Nanodelci hitozana kot potencialni protimikrobni premaz

Chitosan nanoparticles as potential antimicrobial coating

Avtor / Author

Zdenka Peršin Fratnik[1], Uroš Maver[1,2], Metod Kolar[3], Olivera Šauperl[4], Lidija Fras Zemljič[4], Boštjan Vihar[1]

Ustanova / Institute

[1]Univerza v Mariboru, Medicinska fakulteta, Inštitut za biomedicinske vede, Maribor, Slovenija; [2]Univerza v Mariboru, Medicinska fakulteta, Katedra za farmakologijo, Maribor, Slovenija; [3]Vitiva d.d., Markovci, Slovenija; [4]Univerza v Mariboru, Fakulteta za strojništvo, Laboratorij za obdelavo in preskušanje polimernih materialov, Maribor, Slovenija;

[1]University of Maribor, Faculty of Medicine, Institute of Biomedical Sciences, Maribor, Slovenia; [2]University of Maribor, Faculty of Medicine, Department of Pharmacology, Maribor, Slovenia; [3]Vitiva d.d., Markovci, Slovenia; [4]University of Maribor, Faculty of Mechanical Engineering, Laboratory for Characterisation and Processing of Polymers, Maribor, Slovenia;

Ključne besede:
hitozan, nanodelci, protimikrobna aktivnost, premazi, medicinski tekstilni materiali

Key words:
chitosan, nanoparticles, antimicrobial activity, coatings, medical textile materials

Članek prispel / Received
3. 10. 2024
Članek sprejet / Accepted
14. 11. 2024

Naslov za dopisovanje / Correspondence
Assist. dr. Boštjan Vihar, Institute of Biomedical Sciences, bostjan.vihar@um.si, +386 2 234 5872
dr. Zdenka Peršin Fratnik, University of Maribor
zdenka.persin@um.si
+386 2 234 5840

Izvleček

Namen: *Namen študije je bil ugotoviti protimikrobno učinkovitost hitozanskih nanodelcev v primerjavi s hitozansko raztopino in sled temu njihovo učinkovito rabo na medicinskih tekstilnih materialih.*
Metode: *Pripravljena je bila disperzija nanodelcev z različnim masnim deležem hitozana. Na osnovi rezultatov analiz določitve naboja in velikosti delcev, spremembe površinskega naboja (zeta potencial), prostih aminskih skupin (polielektrolitska titracija), morfologije in protimikrobnosti je bil najbolj optimalen sistem hitozanskih nanodelcev uporabljen za nanos na tekstilni material. Učinek nanosa disperzije nanodelcev v primerjavi z nanosom hitozanske raztopine je bil preverjen s testom protimikrobnosti*

Abstract

Purpose: *To evaluate the antimicrobial efficacy of chitosan nanoparticles in comparison to a chitosan solution, as well as to assess their practical application on medical textile materials.*
Methods: *Dispersions of nanoparticles with different weight fractions of chitosan were prepared. An optimal chitosan nanoparticle system for textile material application was identified based on analyses of charge, particle size, changes in surface charge (zeta potential), concentration of free amine groups (polyelectrolyte titration), morphology, and antimicrobial properties. The effect of the nanoparticles dispersion application compared to the chitosan solution was determined using the*

https://doi.org/10.18690/actabiomed.274, CC BY 4.0

*ASTM E2149-01 z rabo Gram-pozitivne (*Staphylococcus aureus*) in Gram-negativne (*Escherichia coli*) bakterije ter glive (*Candida albicans*).*

***Rezultati:** Bolj učinkovit nanos hitozana je bil zaznan pri rabi nanodelcev zaradi njihove večje specifične površine in posledično dostopnosti hitozana, nanesenega tako na notranji kot zunanji strani funkcionaliziranega materiala. Sled temu se je nanos hitozanskih nanodelcev izkazal kot učinkovita protimikrobna zaščita napram vsem trem testiranim mikroorganizmom.*

***Zaključki:** Hitozanski nanos v obliki nanodelcev v primerjavi z raztopino nudi bolj uspešno protimikrobno zaščito in tako funkcionalizirani materiali ponazarjajo perspektivno uporabo na področju medicinskih tekstilnih izdelkov.*

*ASTM E2149-01 antimicrobial test with Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria and a fungus (*Candida albicans*).*

***Results:** More effective application of chitosan was noted with the incorporation of chitosan nanoparticles. Applying chitosan on the inside and outside of the functional material enhanced the specific surface area and improved the accessibility of the chitosan coatings. Applying chitosan nanoparticles provided effective antimicrobial protection against all three microorganisms tested.*

***Conclusions:** Chitosan deposition in the form of nanoparticles compared in form of solution, offered better antimicrobial protection. Such functionalised material presents a promising application in medical textile materials.*

INTRODUCTION

As a derivative of chitin, the second most abundant polysaccharide after cellulose, chitosan is a widely available biomaterial. Due to its biochemical properties, it has gathered much attention (1), especially within the biomedical research community (2) and food (3) and textile (4, 5) industries.

As a natural material, chitosan exhibits excellent biodegradability and good biocompatibility while showing effective antimicrobial properties against bacteria and fungi (1, 2, 6). Chitosan, soluble at low pH due to protonated amino groups, is the focus of most studies investigating its biological functions (7, 8). However, research has also shown that its biological functions can be improved by forming nanoparticles and modifying their size, complementing with other materials (2, 5, 9). One of the simplest and most effective ways of generating chitosan nanoparticles is by ionic gelation with tripolyphosphate (TPP). This technique has been shown to preserve the biological function of chitosan while also improving its effectiveness in drug and nucleic acid delivery systems (10). By creating composites of both components, the positively charged amino groups of chitosan are repulsed by each other and attract the negatively charged phosphate groups of the TPP, forming aggregates. Particle size and suspension stability are a function of surface charge, which correlates with the pH of the solution (11, 12) and, likely, the chitosan to TPP ratio.

In this work, we analyse the effects of varying the chitosan: TPP ratio on the size of the chitosan particles, stability of the suspension, and antimicrobial performance. Our goal was to prepare antimicrobial chitosan-based nanoparticles, which would be ideal as a coating for polymer-based surfaces for medical textile products (Figure 1). The focus of this research was on developing optimal preparation methods that ensure satisfactory stability and antimicrobial properties, thereby enhancing the antimicrobial effectiveness when applied to material surfaces compared to the conventional chitosan solution used for functionalisation in medical textile applications.

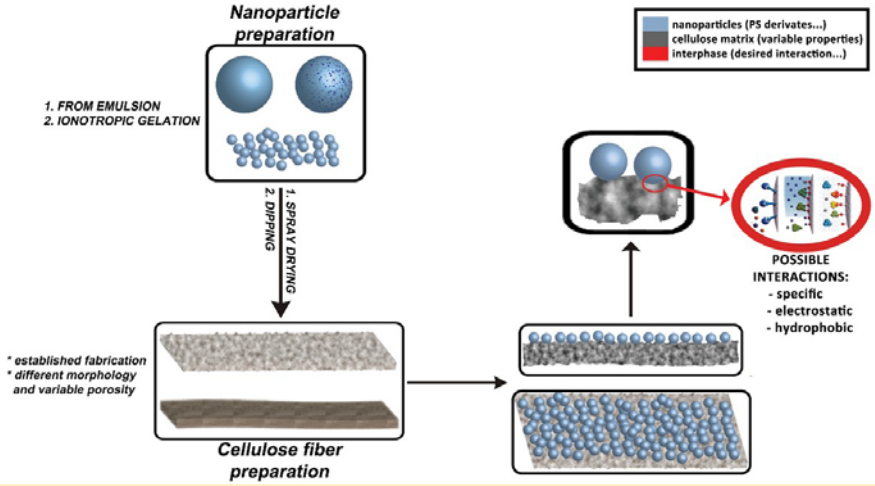


Figure 1. *Schematic depiction of the functionalised material.*

MATERIALS AND METHODS

Materials

Chitosan was obtained from Mahtani Chitosan Pvt. Ltd., India. The average molecular mass was 200,000 g/mol with a 90% degree of deacetylation. It was used without further purification. Sodium triphosphate, poly(vinylsulfonic acid) sodium salt, o-toluidine blue, hydrochloric acid (HCl), 0.1 M potassium hydroxide solution (KOH), and potassium chloride (KCl) were purchased from Merck KGaA, Germany.

Millipore-quality deionised water (Milli-Q® Type 1 Ultrapure Water Systems, Merck KGaA, Germany), with resistivity >18 MΩ was utilised in all experiments.

Chitosan solution preparation

A chitosan solution was prepared using 10 g of chitosan suspended in 990 mL of Milli-Q water. The dissolution was achieved by gradually adding concentrated lactic acid at 40°C with constant agitation for 1 h. Afterwards, the solution was stirred overnight at room temperature. Finally, the pH was adjusted to 3.6 using concentrated lactic acid, and water was added to a final volume of 1 L. The sodium triphosphate (TPP) solution was prepared by dissolving 0.7 g TPP (tripolyphosphate) in 1 L of Milli-Q water.

The poly(vinylsulfonic acid) sodium salt solution (wt = 25%) was prepared by diluting a concentrated solution with Milli-Q water to a final concentration of 3.3 mM.

Chitosan nanoparticles preparation

As reported by Calvo et al. in 1997 (13), nanoparticles can be formed by ionic gelation using specific reactant ratios of chitosan and TPP. Samples with varying mass ratios were prepared according to the equation:

$$V_{CH} = \frac{\gamma_{TPP} \times V_{TPP}}{\gamma_{CH} + \gamma_{TPP} \times z_{(CH:TPP)}}$$

where V_{CH} is the volume of the chitosan solution, γ_{TPP} is the mass concentration of TPP, V_{TPP} is the volume of the TPP solution, γ_{CH} is the mass concentration of chitosan, and $z_{(CH:TPP)}$ is the mass ratio of chitosan to TPP.

During constant stirring, TPP was gradually added to 50 mL of chitosan solution until the desired mass ratio $z_{(CH/TPP)}$ was reached. The process was performed for z = 3, 4, 5, 6, 7, and 8. After stirring for 1 h, the suspensions were centrifuged at 1500 g for 10 min. The pH was then adjusted to 4. The chitosan nanoparticles sample notation with listed mass ratios and the final chitosan (CH) and TPP mass concentrations are listed in Table 1.

Table 1. Final concentrations of chitosan and TPP in nanoparticles dispersions

Sample	$z_{(CH/TPP)}$	γ_{CH}/ (mg/mL)	γ_{TPP}/ (mg/mL)
CHn3	3	1.48	0.49
CHn4	4	1.79	0.45
CHn5	5	2.06	0.41
CHn6	6	2.28	0.38
CHn7	7	2.47	0.35
CHn8	8	2.64	0.33

Legend: CHn = chitosan nanoparticles dispersion; $z_{(CH/TPP)}$ = mass ratio of chitosan:TPP; γ_{CH} = mass concentration; γ_{TPP} = mass concentration of TPP

Functional material preparation

The fibre material sample produced by Lenzing Ag, Austria was used as received for material functionalisation. The length of the fibres was 40 mm, and the linear density was 1.7 dtex. The prepared chitosan solutions and chitosan nanoparticles dispersions were characterised, and the one with the optimal antimicrobial effect was applied to the top side of a 10 g viscose fibre material sample using an airbrush (SP-575 Sparmax, Taiwan). Functionalised material samples were stored in a refrigerator at 5°C. Functional material sample notations and descriptions are listed in Table 2.

Table 2. Textile material functionalised with a chitosan solution and nanoparticles dispersion

Sample	Sample description
CV	Viscose fibres, non-treated
CV+CHs	Viscose fibres sprayed with chitosan solution
CV+CHn	Viscose fibres sprayed with chitosan nanoparticles dispersion depending on used mass ratio chitosan : TPP ($z_{(CH/TPP)}$)

Legend: CV = material sample in the form of viscose fibres, non-treated; CV+CHs = material sample in the form of viscose fibres sprayed with chitosan solution (CHs); CV+CHn = material sample in the form of viscose fibres sprayed with chitosan nanoparticles dispersion

Characterisation of the chitosan solution and nanoparticles dispersion

Determining the particle size, charge, and zeta potential

Particle size distribution in the dispersion sample was measured using dynamic light scattering, while zeta potential was determined with a Zetasizer Nano ZS (Malvern Instruments Ltd, United Kingdom) equipped with a DTS10012 cuvette and DTS1060 capillary cell. The samples were sonicated in an ultrasound bath for 5 min before measurement. The measurement was performed at 25°C, using a He-Ne laser at a wavelength of 633 nm. Three measurements per sample were carried out for 120 s.

Determining the free amino groups by polyelectrolyte titration

The chitosan solution and nanoparticles dispersion samples were measured using the titrator DL53 (Mettler Toledo, USA) equipped with a phototrode D660. Then, 1 mL of sample and 0.5 mL of indicator o-toluidine blue were added to the titration vessel and diluted with water to 40 mL. The prepared sample solutions were titrated with 3.3 mM poly(vinylsulfonic acid) sodium salt solution until a colour change from blue to red was observed.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to confirm the formation of nanoparticles (within the dispersion sample) and evaluate their morphology. A volume of 25 µL was withdrawn from each sample (suspension and dispersion) and carefully dropped onto a scanning electroscope microscope sample holder with attached double-sided adhesive carbon tape (SPI Supplies, USA). The chitosan sample material was dried in an oven at 50°C for 10 min. Chitosan samples were imaged using a field emission scanning electron microscope (Supra 35 VP, Carl Zeiss, Germany) at an operating voltage of 1 keV.

Determining the antimicrobial activity

A modified ASTM E2149-01 standard was employed to assess the antimicrobial properties of the chitosan nanoparticles samples. This method evaluates antimicrobial activity under dynamic contact conditions. Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria, along with the single-celled fungus *Candida albicans*, served as test organisms. A test culture was prepared in nutrient broth and diluted with sterile 0.3 mM phosphate buffer (KH_2PO_4; pH

6.8) to achieve a final concentration of 1.5–3.0 × 10^5 colony-forming units (CFU) per millilitre. This working bacterial dilution was then combined with 1 mL of each nanoparticles sample in 250 mL Erlenmeyer flasks containing 50 mL of the dilution.
The flasks were incubated at 37°C with shaking for 1 h. After a 1:10 dilution, 1 mL of the sample-diluted solution was plated onto nutrient agar and incubated at 37°C for 24 h. The number of formed colonies was counted visually.
Antimicrobial activity was expressed as the percentage of microorganism reduction compared to the control. Microbiological testing was conducted at the National Laboratory for Health, Environment and Food in Maribor, Slovenia.

Characterisation of the functional material

The streaming potential measurement

The zeta potential of the fibre material samples was measured using the streaming potential method with a SurPASS Electrokinetic Analyzer (Anton Paar GmbH, Austria). Circular fibre samples (14 mm diameter) were mounted in a cylindrical cell to create a permeable plug. Consistent packing density was ensured through sample size, weight monitoring, and controlled compression. Samples were pre-soaked in distilled water for one hour before measurement to minimise substrate swelling effects.
A 1 mM KCl solution served as the electrolyte. The initial pH was adjusted to 10 using NaOH, and subsequent pH changes (from 10 to 2) were achieved through automatic titration with 50 mM HCl. Time-dependent zeta potential curves were obtained for various modified material samples at a constant pH of approximately 5.5 (without initial pH adjustment).

Potentiometric titration

A twin burette instrument (Mettler T-70, Mettler Toledo d.o.o., Slovenia) equipped with a combined glass electrode (Mettler T DG 117, Mettler Toledo d.o.o., Slovenia) was used for titrations. Burettes were filled with 0.1 M HCl and 0.1 M KOH solutions prepared with low-carbonate Milli-Q water. A 0.5 g material sample was titrated between pH 3 and 10 in both forward and back runs.
Ionic strength was maintained at 0.1 M by adding KCl. HCl and KOH additions kept the ionic strength within 2% of the initial value. Blank titrations were performed under identical conditions. The equilibrium criterion for timed additions was set at dE/dt = 0.1 mV/min. The total amount of weak acidic groups was calculated from the difference in added KOH volume between the sample and the blank. For more details on charge calculations, please refer to (14).

Scanning electron microscopy

The surface morphology of the material sample was examined using a scanning electron microscope (SUPRA 35 VP, Carl Zeiss, Germany). The sample was mounted onto a sample holder using adhesive carbon tape.

Determining the antimicrobial activity

The antimicrobial properties of the functionalised material were assessed using a similar modified ASTM E2149-01 standard method, as previously applied to chitosan samples.

RESULTS

Chitosan sample

It is known that at $z > 10$, chitosan dissolves completely, while at $z < 3$, the particles aggregate into large clusters. Thus, the suspension with the $z_{(CH/TPP)}$ mixing ratio 3:8 was used to obtain the nanoparticle dispersion. Twenty-four hours after preparation, sedimentation of the chitosan-TPP complex was observed, likely due to saturation of the reagents, followed by agglomeration. However, after centrifugation at 1500 g for 10 min, the particles in the supernatant remained the same quality as directly after preparation. Table 3 summarises the particle size distribution obtained from dynamic light scattering measurements.
Zeta potential measurements (Figure 2) were conducted to explore the potential correlation between particle charge and the presence of free amino groups in the chitosan nanoparticles sample.

The charged amino groups of chitosan are associated with its antimicrobial activity (15-17); thus, their availability

Table 3. The size of the chitosan nanoparticles depending on the chitosan (CH) mass to tripolyphosphate (TPP) ratio

Sample	z (mass ratio CH/TPP)					
z (mass ratio chitosan : TPP)	3	4	5	6	7	8
particle diameter [nm]	314.00	451.80	732.80	976.30	1296.00	1812.00
standard deviation	5.78	8.98	17.07	87.00	76.07	14.14

Legend: $z_{(CH/TPP)}$ = mass ratio of chitosan:TPP in the chitosan nanoparticles samples

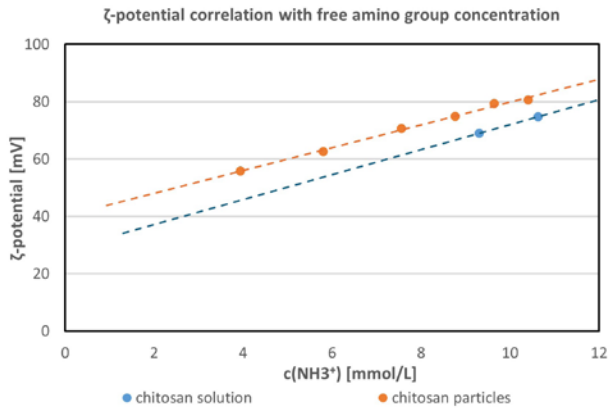


Figure 2. *Correlation between particle charge and free amino groups in the chitosan samples, obtained by zeta measurement.*

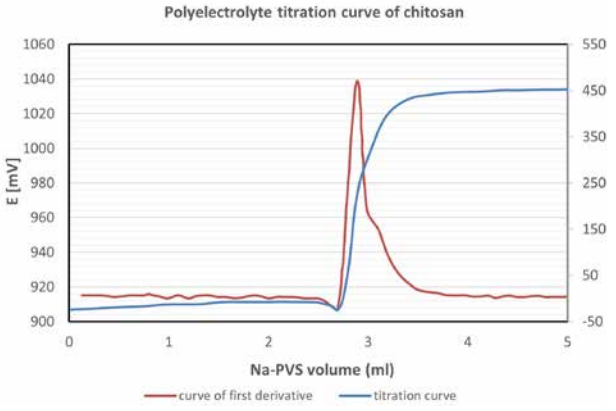


Figure 3. *Polyelectrolyte titration curves. The orange curve represents the calculated first derivative of the data, while the blue curve shows the measured titration data The maximum of blue curve represents the equivalence point.*

after gelation was analysed using polyelectrolyte titration. Na-PVS was added to a diluted chitosan-TPP sample containing the indicator o-toluidine blue. After fully neutralising the available amino groups in the formed Na-PVS chitosan complex, the Na-PVS reacted with the indicator, causing a change in absorbance at 660 nm. A sample diagram of the reaction is shown in Figure 3. The amount of free amino groups was calculated from the Na-PVS required to neutralise the chitosan with the results shown in Figure 4.

The results of the determined amino groups in the nanoparticles dispersions and chitosan solutions are given in Table 4.

A scanning electron microscope was used to define the effect of formed nanoparticles. Figure 5 shows the images of a dried chitosan nanoparticles suspension compared to a chitosan solution.

Chitosan exhibits antimicrobial activity against various bacteria and fungi, employing diverse mechanisms that are not entirely understood (18). Its antimicrobial activity has been investigated in solutions, gels, fibres, and films (19-22). With this in mind, the antimicrobial activity of chitosan nanoparticles dispersed on Gram-positive and Gram-negative bacteria and a fungus is presented in Table 5.

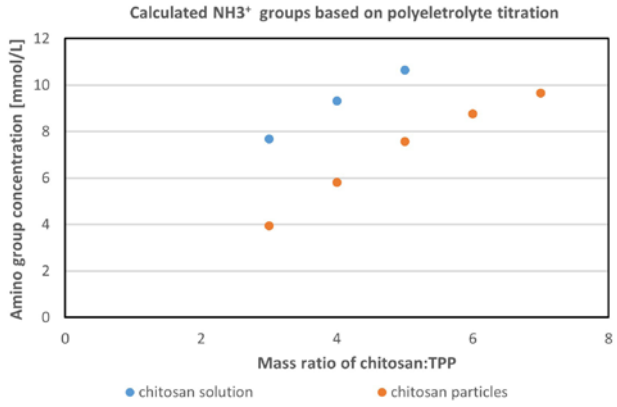


Figure 4. *The calculated concentration of free amino groups based on the Na-PVS required to neutralise the chitosan. Free amino groups of the chitosan-TPP particles are shown in orange. Chitosan solutions with equivalent chitosan concentrations (Z3-5) are shown in blue for comparison.*

Table 4. Amino group content of the chitosan samples based on polyelectrolyte titration

Sample	$n(NH_2)/m(H)$/ (mmol/g ND)	RSD/%
CHs3	5.177	2.35
CHs4	5.182	2.04
CHs5	5.156	3.57
CHn3	1.998	0.00
CHn4	2.585	3.27
CHn5	3.056	7.53
CHn6	3.287	2.17
CHn7	3.405	1.97
CHn8	3.498	1.82
TPP	0	0
H20	0	0

Legend: CHs = chitosan solution; CHn = chitosan nanoparticles dispersion; index = $z_{(CH/TPP)}$ = mass ratio of chitosan:TPP; TPP = sodium triphosphate solution

The most effective chitosan nanoparticles system—i.e. CHn5 $_{(z(CH:TPP=5))}$—was used for the functionalisation of a textile material sample. The effect was then compared to the use of a chitosan solution.

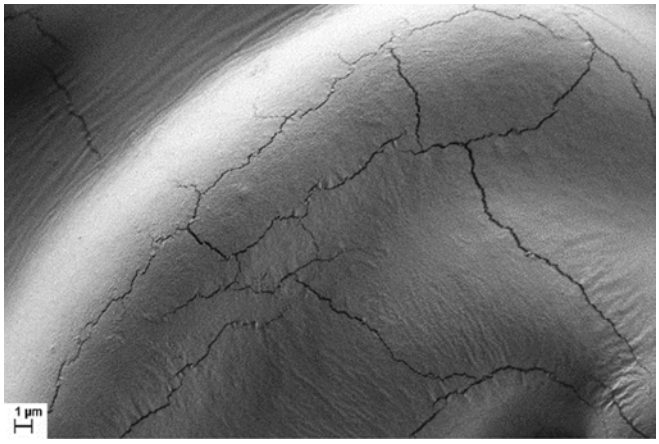
Functional material sample

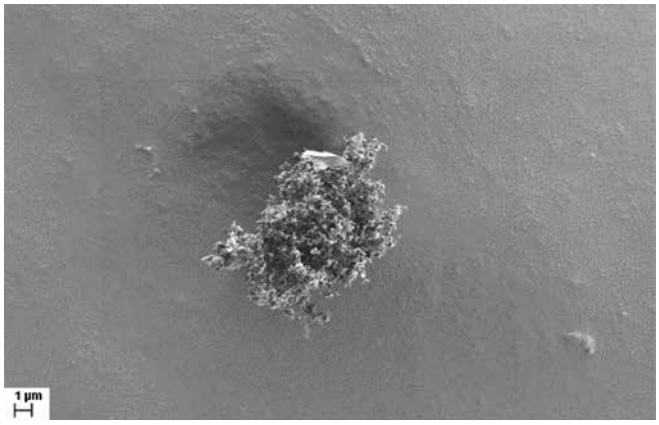
Figure 6 shows the results of the zeta potential of the functional material sample in dependence on the pH of the electrolyte solution.

Figure 7 illustrates the results of the positive charge (protonated amine groups) derived from potentiometric titration for the material sample, depending on the functionalisation used. The positive charge was calculated from isotherm curves of potentiometric titrations, as detailed by Cakara et al. (23).

Figure 8 presents the longitudinal image of the material sample obtained by SEM, depending on the functionalisation used.

The antimicrobial efficiency of the functionalised material was evaluated using the ASTM E 2149-01 standard. A reduction (R) of ≥75% was considered effective. The results of the reduction of two pathogenic bacteria (*S. aureus* and *E. coli*) and yeast (*C. albicans*) on the functional material samples are listed in Table 6. A non-functionalised material sample in the form of viscose fibres was used as a reference.





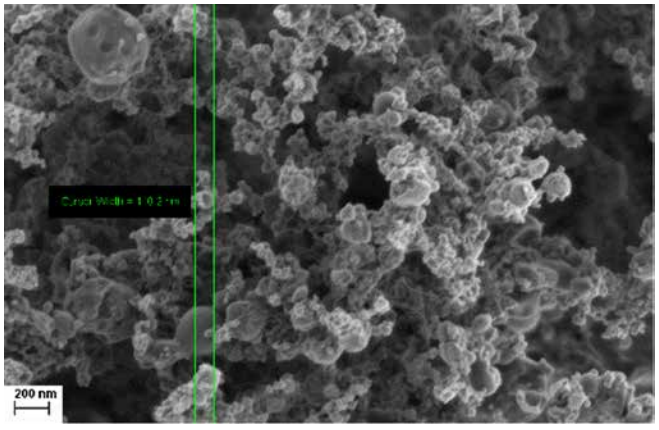


Figure 5. *SEM images of dried chitosan samples; a) CHs, b) and c) CHn at two magnifications.*

Table 5. Antimicrobial activity of the chitosan nanoparticles samples depending on z (mass ratio chitosan :TPP) presented as the reduction (R, %) of the bacteria Staphylococcus aureus *and* Escherichia coli *and the yeast* Candida albicans

Sample	Reduction of tested microorganisms R (%)		
	S. aureus	*E. coli*	*C. albicans*
z3	88	99	95
z4	92	99	98
z5	96	99	100
z6	95	99	98
z7	94	99	94
z8	93	100	91
Control	7	0	-7

Legend: $z_{(CH/TPP)}$ = mass ratio of chitosan:TPP in the chitosan nanoparticles samples

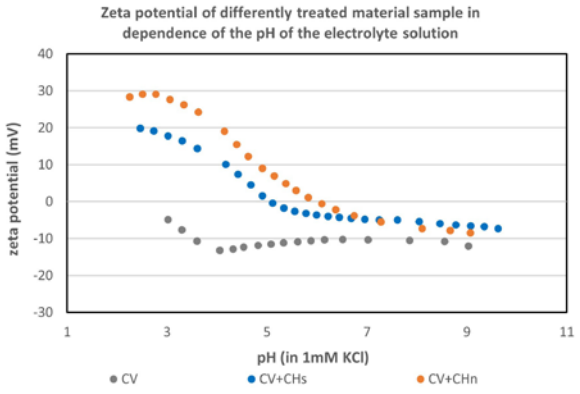


Figure 6. *Zeta potential of treated materials as a function of pH.*

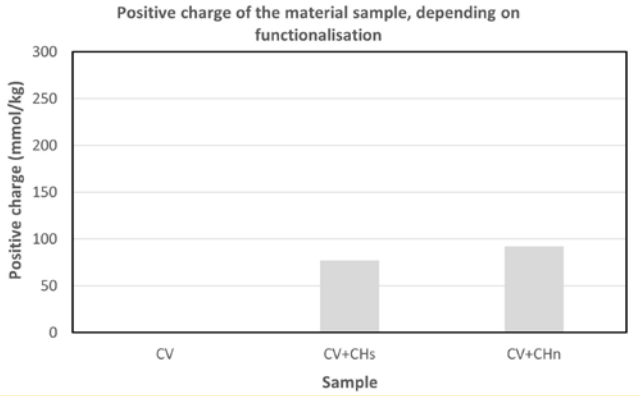


Figure 7. *The positive charge of the material sample depending on the functionalisation.*

Table 6. Reduction (R, %) of the pathogenic microorganisms for the material samples, depending on the functional procedure used

Sample	Reduction of tested microorganisms R (%)		
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*
CV	24	22	1
CV+CHs	78	61	86
CV+CHn	94	81	89

Legend: CV = material sample in the form of viscose fibres, non-treated; CV+CHs = material sample in the form of viscose fibres sprayed with chitosan solution (CHs); CV+CHn = material sample in the form of viscose fibres with chitosan nanoparticles dispersion

DISCUSSION

Chitosan sample

The size of the prepared chitosan nanoparticles increased as a function of the chitosan:TPP ratio. The smallest particles were observed in z = 3 suspensions at around 314 nm and reached 1296 nm by z = 7, as shown in Table 3. The measurements were repeated one week after preparation (not shown here). Changes in size were insignificant.

The zeta potential rose in direct correlation to the amount of free amino groups, suggesting that they are its primary cause. In addition, the zeta potential was above 30 mV in all chitosan samples (solution and dispersion) and was more pronounced in dispersion samples. This suggests that all nanoparticles dispersion samples were physically stable, which is extremely important for its practical use.

The nanoparticles dispersion samples, regardless of the chitosan:TPP ratio, also showed the presence of free amino groups, as in the chitosan solution (Table 4). The amount of amino groups increased with the increasing mass ratio of chitosan to TPP, and was most evident in the chitosan nanoparticles samples. Thus, the highest number of amino groups in the nanoparticles sample was observed at z = 8. Blank TPP and water probes showed no Na-PVS buffering properties, as expected. The difference in the amino group content between the chitosan solution and nanoparticles dispersion samples at the same concentration of chitosan (mass ratios of 3–5) varied from approximately 68% to 150%. The different amounts of amino groups in the chitosan solution and nanoparticles dispersion samples likely account for chitosan reacting with TPP during gelation, promoting lower amino group content.

SEM analysis of the chitosan samples showed that nanoparticles were successfully formed when chitosan was prepared with TPP, unlike the pure chitosan solutions in which such formation did not occur (Figure 5).

The results in Table 5 indicate that the chitosan nanoparticles successfully inhibited the Gram-negative bacteria and yeast, irrespective of the mass fraction of chitosan. Considering all the tested microorganisms, the most promising antimicrobial effect was evident by sample z = 5 (Table 5). A comparison with a chitosan solution with the same chitosan content (z = 5) can be made with our previous work (24, 25). When comparing samples of the solution (24) and nanoparticles dispersion with the same chitosan content (z = 5), the reduction values (R) for *S. aureus* were comparable (R = 98% for the chitosan solution (24) and R = 96% for the chitosan nanoparticles). In contrast, for the Gram-negative bacteria and yeast, the chitosan nanoparticles sample had more effective antimicrobial activity (39% higher reduction of *E. coli* and 16% more effective reduction of *C. albicans*) than chitosan in solution. Control samples containing water and TPP showed no antimicrobial activity.

Figure 9 plots the reduction (R, %) of *S. aureus*, *E. coli*, and *C. albicans* for the chitosan sample (z = 5; solution and nanoparticles dispersion) against the amount of free amino group content. The number of free amino

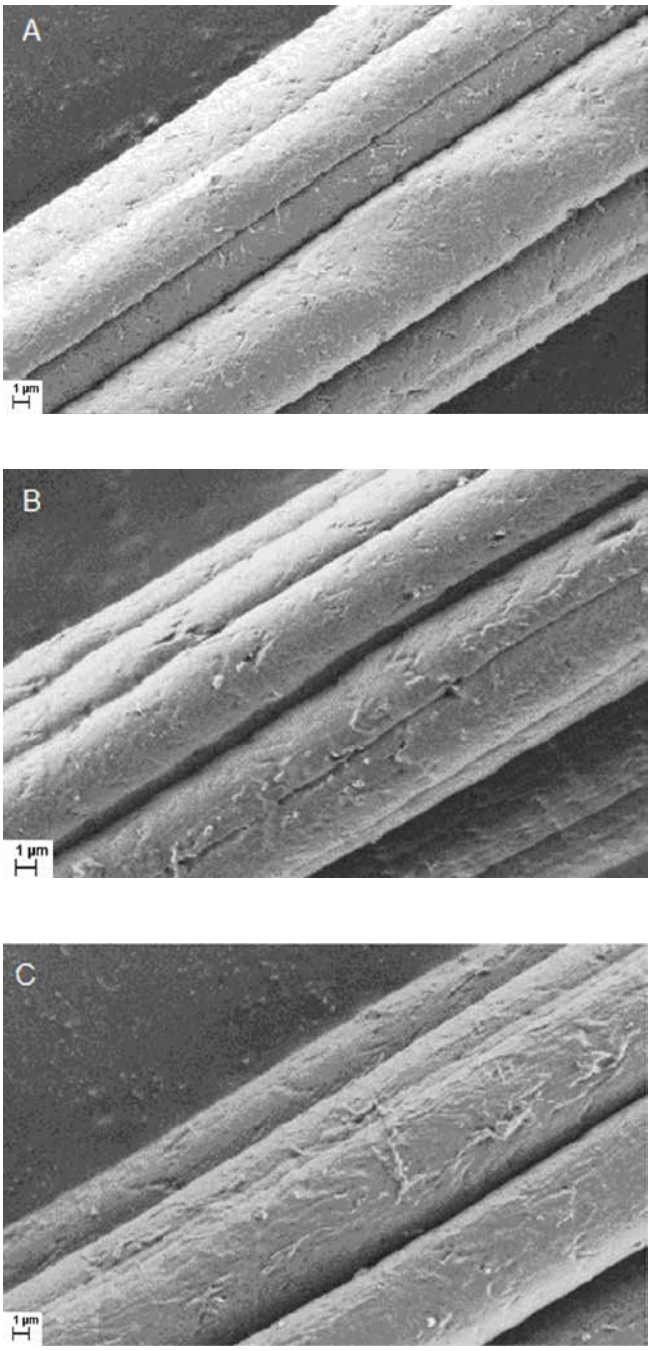


Figure 8. *SEM images of a longitudinal view of the material sample, depending on the functionalisation used; a) viscose fibre non-treated; b) viscose fibre functionalised using chitosan solution (CHs), and c) viscose fibre functionalised using chitosan nanoparticles dispersion (CHn).*

groups in the chitosan solution sample was higher than that of the chitosan nanoparticles dispersion. However, the higher amount of amino group did not reflect better antimicrobial effectiveness toward the tested microorganisms. The charged amino groups of chitosan have been associated with antimicrobial activity (26); thus, the obtained results clearly show that other effects (e.g. particle size and shape, hydrophilicity, and nutrient chelation) must play a role as well, especially in the Gram-negative bacteria and fungicidal activity. Further research will be required to conclusively determine why chitosan:TPP particle dispersions are most effective at z = 5 (6).

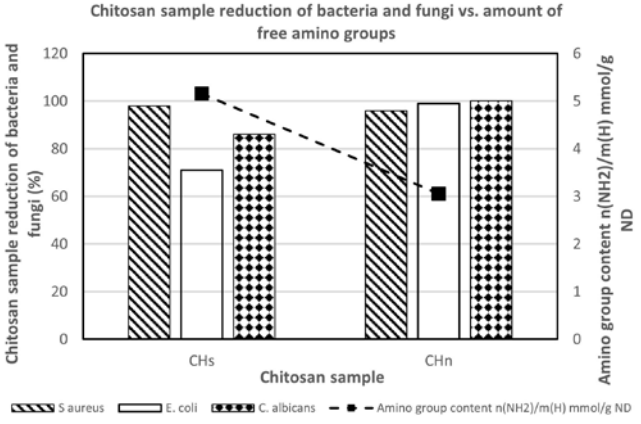


Figure 9. *The reduction of bacteria and yeast by chitosan samples in relation to the quantity of free amino groups.*

Particle size and composition also impact antimicrobial activity; however, this likely correlates to the free reactive groups on the surface of the particles (6, 27). A stable colloidal suspension is an important factor for maintaining the biological function of the nanoparticles. For this, sufficient surface charge is required to reduce agglomeration by electrostatic repulsion (28). Therefore, the ratio of chitosan to TPP needs to be adjusted accordingly.

Functional material sample

As shown in Figure 6, the reference material sample exhibited the typical behaviour of cellulose fibres. Thus, the material sample had a negative potential throughout the measuring range because of deprotonated carboxyl groups. The latter was measured and confirmed by potentiometric titration. The reference material sample showed 32 mmol/kg of deprotonated carboxyl groups. Due to the deprotonation of carboxyl groups above pH 3, the material sample showed anionic character up to this pH value, which is in accordance with a negative zeta potential. Since the material sample of viscose fibres was amphoteric in nature, it was positively charged in a strongly acidic medium, with the anionic character appearing with increasing pH. The functionalisation of the material sample with chitosan led to a positive charge, a consequence of the protonated amino groups of chitosan. The anionic character of the sample was due to the ionisation of the carboxyl groups of the material sample, as mentioned earlier. An increase in the plateau of positive zeta potential indicates an increased amount of amino groups. A rightward shift in the isoelectric point indicates an intense application of chitosan onto the surface of the material sample, namely the fibres. According to Figure 6, it is obvious that the nanoparticles - based coating on the material sample introduced a higher positive charge.

Figure 7 indicates that chitosan was successfully bound to the material sample. The data reveal that the material coated with nanoparticles had a higher availability of amino groups, which can be ascribed to the increased specific surface area and superior accessibility of chitosan on the internal and external surfaces of the fibres.

Figure 8 shows the longitudinal view of the reference and modified viscose fibres, which serves as an overview of the morphology of the bound layers of chitosan and its nanoparticles onto fibres. The image in Figure 8a shows the longitudinal appearance of the reference material sample, while Figure 8b presents the image of the material sample treated with chitosan solution. Figure 8c shows the material sample surface treated with the chitosan nanoparticles dispersion. The SEM images in Figures 8b and 8c show visible changes in the surface of the viscose fibres. Non-treated viscose fibres (Figure 8a) exhibited a smooth surface devoid of features or artefacts. Their longitudinal appearance was characterised by a uniform diameter and parallel interior lines known as striations. In cross-section, regular viscose fibres displayed irregular or serrated edges. The longitudinal appearance and cross-sectional characteristics were the result of the production process of the viscose fibres (wet

spinning). The material sample treated with chitosan solution (Figure 8b) showed a fibre surface characterised by areas that were only partially covered (adsorbed layer of chitosan). Still, the overall longitudinal appearance of the viscose fibres was unchanged. Since chitosan is completely dissolved at pH 4, it can be assumed that a very thin chitosan coating was formed by the adsorption process, which could not be seen with SEM at the magnification used. The application of chitosan nanoparticles to the material sample (Figure 8c) resulted in enhanced coverage of the surface of the viscose fibres. Precipitated chitosan nanoparticles were agglomerated at the fibrillary structure on the surface after chitosan was adsorbed onto the fibres.

The non-treated material sample, as expected, did not possess any antimicrobial effectiveness toward *S. aureus*, *E. coli*, and *C. albicans*, since the reduction was far below 75%. The material sample functionalised with the nanoparticles dispersion exhibited better antimicrobial properties than the material sample functionalised solely with chitosan solution. Microbiological testing indicates that, in addition to the chitosan nanostructure, the amount of amino groups had a significant influence on the antimicrobial properties. Material samples with higher amino group content generally exhibited a superior overall reduction of pathogenic microorganisms. When assessing the antimicrobial action of chitosan (regardless of its form) against the two bacterial strains selected, it becomes evident that chitosan exhibited different actions on the Gram-positive and Gram-negative bacteria. The reason lies in the bacterial cell wall. Chitosan reacts with lipopolysaccharides and proteins (anionic in nature) present on the cell surface of Gram-negative bacteria (29). By contrast, in Gram-positive bacteria, chitosan acts directly with the cell layer containing negatively charged peptidoglycan and teichoic acids (3). In the initial exploration period (30, 31), chitosan was identified as a remarkable antimicrobial agent. However, whether it acted as a bactericide (killing all or part of the bacteria) or as a bacteriostat (preventing bacteria from multiplying but not killing them) was not specified. While chitosan is increasingly favoured for its bacteriostatic properties (32), the precise mechanism of its action against bacteria remains a subject of ongoing research. Chitosan is also effective against fungi, but its fungistatic properties are more important (33).

CONCLUSIONS

Chitosan nanoparticles were successfully synthesised by varying several parameters of chitosan and sodium tripolyphosphate polyelectrolytes. Increasing the mass ratio of chitosan:TPP in the nanoparticles dispersion sample increased the amount of free amino groups and thus the charge, hydrodynamic radius, zeta potential, and the antimicrobial effectiveness toward the tested microorganisms. The dispersion sample of chitosan:TPP with the mass ratio z = 5 was the most promising. Thus, it was used for material sample functionalisation.

One of the study's main objectives was to evaluate the antimicrobial effect of a prepared chitosan nanoparticles dispersion as a coating on a (real) material sample, compared to a material sample surface functionalised by chitosan solution. The chitosan nanoparticles dispersion coating was effective toward the microorganisms tested—i.e., Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria and yeast (*Candida albicans*)— due to the large specific area of nanoparticles, the chitosan could be successfully placed onto the material surface (i.e., the inner and outer side of the fibre). As a result, the greater presence of chitosan on the surface of the material provided a substantial antimicrobial effect, facilitating diffusion into the cells of the pathogenic microorganisms and resulting in more effective degradation.

Prepared chitosan nanoparticles can be applied as a coating to the surface of materials, finding applications in several areas, such as:

- Antibacterial agents in the pharmaceutical industry and cosmetics;
- Antifungal agents in gels and creams, especially those without polyethylene glycol additives;
- Coatings for textiles, which are applicable in hygienic products (such as diapers, wipes, surgical gowns, face masks, and bedding) and medical materials (including tampons, gauze, bandages, and wound dressings).

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support for this study received from the Slovenian Research Agency (grant numbers: P3-0036, I0-0029, P2-0118, J7-4492 and L7-4494).

REFERENCES

1. Zou P, Yang X, Wang J, Li Y, Yu H, Zhang Y, et al. Advances in characterisation and biological activities of chitosan and chitosan oligosaccharides. Food Chem. 2016;190:1174-81.
2. Periayah MH, Halim AS, Saad AZ. Chitosan: A Promising Marine Polysaccharide for Biomedical Research. Pharmacogn Rev. 2016;10(19):39-42.
3. Hosseinnejad M, Jafari SM. Evaluation of different factors affecting antimicrobial properties of chitosan. International Journal of Biological Macromolecules. 2016;85:467-75.
4. Lopes C, Soares J, Tavaria F, Duarte A, Correia O, Sokhatska O, et al. Chitosan Coated Textiles May Improve Atopic Dermatitis Severity by Modulating Skin Staphylococcal Profile: A Randomized Controlled Trial. PLoS One. 2015;10(11):e0142844.
5. Ferrero F, Periolatto M. Antimicrobial finish of textiles by chitosan UV-curing. J Nanosci Nanotechnol. 2012;12(6):4803-10.
6. Ali SW, Joshi M, Rajendran S. Modulation of Size, Shape and Surface Charge of Chitosan Nanoparticles with Reference to Antimicrobial Activity. Adv Sci Lett. 2010;3(4):452-60.
7. Martins DB, Nasario FD, Silva-Goncalves LC, de Oliveira Tiera VA, Arcisio-Miranda M, Tiera MJ, et al. Chitosan derivatives targeting lipid bilayers: Synthesis, biological activity and interaction with model membranes. Carbohydr Polym. 2018;181:1213-23.
8. Xu R, Aotegen B, Zhong Z. Synthesis, characterisation and biological activity of C6-Schiff bases derivatives of chitosan. Int J Biol Macromol. 2017;105(Pt 3):1563-71.
9. Elgadir MA, Uddin MS, Ferdosh S, Adam A, Chowdhury AJK, Sarker MZI. Impact of chitosan composites and chitosan nanoparticle composites on various drug delivery systems: A review. J Food Drug Anal. 2015;23(4):619-29.
10. Bugnicourt L, Ladavière C. Interests of chitosan nanoparticles ionically cross-linked with tripolyphosphate for biomedical applications. Progress in Polymer Science. 2016;60:1-17.
11. Lopez-Leon T, Carvalho EL, Seijo B, Ortega-Vinuesa JL, Bastos-Gonzalez D. Physicochemical characterisation of chitosan nanoparticles: electrokinetic and stability behavior. J Colloid Interface Sci. 2005;283(2):344-51.
12. Gan Q, Wang T, Cochrane C, McCarron P. Modulation of surface charge, particle size and morphological properties of chitosan-TPP nanoparticles intended for gene delivery. Colloid Surface B. 2005;44(2-3):65-73.
13. Calvo P, RemunanLopez C, VilaJato JL, Alonso MJ. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. J Appl Polym Sci. 1997;63(1):125-32.
14. Laine J, Lovgren L, Stenius P, Sjoberg S. Potentiometric Titration of Unbleached Kraft Cellulose Fiber Surfaces. Colloid Surface A. 1994;88(2-3):277-87.

15. Li ZH, Yang F, Yang RD. Synthesis and characterisation of chitosan derivatives with dual-antibacterial functional groups. International Journal of Biological Macromolecules. 2015;75:378-87.
16. Qin CQ, Li HR, Xiao Q, Liu Y, Zhu JC, Du YM. Water-solubility of chitosan and its antimicrobial activity. Carbohyd Polym. 2006;63(3):367-74.
17. Raafat D, Sahl HG. Chitosan and its antimicrobial potential - a critical literature survey. Microb Biotechnol. 2009;2(2):186-201.
18. Ke CL, Deng FS, Chuang CY, Lin CH. Antimicrobial Actions and Applications of Chitosan. Polymers-Basel. 2021;13(6).
19. Goy RC, de Britto D, Assis OBG. A Review of the Antimicrobial Activity of Chitosan. Polimeros. 2009;19(3):241-7.
20. Kong M, Chen XG, Xing K, Park HJ. Antimicrobial properties of chitosan and mode of action: A state of the art review. Int J Food Microbiol. 2010;144(1):51-63.
21. Sahariah P, Másson M. Antimicrobial Chitosan and Chitosan Derivatives: A Review of the Structure-Activity Relationship. Biomacromolecules. 2017;18(11):3846-68.
22. Abd El-Hack ME, El-Saadony MT, Shafi ME, Zabermawi NM, Arif M, Batiha GE, et al. Antimicrobial and antioxidant properties of chitosan and its derivatives and their applications: A review. International Journal of Biological Macromolecules. 2020;164:2726-44.
23. Cakara D, Fras L, Bracic M, Kleinschek KS. Protonation behavior of cotton fabric with irreversibly adsorbed chitosan: A potentiometric titration study. Carbohyd Polym. 2009;78(1):36-40.
24. Ristic T, Hribernik S, Fras-Zemljic L. Electrokinetic properties of fibres functionalised by chitosan and chitosan nanoparticles. Cellulose. 2015;22(6):3811-23.
25. Ristic T, Persin Z, Kuncic MK, Kosalec I, Zemljic LF. The evaluation of the in vitro antimicrobial properties of fibers functionalised by chitosan nanoparticles. Text Res J. 2019;89(5):748-61.
26. Guarnieri A, Triunfo M, Scieuzo C, Ianniciello D, Tafi E, Hahn T, et al. Antimicrobial properties of chitosan from different developmental stages of the bioconverter insect. Sci Rep-Uk. 2022;12(1).
27. Du WL, Niu SS, Xu YL, Xu ZR, Fan CL. Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions. Carbohyd Polym. 2009;75(3):385-9.
28. Sonam, Chaudhary H, Arora V, Kholi K, Kumar V. Effect of Physicochemical Properties of Biodegradable Polymers on Nano Drug Delivery. Polym Rev. 2013;53(4):546-67.
29. Nikaido H, Vaara M. Molecular-Basis of Bacterial Outer-Membrane Permeability. Microbiol Rev. 1985;49(1):1-32.
30. Young DH, Kohle H, Kauss H. Effect of Chitosan on Membrane-Permeability of Suspension-Cultured Glycine-Max and Phaseolus-Vulgaris Cells. Plant Physiol. 1982;70(5):1449-54.
31. Kumar ABV, Varadara MC, Gowda LR, Tharanathan RN. Characterisation of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and Pronase, and their bactericidal action against Bacillus cereus and Escherichia coli. Biochem J. 2005;391:167-75.
32. Coma V, Martial-Gros A, Garreau S, Copinet A, Salin F, Deschamps A. Edible antimicrobial films based on chitosan matrix. J Food Sci. 2002;67(3):1162-9.
33. Raafat D, von Bargen K, Haas A, Sahl HG. Insights into the mode of action of chitosan as an antibacterial compound. Appl Environ Microb. 2008;74(12):3764-73.