Colloids and Surfaces B: Biointerfaces 145 (2016) 740–747

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Innovative bionanocomposite films of edible proteins containing liposome-encapsulated nisin and halloysite nanoclay

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ARTICLE INFO

Article history:
Received 20 January 2016
Received in revised form 15 April 2016
Accepted 27 May 2016
Available online 28 May 2016

Keywords:
Active packaging
Antimicrobial film
Nanoencapsulation
Nanoclay
Nanocomposite

ABSTRACT

Films and coatings based on natural polymers have gained increased interest for food packaging applications. In this work, halloysite and phosphatidylcholine liposomes encapsulating nisin were used to develop nanocomposite films of gelatin and casein. Liposomes prepared with either soybean lecithin or Phospholipon® showed particle size ranging from 124 to 178 nm and high entrapment efficiency (94–100%). Considering their stability, Phospholipon® liposomes with 1.0 mg/ml nisin were selected for incorporation into nanocomposite films containing 0.5 g/l halloysite. The films presented antimicrobial activity against Listeria monocytogenes, Clostridium perfringens and Bacillus cereus. Scanning electron microscopy revealed that the films had a smooth surface, but showed increased roughness with addition of liposomes and halloysite. Casein films were thinner and slightly yellowish, less rigid and very elastic as compared with gelatin films. Thermogravimetric analysis showed a decrease of the degradation temperature for casein films added with liposomes. The glass transition temperature decreased with addition of liposomes and halloysite. Gelatin and casein films containing nisin-loaded liposomes and halloysite represent an interesting alternative for development of active food packaging.

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1. Introduction

The increasing consumer demand for natural foods, with extended shelf life but without the addition of chemical preservatives, motivate the development of new conservation technologies. Biopreservation emerges as an alternative, in which a protective microbiota or their metabolites are employed aiming the control of pathogenic and spoilage microorganisms in food products [1,2].

Bacteriocins are recognized as safe and natural antimicrobial substances, especially useful when applied in hurdle technology [3]. Nisin is a bacteriocin known since the 1920s and currently approved as biopreservative in more than 50 countries [4]. This bacteriocin has received great attention because of its broad inhibition spectrum against Gram-positive bacteria, such as *Listeria* and *Staphylococcus*, and the effective control of *Bacillus* and *Clostridium* spores [5]. However, the application of nisin in its free form can result in undesirable interactions with the food matrix, leading to loss of activity [6,7].

Encapsulation of bioactive compounds into nanovesicles may promote a number of beneficial effects by protecting them against degradation and undesirable interactions, and increasing their stability, apparent solubility and efficiency [8,9]. Besides, the amount of encapsulated bioactive required for a specific effect is often much less than the amount required when non-encapsulated [10]. Liposomes have been used as an interesting platform to deliver bioactive compounds, such as antimicrobials, antioxidants, vitamins in food systems [7,11].

Most traditional materials used for packaging are not biodegradable, pollute the environment and consume fossil fuels for their production. A biocompatible alternative is the use of natural polymers such as starch, gelatin and chitosan [12,13]. Despite the intensive research performed in the last decade on development of biopolymer-based food packaging, this topic still needs significant advances [14,15]. To be competitive with traditional thermoplastics, biopolymers may have its mechanical and barrier properties improved with the addition of plasticizers, salts or other compounds. Nanoclays have been successfully used as reinforcement

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http://dx.doi.org/10.1016/j.colsurfb.2016.05.080
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in biopolymer-based composites, frequently providing better characteristics to the films [16,17].

Previous investigations on edible films containing nisin have been reported [18–20]. However, the combination of liposome-encapsulated nisin with nanoclays in protein-based films has not been investigated yet. Thus, the objective of the present study was to develop edible antimicrobial films of gelatin and casein incorporating nanoclays as reinforcer and phosphatidylcholine liposomes containing nisin. Phosphatidylcholine liposomes made of Phospholipon® 90G were compared with liposomes made with soy lecithin, and then used to prepare gelatin and casein films in the presence of bentonite, surface-modified montmorillonite or halloysite. The antimicrobial, mechanical, thermal and morphological properties of the films were evaluated.

2. Materials and methods

2.1. Materials

Phospholipon®90G (pure phosphatidylcholine stabilized with 0.1% ascorbyl palmitate) was supplied by Lipoid GMBH (Ludwigshafen, Germany). Soybean lecithin was provided by Solae S.A. (Esteio, Brazil). Gelatin was from Oetker Brasil (Cotia, Brazil) and casein was obtained from Synth (Diadema, Brazil). Ethyl acetate used for purification of crude soybean lecithin, chloroform used in liposome production and glycerol used as a plasticizer in films were purchased from Merck (Darmstadt, Germany). Nylon membrane filters (0.22 μm) were obtained from TPP (Trasadingen, Switzerland). The nanoclays hydrophilic bentonite (Nanomer—PGV), montmorillonite (MMT) surface modified with 25–30% octadecylamine (Nanomer—I.30E) and halloysite (HNT), were from Sigma-Aldrich (St. Louis, MO, USA).

Commercial nisin (Nisaplin®) was obtained from Danisco Brasil Ltda (Cotia, Brazil). According to the manufacturer, the formulation contains NaCl and denatured milk solids as fillers, and 2.5% (w/w) pure nisin. The stock solution was prepared by solubilizing Nisaplin with 0.01 M HCl to obtain a nisin concentration of 2.5 mg/ml. This solution was filter sterilized through 0.22 μm membranes, and further diluted in 10 mM phosphate buffer pH 7.0 to reach working concentrations. Nisin is expressed as levels of pure nisin (mg/ml). To convert these units to International Units (IU/ml) or to equivalent Nisaplin® levels (mg/ml), the levels of pure nisin should be multiplied by 40 [21].

2.2. Purification of crude soybean lecithin

Crude soybean lecithin (5 g) was dissolved in 25 ml ethyl acetate. Then, distilled water (1 ml) was slowly added under gentle agitation, resulting in the formation of two phases. The upper phase was discarded. The lower phase, having a gel aspect, was dispersed in 30 ml acetone, forming clusters that were crushed using a glass stick. Then, the acetone was separated by decanting and a new aliquot of 30 ml acetone was added, repeating the shredding process. The precipitate was vacuum filtered and dried in a desiccator.

2.3. Liposome production by film hydration

Liposome production was performed according to the film hydration method [22], with some modifications. Phosphatidylcholine (76 mg) was dissolved in chloroform (15 ml) in a round-bottom flask and the organic solvent was removed using a rotary evaporator until a thin film was formed on the walls. Traces of organic solvents were removed by storage in desiccator under vacuum for 18 h. The nisin stock solution was diluted in phosphate buffer to reach 0.5, 1.0 and 1.5 mg/ml and 5 ml were added to disperse the resulting dried lipid film. These mixtures were then homogenized above their phase transition temperature (60 °C) to produce multilamellar lipid vesicles (MLVs). In order to reduce the size and homogenize the liposomes, sonication of the MLVs was carried out in an ultrasonic cell disrupter (Unique, Brazil) by five cycles of 1 min at intervals of 1 min, during which the samples were kept in ice. Then, the solution was filtered through 0.22 μm membranes.

2.4. Liposome characterization

Liposome size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) in a particle size analyzer (BI–200 M goniometer, BI-9000AT digital correlator, Brookhaven Instruments, Holtsville, NY, USA) essentially as described elsewhere [23]. DLS measurements were performed immediately after the liposome preparation. Samples (300 μl) were diluted in 10 ml of 10 mM phosphate buffer pH 7.0 prior analysis. The zeta potential of the nanovesicles was determined after dilution of the formulations in 1 mM NaCl using a Zetasizer Nano-ZS ZEN 3600 equipment (Malvern Instruments, Herrenberg, Germany).

2.5. Antimicrobial activity

The antimicrobial activity was detected by agar diffusion assay as described previously [24]. Aliquots (10 μl) of free and liposome-encapsulated nisin were applied onto BHI agar plates previously inoculated with a swab submerged in a suspension to a final OD_{600}nm of 0.15 of *Listeria monocytogenes* ATCC 7644 (approximately 10^7 UFC ml^{-1}). After incubation at 37 °C for 24 h, the plates were checked for zones of inhibition. The reciprocal value of the highest dilution that produced a definite inhibition zone was taken as the activity units (AU). Antimicrobial activity, in AU/ml, was calculated as D/V (ml), where D is the dilution factor and V is the sample volume. Antimicrobial activity was evaluated immediately after the liposome preparation and aliquots were stored at 4 °C and at room temperature (25 ± 2 °C) to analyze the activity and stability of liposomes over time.

2.6. Entrapment efficiency

The entrapment efficiency (EE) of liposomes was determined by evaluation of nisin antimicrobial activity [22]. The liposome suspension was subjected to ultrafiltration (Amicon Ultracel YM-10, Millipore, Billerica, MA, USA) at 10,000*g* for 20 min. The antimicrobial activities of encapsulated nisin (retentate) and free nisin (filtrate) were measured as described above, and EE was calculated using the following equation [6]:

$$EE\ (\%) = \frac{encapsulated\ nisin\ (AU/ml)}{encapsulated\ nisin\ (AU/ml) + free\ nisin\ (AU/ml)} \times 100$$

2.7. Film preparation

Film formulations were tested for each polymer (casein and gelatin) with the addition of plasticizer (glycerol), liposomes and nanoclays (montmorillonite, known as hydrophilic bentonite (MMT), montmorillonite modified with octadecylamine and halloysite, tested at 0.5 and 1.0 g/l).

Casein films were obtained from a 30 g/l casein solution in distilled water. As casein is poorly soluble in distilled water, the pH solution was adjusted to 7.0 with the addition of 1 M NaOH and then heated to 70 °C under stirring. This process was repeated and the solution was centrifuged at 6000*g* for 5 min to remove insoluble material. The supernatant was then diluted in distilled water at a 3:1 ratio. Gelatin films were made of a 40 g/l commercial colorless

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