chitosan with a higher deacetylation degree are the most suitable for wound healing application.

EXPERIMENTAL SECTION

Materials

In this study, we used five types of chitosan, with different molecular weights (MW) and different deacetylation degrees (DDA%) or fraction of acetylation (FA), expressed differently due to the provider choice of describing the product. As described in literature, the fraction of acetylation of chitosan is situated in 0 to 1 range, 0 FA meaning polyglucosamine while 1 FA is considered chitin [14].

Three types of chitosan of different MW and FA were obtained from Chitinor (Chitopharm S – MW 198 kDa, FA 0.19, Chitopharm L – MW 604 kDa, FA 0.17, Chitopharm 50 – 190 kDa, FA 0.52), during one research project. One chitosan sample was obtained from Primex (ChitoClear 43010 –

MW 270 kDa, 95%DDA), and one chitosan powder was purchased from Sigma Aldricht (Chitosan low molecular weight – 50-190 kDa, 75-85% DDA). Acetic acid (CH_3COOH) p.a., sodium hydroxide (NaOH) p.a ISO, sodium chloride (NaCl) p.a. ACS ISO, potassium chloride (KCl) p.a. ACS ISO, di-sodium hydrogen phosphate (Na_2HPO_4) p.a. ACS, and potassium dihydrogen phosphate (KH_2PO_4) p.a. ACS, were purchased from Carl Roth. Nisin was purchased from MP Biomedicals. Mueller Hinton Broth, Mueller Hinton Agar was purchased from Carl Roth, hydrogen peroxide (H_2O_2) 30% puriss. p.a., reag. ISO, reag. Ph. Eur. and lysozyme (~100000 U/mg) were purchased from Sigma Aldrich.

Preparation of chitosan membranes

All chitosan (Cs) solutions were prepared as follows: 1g of chitosan powder was added to 100 mL of 1% CH_3COOH solution (w/v). The mixtures were magnetically stirred (Witeg SMHS-3) at room temperature (RT) and 300 rpm until complete dissolution. The solutions were filtered through six layers of sterile filter gauze to remove any undissolved particles. All chitosan solutions were left overnight for deaeration. Nisin (N) was dissolved in 1% CH_3COOH solution (w/v) [27] and mixed with chitosan solution to give a final concentration of 10 mg/mL. The Cs and Cs-N membranes were prepared by the method of casting and evaporation of the solvent [26,33] with minor modifications: 10 mL of solution was cast in glass Petri plates (60 mm diameter) and dried at 37°C (Memmert UF 55 oven) overnight.

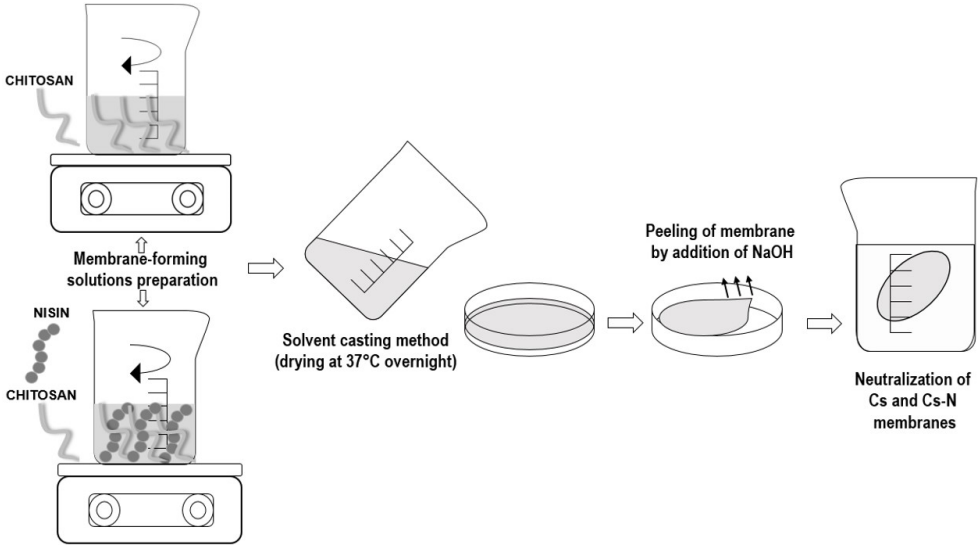

Figure 11. Schematic representation of membrane preparation process

The resulting membranes were subjected to alkali treatment by pipetting 15 mL of 1M NaOH and mixed on a rotary shaker (BIOSAN Sunflower Mini-Shaker 3D) until the membranes were pealed off easily from the Petri plate surface. Thereafter, the membranes were washed with distilled water until neutralization. pH paper was immersed in the wash water to check the neutralization state [34]. Finally, all membranes were dried at RT for 24 hours in a pressed condition to avoid wrinkle formation as much as possible [35].

Moisture content and total soluble matter

The moisture content of the Cs and Cs-N membrane was determined by the method of Yu et al. [36]. Membranes were weighed and dried overnight in a convection oven (Memmert UF 55 oven) at 105°C. The moisture content (MC%) was determined using the following formula: $MC\% = (m_1 – m_2)/m_1 \times 100$, meaning m_1 and m_2 are the initial and final dry weight (g) of the chitosan membranes.

Afterward, the same dry membranes were used for the determination of total soluble matter (TSM) according to the method reported previously in the literature [37]. Therefore, the previously dried membranes of known weight were submerged in distilled water. After incubation at RT for 24 hours, the membranes were taken out and dried overnight at 105°C. The weight of dry matter, that was not solubilized in water, was determined as follows: $TSM\% = (W_i\text{-}W_f)/W_i \times 100$, where W_i and W_f are the initial and final mass of the membranes.

Swelling measurements

Cs and Cs-N membranes, dried at a constant weight, were immersed in phosphate buffer solution at RT. Their weight was measured after 24 hours, by removing the membranes from liquid and blotting with filter paper the excess of liquid. The swelling ratio (S), after 24 hours, was determined by the following equation: $S = (M_t – M_0)/M_0$, where M_0 is represented by the mass of the dry membrane and M_t is the mass of the swollen membrane at 24 hours. The results are expressed as gram of solvent sorbed per gram of dry membrane, S ($g \cdot g^{-1}$) [15].

Water vapor permeability

Chitosan membranes were fixed over the opening of a glass bottle containing 5 mL of distilled water. The system was weighed and the water vapor permeability was measured over time, at 37°C, by measuring the weight of the system. The water vapor transmission rate (WVTR) was calculated using this formula: $WVTR = (W_0 – W_t)/(tA)$ where W_0 is the mass of the system at the initial weighing, W_t is the mass of the system at certain time t, while t is the measurement time and A represents the open area of the glass bottle. The results are expressed as grams per square meter per day ($g \cdot m^{-2} \cdot d^{-1}$) [18,33].

Optical characteristics and thickness of membranes

A Shimadzu UV-1900i UV-VIS spectrophotometer was used for measuring the transmittance and opacity of Cs and Cs-N membranes. Membranes were cut in strips and attached to the wall of the cuvette, while an empty cuvette was used as blank. Measurements were made in the wavelength range from 200 to 800 nm. The opacity of the membranes (Om) was determined by measuring the absorbance at 600 nm. The Om value, a parameter positively linked to film opacity, was calculated by the following equation: $Om = A_{600nm}/N$, where A600nm is the absorbance of chitosan membrane at 600 nm and N is the membrane thickness (mm), measured by Vernier digital caliper [36].

The thicknesses of chitosan membranes were measured using a Vernier digital caliper with a measuring accuracy of 0.01 mm. The thickness of each membrane was measured at six random points and the average thickness of membranes was calculated [37].

In vitro degradation

In vitro degradation of the membranes was determined according to Mishra et al. [23] and Ma et al. [18].

The degradation of Cs and Cs-N membranes at physiological conditions was made using PBS (phosphate buffered saline) solution (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.245 g/L, pH 7.4) and PBS solution with lysozyme and H_2O_2. Samples of dried membranes were placed in 10 mL PBS solution (as control) and to mimic *in vivo* physiological conditions, PBS/lysozyme solution of 20,000 U/mL and 3.5% H_2O_2 in PBS were used. The samples were incubated at 37°C for 24 hours. Afterward, membranes were dried in an oven at 105°C for 24 hours and weighed again.

The degradation at physiological conditions was calculated as follows: $(W_{dry\ f}/W_{dry\ i}) \times 100$, where $W_{dry\ i}$ is the initial mass and $W_{dry\ f}$ is the final mass of membranes, after 24 hours.

Antibacterial testing

Antibacterial activity of chitosan membranes was tested using Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853) and Gram-positive bacteria *Staphylococcus aureus* MRSA (ATCC 43300) by agar disk diffusion method [38]. Bacterial inoculum of 0.1 a.u. (OD_{620nm}), cultivated in Mueller Hinton Broth (beef infusion solids 2.0 g/L, casein hydrolysate 17.5 g/L, starch 1.5 g/L), was spread by swabbing on Petri plates containing Mueller Hinton Agar (beef infusion solids 2.0 g/L, casein hydrolysate 17.5 g/L, starch 1.5 g/L, agar 17 g/L). Disk diffusion method was applied to both chitosan and chitosan-nisin forming solutions and membranes. The antibacterial testing of membrane-forming solutions was tested using sterilized filter paper disks

of 7 mm diameter, which were placed onto the inoculated agar surface, and 20 µL of the solution was pipetted on each disk. The antibacterial effect of membranes was tested similarly. Therefore, sterilized disks of chitosan and chitosan-nisin membranes (7 mm diameter) were placed carefully onto the inoculated agar surface. The Petri plates were placed at 4°C for two hours prior to the incubation step, at 37°C for 24 hours.

ACKNOWLEDGMENTS

This work was supported by the ERA-Net COFASP Project "Biotechnological tools implementation for new wound-healing applications of byproducts from the crustacean seafood processing industry-Chitowound (project no. PN3-P3-284/4/2017 program H2020, financed by the Romanian National Authority for Scientific Research UEFISCDI).

REFERENCES

1. A. Matica; G. Menghiu; V. Ostafe; *New Front Chem,* **2017**, *26*, 55-63.
2. A. Matica; G. Menghiu; V. Ostafe; *New Front Chem,* **2017**, *26*, 39-54.
3. A. Matica; G. Menghiu; V. Ostafe; *New Front Chem,* **2017**, *26*, 75-86.
4. V. Patrulea; V. Ostafe; G. Borchard; O. Jordan; *Eur J Pharm Biopharm,* **2015**, *97*, 417-426.
5. M.A. Matica; F.L. Aachmann; A. Tøndervik; H. Sletta; V. Ostafe; *Int J Mol Scie,* **2019**, *20*, 5889.
6. C. Moraru; M. Mincea; G. Menghiu; V. Ostafe; *Molecules,* **2020**, *25*, 4758.
7. M. Mahlapuu; J. Håkansson; L. Ringstad; C. Björn; *Front Cell Infect Microbiol,* **2016**, *6*, 194-194.
8. J.C.P. Santos; R.C.S. Sousa; C.G. Otoni; A.R.F. Moraes; V.G.L. Souza; E.A.A. Medeiros; P.J.P. Espitia; A.C.S. Pires; J.S.R. Coimbra; N.F.F. Soares; *Innov Food Sci Emerg Technol,* **2018**, *48*, 179-194.
9. Z. Najmi; A. Kumar; A.C. Scalia; A. Cochis; B. Obradovic; F.A. Grassi; M. Leigheb; M. Lamghari; I. Loinaz; R. Gracia; L. Rimondini; *Front. Bioeng. Biotechnol.,* **2020**, *8*.
10. J.M. Shin; J.W. Gwak; P. Kamarajan; J.C. Fenno; A.H. Rickard; Y.L. Kapila; *J Appl Microbiol,* **2016**, *120*, 1449-1465.
11. C. Schmitz; L.G. Auza; D. Koberidze; S. Rasche; R. Fischer; L. Bortesi; *Mar Drugs,* **2019**, *17*, 452.
12. A. Aljawish; L. Muniglia; A. Klouj; J. Jasniewski; J. Scher; S. Desobry; *Food Hydrocoll.,* **2016**, *60*, 551-558.

13. A. Jozala; L. de Lencastre Novaes; A. Pessoa. Nisin. In *Concepts, Compounds and the Alternatives of Antibacterials*, V. Bobbarala, Ed. InterchOpen: 2015; pp. 103-123.
14. J. Wattjes; S. Sreekumar; C. Richter; S. Cord-Landwehr; R. Singh; N.E. El Gueddari; B.M. Moerschbacher; *React Funct Polym,* **2020**, *151*, 104583.
15. J. Ostrowska-Czubenko; M. Gierszewska; M. Pieróg; *J. Polym. Res,* **2015**, *22*, 153.
16. F. Blind; S. Fränzle; *Polysaccharides,* **2021**, *2*, 773-794.
17. J.D. Bumgardner; V.P. Murali; H. Su; O.D. Jenkins; D. Velasquez-Pulgarin; J.A. Jennings; A. Sivashanmugam; R. Jayakumar. Characterization of chitosan matters. In *Chitosan Based Biomaterials Volume 1*, J.A. Jennings, J.D. Bumgardner, Eds. Woodhead Publishing: 2017; pp. 81-114.
18. Y. Ma; L. Xin; H. Tan; M. Fan; J. Li; Y. Jia; Z. Ling; Y. Chen; X. Hu; *Mater. Sci. Eng. C,* **2017**, *81*, 522-531.
19. 19.M.J. Bof; V.C. Bordagaray; D.E. Locaso; M.A. García; *Food Hydrocoll.,* **2015**, *51*, 281-294.
20. J. Kingkaew; S. Kirdponpattara; N. Sanchavanakit; P. Pavasant; M. Phisalaphong; *Biotechnol Bioprocess Eng.,* **2014**, *19*, 534-544.
21. D. Yarboro; A. Millar; R. Smith; *Wound management & prevention,* **2019**, *65*, 16-22.
22. A. Gupta; P. Avci; T. Dai; Y.-Y. Huang; M. Hamblin; *Adv Wound Care,* **2013**, *2*, 422-437.
23. S.K. Mishra; S. Raveendran; J.M. Ferreira; S. Kannan; *Langmuir.,* **2016**, *32*, 10305-10316.
24. A.I. Visan; G. Popescu-Pelin; G. Socol; *Polymers,* **2021**, *13*.
25. C. Wu; T. Wu; Z. Fang; J. Zheng; S. Xu; S. Chen; Y. Hu; X. Ye; *RSC Adv.,* **2016**, *6*, 46686-46695.
26. L. Remedio; J. Santos; V. Maciel; C. Yoshida; R. Carvalho; *Food Hydrocoll.,* **2018**, *87*.
27. X. Zhu; H. Wu; J. Yang; J. Tong; J. Yi; Z. Hu; J. Hu; T. Wang; L. Fan; *React. Funct. Polym,* **2015**, *91-92*, 71-76.
28. N. Cé; C.P.Z. Noreña; A. Brandelli; *CYTA J Food.,* **2012**, *10*, 21-26.
29. J.L. Webber; R. Namivandi-Zangeneh; S. Drozdek; K.A. Wilk; C. Boyer; E.H.H. Wong; B.H. Bradshaw-Hajek; M. Krasowska; D.A. Beattie; *Sci. Rep.,* **2021**, *11*, 1690.
30. M.L. Mangoni; A.M. McDermott; M. Zasloff; *Exp Dermatol,* **2016**, *25*, 167-173.
31. H. Felgueiras; M.T. Amorim; *Colloids Surf B Biointerfaces,* **2017**, *156*.
32. L.W. Place; T. Helmonds; S.F. Filocamo. *Development and Characterization of Methods to Encapsulate Nisin for Use as an Antimicrobial Agent*; Natick Soldier Research, Development and Engineering Center: 2018.
33. L. Cui; S. Gao; X. Song; L. Huang; H. Dong; J. Liu; F. Chen; S. Yu; *RSC Advances,* **2018**, *8*, 28433-28439.
34. R.I. Nakayama; K. Katsumata; Y. Niwa; N. Namiki; *Membranes,* **2020**, *10*.
35. A.K. Azad; N. Sermsintham; S. Chandrkrachang; W.F. Stevens; *Journal of biomedical materials research. Part B, Applied biomaterials,* **2004**, *69*, 216-222.
36. Z. Yu; F.K. Alsammarraie; F.X. Nayigiziki; W. Wang; B. Vardhanabhuti; A. Mustapha; M. Lin; *Food Res. Int.,* **2017**, *99*, 166-172.
37. S. Ahmed; S. Ikram; *J. Photochem. Photobiol. B, Biol.,* **2016**, *163*, 115-124.
38. A. Nouri; M.T. Yaraki; M. Ghorbanpour; S. Agarwal; V.K. Gupta; *Int. J. Biol. Macromol.,* **2018**, *109*, 1219-1231.