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A Comparative Study Between the Antibacterial Effect of Nisin and Nisin-Loaded Chitosan/Alginate Nanoparticles on the Growth of *Staphylococcus aureus* in Raw and Pasteurized Milk Samples

Maryam Zohri · Mohammad Shafiee Alavidjeh · Ismaeil Haririan · Mehdi Shafiee Ardestani · Seyed Esmaeil Sadat Ebrahimi · Hadi Tarighati Sani · Seyed Kazem Sadjadi

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Abstract The aim of this study was to evaluate the antibacterial effect of nisin-loaded chitosan/alginate nanoparticles as a novel antibacterial delivery vehicle. The nisin-loaded nanoparticles were prepared using colloidal dispersion of the chitosan/alginate polymers in the presence of nisin. After the preparation of the nisin-loaded nanoparticles, their physicochemical properties such as size, shape, and zeta potential of the formulations were studied using scanning electron microscope and nanosizer instruments, consecutively. FTIR and differential scanning calorimetery studies were performed to investigate polymer–polymer or polymer–protein interactions. Next, the release kinetics and entrapment efficiency of the nisin-loaded nanoparticles were examined to assess the application potential of these formulations as a candidate vector. For measuring the antibacterial activity of the nisin-loaded nanoparticles, agar diffusion and MIC methods were employed. The samples under investigation for total microbial counts were pasteurized and raw milks each of which contained the nisin-loaded nanoparticles and inoculated *Staphylococcus aureus* (ATCC 19117 at 10^6 CFU/mL), pasteurized and raw milks each included free nisin and *S. aureus* (10^6 CFU/mL), and pasteurized and raw milks each had *S. aureus* (10^6 CFU/mL) in as control. Total counts of *S. aureus* were measured after 24 and 48 h for the pasteurized milk samples and after the time intervals of 0, 6, 10, 14, 18, and 24 h for the raw milk samples, respectively. According to the results, entrapment efficiency of nisin inside of the nanoparticles was about 90–95%. The average size of the nanoparticles was 205 nm, and the average zeta potential of them was −47 mV. In agar diffusion assay, an antibacterial activity (inhibition zone diameter, at 450 IU/mL) about 2 times higher than that of free nisin was observed for the nisin-loaded nanoparticles. MIC of the nisin-loaded nanoparticles (0.5 mg/mL) was about four times less than that of free nisin (2 mg/mL). Evaluation of the kinetic of the growth of *S. aureus* based on the total counts in the raw and pasteurized milks revealed that the nisin-loaded nanoparticles were able to inhibit more effectively the growth of *S. aureus* than free nisin during longer incubation periods. In other words, the decrease in the population of *S. aureus* for free nisin and the nisin-loaded nanoparticles in pasteurized milk was the same after 24 h of incubation while lessening in the growth of *S. aureus* was more marked for the nisin-loaded nanoparticles than the samples containing only free nisin after 48 h of incubation. Although the same growth reduction profile in *S. aureus* was noticed for free nisin and the nisin-loaded nanoparticles in the raw milk up to 14 h of

M. Zohri (✉)
Science and Research Branch, Islamic Azad University, Tehran, Iran
e-mail: mzohri@srbiau.ac.ir

M. S. Alavidjeh · I. Haririan
Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Enghelab Street, Tehran, Iran

M. S. Ardestani
Research and Development and Hepatitis B Department, Research and Production Complex, Pasteur Institute of Iran, Tehran, Iran

S. E. S. Ebrahimi
Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

H. T. Sani
Research Dairy Laboratory of Mihan Factory, Islamshahr, Iran

S. K. Sadjadi
Pharmaceutical Science Branch, Islamic Azad University, Tehran, Iran

incubation, after this time the nisin-loaded nanoparticles showed higher growth inhibition than free nisin. Since, generally, naked nisin has greater interactions with the ingredients present in milk samples in comparison with the protected nisin. Therefore, it is concluded that the antibacterial activity of nisin naturally decreases more during longer times of incubation than the protected nisin with the chitosan/alginate nanoparticles. Consequently, this protection increases and keeps antibacterial efficiency of nisin in comparison with free nisin during longer times of storage. These results can pave the way for further research and use of these nanoparticles as new antimicrobial agents in various realms of dairy products.

Keywords Nisin · Chitosan/Alginate nanoparticles · *Staphylococcus aureus* · Antibacterial activity · Raw milk · Pasteurized milk

Introduction

Among controlled release formulations, polymeric nano and micro particles have shown great promise for protein delivery [14]. One of the most attractive areas of research in drug delivery today is the design of nanosystems that are able to deliver drug/drug-like agents at the right dosage to the right place at favorable times. These nanosystems are often submicron particles containing entrapped drugs intended for enteral or parenteral administration. They may prevent or minimize biological degradation of drug/drug-like agents. One of the agents that consists of several closely related polypeptide antibiotics and is widely used as a preservative in several food products such as processed cheese, dairy desserts, and canned foods is nisin. This antimicrobial protein exhibits inhibitory activity against spore-forming bacteria and other Gram-positive spoilage and pathogenic bacteria including *Listeria monocytogenes* [15]. Kelly et al. investigated antimicrobial action of nisin against *Salmonella typhimurium* lipopolysaccharide mutants. They noticed that nisin sensitivity was associated with the extent of saccharide deletions from the outer membrane core oligosaccharide. Results of their work showed that the core oligosaccharide in lipopolysaccharide plays a role in nisin sensitivity. Schillinger et al. used bacteriocinogenic lactic acid bacteria to inhibit spontaneous nisin-resistant mutants of *Listeria monocytogenes* Scott A [17]. They found that the use of nisin in combination with a starter culture producing a non-nisin antilisterial bacteriocin may prevent the emergence of nisin-resistant mutants of *L. monocytogenes* [15]. Davies et al. [6] used nisin as a preservative in ricotta type cheeses to control the food-borne pathogen *L. monocytogenes* and obtained a high level of retention over the 10-week incubation period at 6–8 °C, with only 10–32% nisin loss. Benech et al. [3] studied the inhibition of *Listeria innocua* in Cheddar cheese by either adding of nisin Z in liposomes or by its in situ production in mixed culture. They noticed that encapsulation of nisin Z in liposomes can provide a powerful tool for improving nisin stability and inhibitory action in the cheese matrix while protecting the cheese starter from the detrimental action of nisin during cheese production. Also, they studied the impact of nisin producing culture and liposome-encapsulated nisin on ripening of *Lactobacillus casei* added Cheddar cheese and found that incorporating *L. casei* and the nisinogenic culture into cheese produced a debittering effect and improved cheese flavor quality. Cheeses with added *L. casei* and liposome-encapsulated nisin Z exhibited the highest flavor intensity and was first ranked for sensory characterizations [2]. Mellite et al. evaluated inhibitory effect of the growth of *S. aureus* by nisin-containing modified alginate films and beads on beef. They suggested that sterile, hydrophobic, and biodegradable films or beads incorporating various amounts of nisin could be used to control the growth of pathogens or microorganisms responsible for spoilage at the surface of round beef or other meat products [10]. Wan et al. [18] studied the incorporation of nisin in micro particles of calcium alginate and obtained an incorporation efficiency of 87–93% besides the loaded form was 100% active against an indicator culture of *Lactobacillus curvatus* both in MRS broth and reconstituted skim milk. Mirdamadi et al. studied the effect of liposomes containing nisin and a culture producing nisin against *L. monocytogenes* (ATCC 19117), *S. aureus* (ATCC 25923) and *Eshershia coli* (ATCC 25922) and found that nisin-loaded liposomes were more effective than free nisin [11]. The results of others showed that encapsulation of nisin can increase antimicrobial properties of nisin and prevent from unwilling interactions with other ingredient in food materials. Carriers that are currently used for this purpose can interact with nisin, and this is unfavorable due to reduction in its antibacterial strength [2, 3]. Staphylococcal food poisoning is caused by the consumption of food containing staphylococcal entero toxins. For toxic levels of enterotoxin to occur, extensive multiplication of staphylococci cells generally needs to have taken place in the food. *S. aureus* causes several infections that compromise food safety because of their frequency and the fact that they do not necessarily prevent the infected person from working. Various types of skin eruptions and inflammations (boils, acne, styes, etc.) and wounds, sometimes as small as minor damage around fingernails, can harbor large numbers of the organisms. *S. aureus* can also be the cause of respiratory infections or may become established in the gut, causing enteritis. Due to the high incidence of *S. aureus* carriage by humans, prevention of staphylococcal food poisoning relies on good hygienic practices to reduce the incidence of

contamination of food by food handlers. Therefore, growth prevention of *S. aureus* can increase the shelf-life of the products probably containing *S. aureus* contamination. Among controlled release formulations, polymeric nano- and micro particles have shown great promise for protein delivery [10]. Previous works focused on the systems such as liposomes, alginate films, or other micro particles that exhibited different disadvantage, For example, liposomes can interact with nisin, and this leads to a decrease in its antimicrobial activity. Poly ionic complexes of chitosan/alginate are formed through ionic gelation due to interactions between the carboxyl groups of alginate and the amine groups of chitosan. Chitosan/alginate nanoparticle has biocompatible and biodegradable characteristics. The formed complex hydrogel protects the encapsulant and controls the release of the encapsulated material better than either of the alginate or chitosan alone. Another advantage of this delivery system is its safety, which makes possible the repeated administration of chitosan/alginate micro- and nanoparticles, which have been widely used for the encapsulation of drugs, proteins, and oligonucleotides [16]. Li et al. studied quaternized chitosan/alginate nanoparticles for protein delivery. They reported that the loading efficiency of bovine serum albumin (BSA) was affected by the concentration, size, and the molecular parameters of the polymers and protein [9]. Regarding the advantages of the chitosan/alginate nanoparticles and their suitability as a vector for drug-like agents, it was decided to further examine the physicochemical properties of the chitosan/alginate nanoparticles as a potential carrier for nisin. Characteristics such as size, shape, zeta potential, their interactions with each other by FTIR (Fourier transform infrared spectroscopy) and DSC methods (differential scanning calorimetry), entrapment efficiency of nisin inside, and finally the kinetics of nisin release from the nanoparticles were investigated. In addition, inhibitory effects of nisin and the nisin-loaded chitosan/alginate nanoparticles on the harmful growth of *S. aureus* were evaluated and compared with through the agar diffusion and serial dilution methods in raw and pasteurized milk samples. Raw milk was chosen for the reason that some products being made from raw milk such as traditional cheese can have *S. aureus* pollution, and the nanoparticle formulations may help to decrease the *S. aureus* population in raw milk products more effectively than the formulation containing only free nisin. Choosing pasteurized milk is for the fact that the incorporation of nisin into the nanoparticles may be able to decrease the population of *S. aureus* in post-contamination processes and increase the shelf life of pasteurized milk and other related products as well. These findings may possibly help with the designing and employing of better carriers for boosting the antibacterial effects of safe biologics in food industry in the near future.

Materials and Methods

Materials

Acetic acid (Merck Co., Germany), sodium alginate (BDH Co., UK), low molecular weight chitosan (Sigma–Aldrich Co., USA), Coomassie Brilliant Blue G250 (Sigma Chemical Co., USA), phosphoric acid 85% (Sigm–Aldrich Co., USA), and nisin (as Nisaplin form containing 2.5% w/w of nisin, Merck Co., Germany) were purchased and used as received. *S. aureus* ATCC (19117) and *Micrococcus luteus* ATCC 10240 were prepared from Persian collection of bacteria in Iranian Research Organization for Science and Technology.

Method of Producing the Nisin-Loaded Chitosan/Alginate Nanoparticles

Polymer Solutions

Stock solutions of sodium alginate and chitosan were prepared by dissolving 250 mg of each one in 50 mL of deionized water. Chitosan is insoluble in deionized water. For increasing its solubility, 2 mL of acetic acid 2% (w/w) was added to the chitosan stock. Then, it was filtered with 0.22-μm syringe filter prior to use. The pH of the chitosan and alginate working solutions was adjusted to 5.4 and 5.2, respectively [1, 14].

Preparation of Nisin-Loaded Nanoparticles

The nisin-loaded nanoparticles were prepared by a two-step method adapted from Rajaonarivony's method of preparing poly-L-lysine nanoparticles [13]. Briefly, for preparing the nanoparticles, 100 μl of nisin solution (10 mg/mL) was added drop-wise to 8 mL aqueous solution of sodium alginate (250 mg/mL) and stirred for 30 min. Then, 4 mL of the chitosan solution (250 mg/mL) was added to the resulting alginate solution and stirred for an additional 1 h. The nanoparticles were obtained by ultrafiltration technique (Amicon, Ultracel-100 K, 100-kDa cutoff) after centrifuging at 2,500×*g* and 4 °C for 10 min to separate the free polymers from the nanoparticles [1, 14].

Nanoparticle Characterization

Morphology of the Nanoparticles

The nanoparticles morphology such as shape and occurrence of aggregation phenomena was studied by SEM (Scanning Electron Microscopy). Prior to observation, the samples of the nanoparticle suspensions (5–10 μl) were mounted on metals stubs, plating coated under vacuum,

and then examined by scanning electron microscopy (SEM, LEO 440 I, 10 kV, UK) [3].

Size Determination of the Nanoparticles

The size and surface zeta potential of the nanoparticles were evaluated by Zetasizer 3000 HS (Malvern Instruments, UK). Diluted aqueous dispersion of the nanoparticles was measured by the device. Mean and standard deviation of each of the samples were measured and presented for comparison [7].

Determination of Loading Efficiency of the Nanoparticles

The nisin-loaded nanoparticles dispersions were centrifuged using a 100-kDa molecular weight cutoff ultrafilter, (Amicon Ultra-15 Ultracel-100 K) at 11,000×*g*, 25 °C for 10 min. The protein content in the outer tube was analyzed by Bradford protein assay. The samples were analyzed in triplicates; the nisin loading efficiency (LE) was calculated using Eq. 1: [6, 12].

$$\mathrm{LE}(\%) = \frac{\mathrm{Nisin}_{\mathrm{total}} - \mathrm{Nisin}_{\mathrm{supernatant}}}{\mathrm{Nisin}_{\mathrm{total}}} \quad (1)$$

Calibration Curve for the Release of Nisin

Bradford reagent was used to determine the concentration of proteins in solution based on the formation of a complex between a dye, Brilliant Blue G, and proteins in solution. The protein–dye complex has a maximum absorption at 595 nm. The Bradford reagent used in this study has a high sensitivity and hence is able to detect protein molecules at a low concentration of 0.02 mg/mL. The linear concentration for the protein molecule, i.e. nisin as the standard protein one, ranged from 0.1 to 1.4 mg/mL. After this stage, the calibration curve for nisin was depicted ($R^2 = 0.982$) [12].

Release of Nisin from the Nanoparticles

The 12-kDa (MWCO) membrane was selected as the MWCO being sufficiently large to allow passage of nisin. For the experiment, 5 mL of a 1 mg/mL solution of phosphate-buffered saline (PBS) at pH 7.4 (release media) was poured into the inner tube of the dialyzer. The dialyzer tube was placed into a 50-mL glass cylinder containing release media, which was continually stirred at 300 rpm using a small magnetic stir bar to prevent the formation of an unstirred water layer at the membrane/outer solution interface. Diffusion to the outer solution at 37 °C was assessed by sampling the contents of the outer solution at periodic intervals of 0, 15, 30, 60, 120, 240, 300, 360, 420 and 520 min [8].

Fourier Transform Infrared Spectroscopy Analysis

FTIR (Thermo Nicolet, Nexus 870 FTIR, USA) was applied for the analysis of native nisin powder, chitosan powder, powder of chitosan/alginate nanoparticles, and nisin-loaded chitosan/alginate nanoparticles. At ratio of 2% (w/w), the samples were mixed with dry KBr (Fine Chem Ltd., Mumbai, India). The mixture was ground into fine powder using an agate mortar before compressing into KBr disc under a hydraulic press at 10,000 psi. Each KBr disc was scanned at 4 mm/s at a resolution of 2 cm over a wave number region of 400–4,000 cm^{-1}. The peaks were recorded for the different samples [8].

DSC Analysis

DSC thermograms were obtained using DSC-60 system (Shimadzu Co., Japan). The samples were dried in vacuum desiccators, and then the dried powders were crimped in a standard aluminum pan and heated from 20 °C to 350 °C at a heating rate of 10 °C/min under constant purging of nitrogen [7].

Microbial Assays

Bacterial Strain and Growth Conditions

S. aureus ATCC 19117 was obtained from the Iranian Bacteria Collection. The stock cultures were maintained by regular subculture to BHI agar broth and then incubated at 37 °C overnight. This culture served as the inoculums for the susceptibility studies, starting with approximately 10^6 CFU/mL in the test tubes. These CFU counts were accurately and reproducibly obtained by inoculation of 0.1 mL of the culture having an absorbance value of 0.2 as determined by optical density measurement at 600 nm using a UV/VIS spectrophotometer (Spectronic 20 D, USA) [10, 11].

Antibacterial Assay of the Nisin-Loaded Nanoparticles

Agar diffusion assay is a standard method for the activity evaluation of antibacterial agents.

Standard Calibration Curve of Nisin

First, nisin stock solution was prepared using Nisaplin as the source at concentration of 1000 IU/mL (equivalent to 10 mg/mL) in sterile water [11]. Then for quantification, standard solutions of purified nisin with concentrations ranging from 0 up to 1000 IU/mL were prepared. *M. luteus* ATCC 10240 was used as the indicator organism. This organism is the most sensitive to nisin and chosen based on

the reference [11]. After incubation at 37 °C for 24 h, the diameters of the inhibition zone were recorded, and nisin standard calibration curve obtained [11].

Activity Assay of Nisin-Loaded Nanoparticles with Agar Diffusion Assay

Agar diffusion assay was done for the nisin-loaded nanoparticles at the activity of 450 IU/mL proportionate to (mass equivalent) that of free nisin. After comparison with the standard calibration curve of nisin, the strength (inhibition zone) of the nisin-loaded nanoparticles was calculated and compared with that of free nisin [11].

Minimum Inhibitory Concentration of Nisin

For determining MIC (minimum inhibitory concentration) of nisin, broth dilution technique was employed. Dilutions from BHI agar 2.5, 3, 3.5, 4, 4.5, and 4.75 mL poured into the test tubes. Next, dilutions of the nisin stock (10 mg/mL) were prepared in the test tubes at final concentrations of 0.5, 1, 2, 4, and 5 mg/mL. Afterward, 10 µl of *S. aureus* was added to the test tubes, and the samples were incubated at 37 °C. Then, growth of *S. aureus* at time intervals of 24, 48, and 72 h was examined, and the MIC was calculated after 72 h [11].

MIC of the Nisin-Loaded Nanoparticles

For the calculation of minimum inhibitory concentration of the nisin-loaded nanoparticles, serial dilutions from BHI agar 2.5, 3, 3.5, 4, 4.5, and 4.75 mL were purred into test tubes. Next, dilutions of the nisin-loaded nanoparticles at final concentrations of 0.5, 1, 2, 4, and 5 mg/mL (mass equivalent to nisin) were prepared in the test tubes. Then, 10 µl of *S. aureus* culture was added to the test tubes, and the samples were incubated at 37 °C. Then, the growth of *S. aureus* at time intervals of 24, 48, and 72 h was observed, and the MIC was measured, accordingly [11].

Microbial Count Tests on the Samples

Raw and pasteurized milks were prepared from Mihan Factory. Three treatments of different samples, i.e. *S. aureus*, *S. aureus* plus free nisin, and *S. aureus* plus the nisin-loaded nanoparticles, were considered and added to the milks. After this step, microbial count of raw milk was measured at time intervals of 0, 6, 10, 14, 18, and 24 h with Bactoscan FC (Conveyor 5000, Germany), and the pasteurized milk samples were cultivated for 24 and 48 h and transferred to the incubator at 37 °C. After this time, the microbial counts for the pasteurized milk were done visually in triplicates, and the average of each treatment was plotted at 24 and 48 h.

Statistical Analysis

All of the experiments were done in triplicates, and the averages of the data were compared with independent *t*-test. A p value of < 0.05 was considered as statistical significance.

Results and Discussion

Microscopic Image Assessment

SEM confirmed the presence of the nanoparticles and provided morphological information on the nisin-loaded chitosan/alginate nanoparticles. Spherical, distinct, regular, and smooth shape was seen for the nanoparticles through the SEM images (Fig. 1). The particle size range of the nanoparticles was 50–250 nm.

Evaluation of the Size Distribution

From the results gained and summarized in Table 1, it can be observed that the mean size of nanoparticles is 205 nm and the mean of PDI (polydispersity index) of the nanoparticles was 0.34. PDI is related to homogeneity of the particles. The zeta potential of nanoparticles prepared by

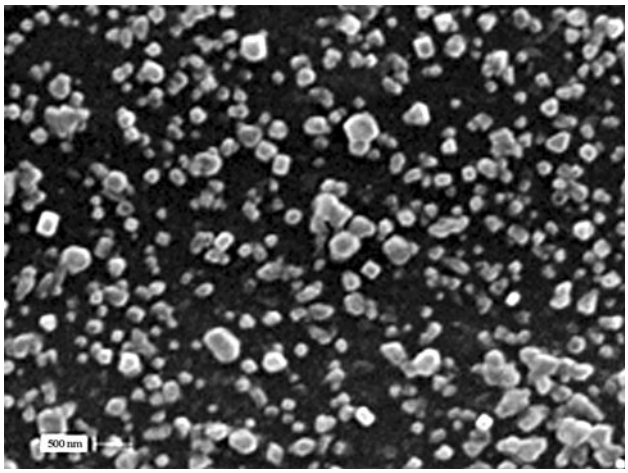

Fig. 1 SEM image of the nisin-loaded chitosan/alginate nanoparticles

Table 1 The averages of size, zeta potential and PDI of the nisin-loaded chitosan/alginate nanoparticles (SD = standard deviation; $n = 3$)

Properties	Mean ± SD
Size (nm)	205 ± 31
Zeta potential (mV)	−47 ± 11
PDI (polydispersity index)	0.34 ± 0.01

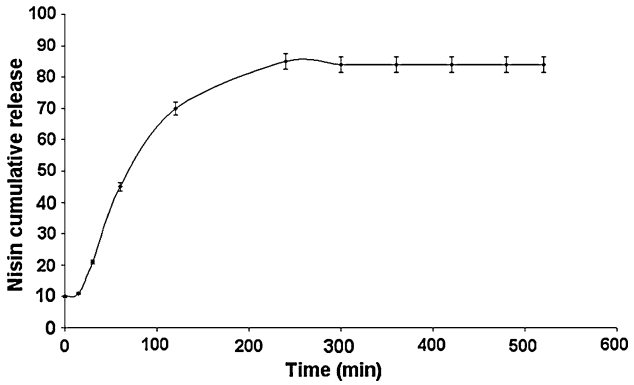

Fig. 2 Release diagram of nisin from the nisin-loaded nanoparticles at different times

this method was about −47 mV which could result in good stability of the nanoparticles during the manipulation and storage.

Release Profile and Loading Efficiency Evaluation of the Nanoparticles

Release of an entrapped protein from particle involves the swelling and loosening of the compact structure of the particle, allowing protein molecules entrapped at sub-layers of the particles to dissociate under hydrolytic actions and then diffuse through and detach from the particle structure. This transitional release happened for 240 min from the initiation. It is interesting to note that during the initial and transitional fast release, over 85% protein molecules were released, which was much higher than the release observed for small drug molecules from dense polymer devices, which typically release around 30% of drugs in the initial burst stages. After this stage, the release slows until reaches to steady state as seen in diagram in Fig. 2 as was expected [19]. Loading efficiency was 90–95% which was determined using the equation mentioned in Sect. "Determination of Loading Efficiency of the Nanoparticles".

FTIR Evaluation

In order to examine the interaction between components of nano particulate systems, preliminary study was taken over poly electrolytes interactions. It is well established that the carboxyl group of the anionic polymer may interact with the amino group of chitosan and forms an ionic complex [10]. This was reflected by changes in the absorption bands of amino groups, carboxylic groups, and amide bonds in the FTIR spectra (Fig. 3). The more intensity observed at about 3,437 cm^{-1} resulting from N–H and O–H stretching vibrations for the nisin-loaded nanoparticles than that of the chitosan/alginate nanoparticles is due to the presence and incorporation of nisin inside of the nanoparticle

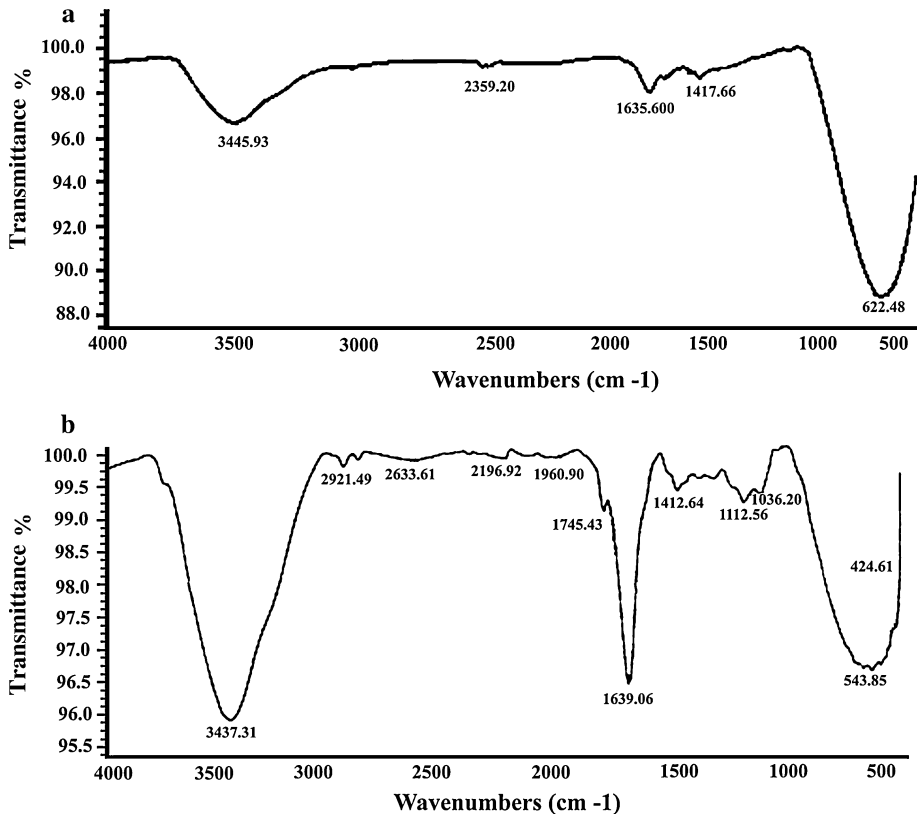

Fig. 3 FTIR spectra of the chitosan/alginate nanoparticles and the nisin-loaded chitosan/alginate nanoparticles: **a** the chitosan/alginate nanoparticles; **b** the nisin-loaded chitosan/alginate nanoparticles

mixture via possibly physicochemical interactions. The same behavior for the nisin-incorporated nanoparticles, i.e. the more intensity in the absorption band at 1,639 cm^{-1} (Fig. 3b) due to C = O stretching vibration, with a little shift about 4 cm^{-1} from the 1,635 cm^{-1} (Fig. 3a) to 1,639 cm^{-1} was also observed compared with the chitosan/alginate nanoparticles alone. This could be another confirmatory evidence for the presence of nisin inside of the nanoparticles through the physicochemical interactions.

DSC Assessment

In the DSC thermograms of the chitosan/alginate nanoparticles, four exothermic peaks were found respectively in 50, 250, 300, and 350 °C. But in thermogram of the nisin-loaded chitosan/alginate nanoparticles, two endothermic peaks and three exothermic peaks were found in 250, 350, and 400 °C (Fig. 4). Thermograms of the chitosan/alginate nanoparticles showed a broader endothermic peak at 400 °C, which probably represents the combination of the two endothermic polymer peaks. Endothermic peaks corresponded with loss of water associated with hydrophilic groups of polymers, while exothermic peaks result from degradation of polyelectrolytes due to dehydration and depolymerization reactions most probably partial decarboxylation of the protonated carboxylic groups and oxidation reactions of the polyelectrolytes. Exothermic peak of the chitosan/alginate nanoparticles was recorded at 70 °C, and exothermic peak of the nisin-loaded chitosan/alginate nanoparticles was recorded at 80, 260, and 340 °C. In these thermograms, changes in these peaks were corresponding to the interaction between nisin and mixture of the two polymers [7].

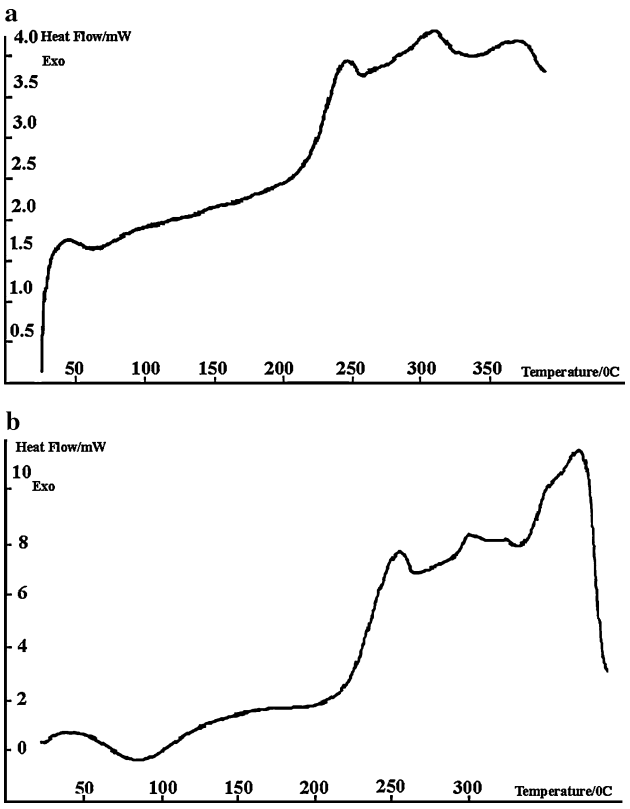

Fig. 4 DSC thermograms of the chitosan /alginate nanoparticles and the nisin-loaded chitosan/alginate: **a** the chitosan/alginate nanoparticles; **b** the nisin-loaded chitosan/alginate nanoparticles

Standard Curve for Nisin by Agar Diffusion Assay Method

Nisin standard curve was obtained according to the diameter of the inhibition zone *vs* log of nisin concentration ($R^2 = 0.99$).

Activity Evaluation of the Nisin-Loaded Nanoparticles by Agar Diffusion Assay

The nisin-loaded nanoparticles activity relative to standard calibration curve showed more inhibition zone about 2 times than that of free nisin at 450 IU/mL.

Evaluation of Minimum Inhibitory Concentration of Free Nisin

The MIC of nisin was 2 mg/mL after 72 h of incubation period with the *S. aureus* samples.

Evaluation of Minimum Inhibitory Concentration of the Nisin-Loaded Nanoparticles

MIC of the nisin-loaded nanoparticles was 0.5 mg/mL after 72 h of incubation with the *S. aureus* samples. With attention to the significant results gained ($p < 0.05$), MIC of the nisin-loaded nanoparticles (0.5 mg/mL) was lower about four times than free nisin (2 mg/mL). Many factors can be attributed to this observation. One of these factors is the controlled release of nisin by the nanoparticles and protecting nisin during this period against the unfavorable reactions with other ingredients inside [11].

Microbial Count Evaluation for Raw and Pasteurized Milks

In the pasteurized milk samples as can be seen in Fig. 5, the growth of *S. aureus* in control sample is fast and increasing over time. *S. aureus* growth was equal, and the same during 24 h of incubation with samples included free nisin and the nisin-loaded nanoparticles; however, it showed a significant difference to the nisin-loaded nanoparticles after 48 h of incubation period. In the raw milk samples, *S. aureus* growth in the absence of nisin/the nisin-loaded nanoparticles

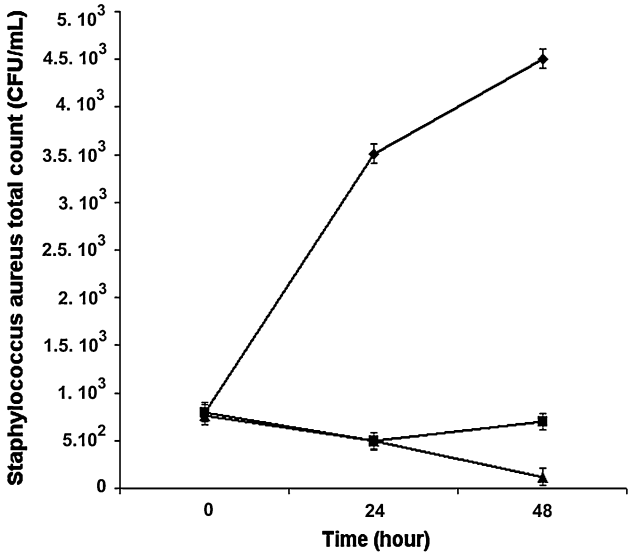

Fig. 5 Microbial count for S*taphylococcus aureus* in the pasteurized milk samples: (*filled diagnol*) i.e. pasteurized milk + *Staphylococcus aureus*; (*filled square*) i.e. pasteurized milk + *Staphylococcus aureus* + free nisin; (*filled triangle*) i.e. pasteurized milk + *Staphylococcus aureus* + nisin-loaded nanoparticles

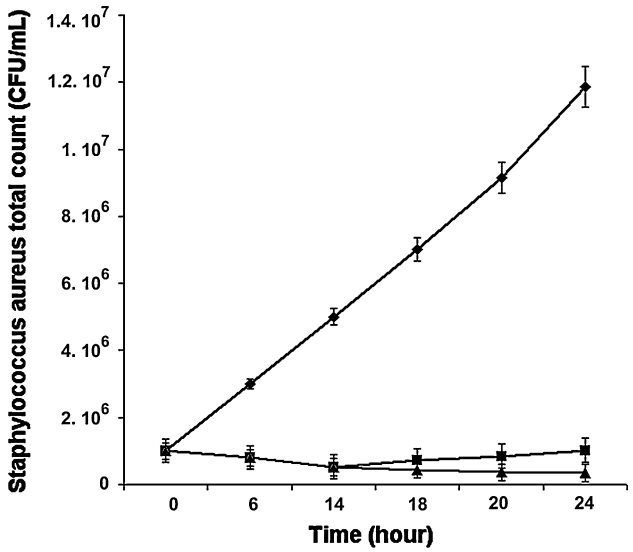

Fig. 6 Microbial count for *Staphylococcus aureus* in the raw milk samples: (*filled diagnol*) i.e. raw milk + *Staphylococcus aureus*; (*filled square*) i.e. raw milk + *Staphylococcus aureus* + free nisin; (*filled triangle*) i.e. raw milk + *Staphylococcus aureus* + nisin-loaded nanoparticles

showed a continuous increase in all times. However, in the presence of free nisin/the nisin-loaded nanoparticles, a decreasing and harmonious growth was first observed up to 14 h of incubation time. But after the consumption of free nisin, more growth was naturally observed in *S. aureus* population compared to nisin-loaded nanoparticles. In sum, the nisin-loaded nanoparticles exhibited a significant decrease in the growth of *S. aureus* when compared to free nisin (Fig. 6).

Conclusion

In the current study, the nisin-loaded nanoparticles were prepared by colloidal dispersion method, characterized through physicochemical tests, and its antibacterial efficiency compared with free nisin against *S. aureus*. The nisin-loaded nanoparticles produced in this experiment exhibited more efficiency in antibacterial activity than that of the nisin-loaded alginate microparticles generated by Wan et al. due to the contribution of chitosan in antibacterial strength [18]. This higher efficacy may be related to the egg-structure of alginate, chitosan, and the electrostatic charge balance between the two polymers. Nisin has a positive electrostatic charge which causes the charge of the resulting nanoparticles becomes positive, and this charge is the main factor related to the good physical stability of the formed nanoparticles. Another proof toward the importance of the electrostatic charge of the nanoparticles can be better loading capacity and higher release. The loaded nanoparticles showed the most liberated nisin after 420 min of incubation in the release medium. It probably relates to burst release of nanoparticles [10]. Burst release is related to swelling of the chitosan/alginate nanoparticles due to its hydrophilic properties. Release of nisin is a pH-dependent factor and with attention to the in vitro release results, about 90–95% release, in the medium with pH of 7.4 (because this pH leads to the more positive charge of nisin and consequently more partitioning of nisin into the release medium). Considering these parameters, it is estimated that in raw and pasteurized milks whose pH was less than the in vitro release conditions, release may be near or even more than the in vitro results [9]. After this time, the rate of release became slow compared with the initial burst release. This suggests that the remaining protein molecules were tightly bounded inside the particle structure and had to undergo a complete dissociation from the newly formed swollen structure but unable to escape readily owing to the formation of multiple hydrogen bonds along the chitosan polymer chain, which have to be broken into segments before the protein molecule could be released from the particle mass [17]. The initial release of nisin may result from the rapid hydration of nanoparticles due to the hydrophilic nature of chitosan and alginate nanoparticles. However, in this structure, nisin has a positive charge, and it can affect the release mechanism. The release medium penetrates into the particles and dissolves the entrapped nisin and, therefore, it could be proposed that the major factor determining the drug release from nanoparticles is its solubilization or dissolution rate in the release medium.

Further, it has been known that the solubility of nisin depends on pH (highest solubility at the concentrations of 40–60 mg/mL and the pH ranging from 2 to 5). Therefore, considering the pH of PBS = 7.4, this higher pH can be the cause of faster initial release. Changes were seen in the FTIR and DSC diagrams before and after the loading of nisin into the nanoparticles. These variations can result from the physicochemical interactions between the chitosan/alginate nanoparticles and nisin [7]. The MIC of the nisin-loaded nanoparticles was four times lower than that of free nisin. This will be due to the synergistically antimicrobial effect of chitosan nanoparticles with nisin [4]. The growth of *S. aureus* was inhibited equally by free nisin/the nisin-loaded nanoparticles at times of 24 and 14 h in the cases of the pasteurized and the raw milks, respectively. But more decrease in *S. aureus* growth was observed after this time for both raw and pasteurized milks in the presence of the nisin-loaded nanoparticles than samples containing only free nisin. Antimicrobial count tests revealed that free nisin cannot be as effective as the nisin-loaded nanoparticles at some times (Figs. 5, 6). This ineffectiveness may be a result of the unwanted interactions with protein, lipid, and other materials onside the milk samples, which are able to reduce nisin antimicrobial activity. However, the nisin-loaded nanoparticles with high loading efficiency show more effectiveness than free nisin [3]. This is for the reason that chitosan/alginate structures have protected nisin against the unwilling interactions with possible ingredients mentioned, and the activity of nisin will not be influenced during time. Benech and et al. also found the same results in this respect that encapsulation of nisin Z in liposomes can provide a powerful tool to improve nisin stability and inhibitory action in the cheese matrix while protecting the cheese starter from the detrimental action of nisin during cheese production [5, 10]. Therefore, structures like liposomes but more stable can also be employed as an effective bio preservative for increasing the shelf-life of food products against *S. aureus* pollution [2]. The results of this study suggest that incorporation of nisin into chitosan/alginate nanoparticles could possibly provide a potential delivery technology for improving the efficacy of nisin in foods. Incorporation or encapsulation would also provide a possible delivery technology for improving methodology for the co-delivery of nisin. Incorporation of the nanoparticles made from food-grade polymers such as the chitosan/alginate polymers offers a more stable system than liposomes encapsulation, but substantial research and development is required to realize the full potential of this technology for bacteriocins in the future as well.

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