Carbohydrate Polymers 103 (2014) 573–580

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Preparation and characterization of alginate and alginate-resistant starch microparticles containing nisin

Seyede Marzieh Hosseini[a], Hedayat Hosseini[a], Mohammad Amin Mohammadifar[a], J. Bruce German[b], Amir Mohammad Mortazavian[a], Abdorreza Mohammadi[a], Kianoosh khosravi-Darani[c], Saeedeh Shojaee-Aliabadi[a], Ramin Khaksar[a,*]

[a] Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Foods for Health Institute, Department of Food Science & Technology, University of California, Davis, CA, USA
[c] Research Department of Food Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Science, Tehran, Iran

ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form
22 December 2013
Accepted 26 December 2013
Available online 5 January 2014

Keywords:
Alginate
Resistant starch
Microparticle
Nisin

ABSTRACT

Delivery systems with sustained release of nisin have been proposed to improve stability and long-term effectiveness of this bacteriocin in foods. In this study, nisin was encapsulated in alginate (Alg) and alginate-resistant starch (Alg-RS) microparticles and its release was investigated. Studies found that the nisin concentration has significant influence on encapsulation efficiency (EE), loading capacity (LC) and size of both microparticles. Furthermore, encapsulation efficiency and loading capacity values were more increased by the addition of resistant starch to the alginate formulation. The highest encapsulation efficiency was obtained with Alg-RS microparticles prepared using initial nisin to alginate weight ratio of 25% w/w (59.77 ± 2.26%). Fourier transform-infrared (FT-IR) spectroscopy, X-ray diffraction (XRD) and differential scanning calorimetry (DSC) results confirmed the presence of nisin in the microparticles. The in vitro nisin release from these microparticles followed a controlled-release pattern consistent with a Fickian diffusion mechanism. The release rate from Alg-RS microparticles was less than that from the Alg microparticles.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Nisin is an antimicrobial peptide with 34 amino acids and a molecular weight of 3.5 kDa produced by strains of *Lactococcus lactis* subsp. *lactis* (Mulders, Boerrigter, Rollema, Siezen, & Devos, 1991). This bacteriocin has been extensively studied because of its potential applications as a natural preservative in the food industry. However, several studies have reported that the antimicrobial efficacy of nisin is reduced when it is applied in foods, because of its structural instability, interaction with the food constituents such as proteins and lipids, inactivation by enzymatic degradation, and development of nisin-resistant bacterial strains (Benech, Kheadr, Laridi, Lacroix, & Fliss, 2002; Bhatti, Veeramachaneni, & Shelef, 2004; Laridi et al., 2003).

In order to overcome these problems, alternative procedures that target the sustained release of nisin have been proposed. Many studies applied liposomes, polymeric capsules or films to improve nisin biological activity (Ercolini et al., 2010; Laridi et al., 2003; Salmaso, Elvassore, Bertucco, Lante, & Caliceti, 2004; Silva Malheiros, Serafini Micheletto, Pesce da Silveira, & Brandelli, 2010; Xiao, Davidson, & Zhong, 2011; Zohri et al., 2010). Encapsulation of nisin represents an efficient approach to increase its stability, protects nisin from the interactions with the food components and allows for a controlled release (Laridi et al., 2003; Xiao et al., 2011). Most of researchers have been used liposomes for this bacteriocin encapsulation. However some drawbacks are attributed to liposomes as a nisin carrier including inactivation and fast leakage due to interaction of nisin with liposomes, low stability and high cost (El Jastimi, Edwards, & Lafleur, 1999; Kopermsub, Mayen, & Warin, 2011).

Recently, the use of biopolymers in the design of biodegradable particles from nanometer to micrometer length scales has received much attention as delivery systems. Alginate is an anionic biopolymer containing 1,4-linked β-mannuronic and α-guluronic acid residues. It is one of the most biopolymeric matrices used in delivery systems because of its biodegradability, biocompatibility, structural simplicity, and ability to form three dimensional gels in the presence of metal cations notably calcium, in aqueous

* Corresponding author at: Food Science and Technology Department, Faculty of Nutrition and Food Science, Shahid Beheshti University, P.O. Box 19395-4741, Tehran, Iran. Tel.: +98 21 22376480; fax: +98 21 22376480.
E-mail addresses: r.khaksar@sbmu.ac.ir, ramin.khaksar@gmail.com (R. Khaksar).

0144-8617/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.12.078

solutions (Alipour, Montaseri, & Tafaghodi, 2010; Chan et al., 2011; Martinsen, Storro, & Skjakbraek, 1992). However alginate microparticles exhibit a low physical stability, porous and hydrophilic structure which causes rapid release of entrapped materials (Chan et al., 2011; Lopez Cordoba, Deladino, & Martino, 2013; Martin, Lara-Villoslada, Ruiz, & Morales, 2013).

Blending with other many different materials such as polymers, carbon nanotube (Zhang et al., 2010), polysaccharide nanocrystals (Lin, Huang, Chang, Feng, & Yu, 2011), and clay materials (Kaygusuz & Erim, 2013) have been used to improve the structural and functional properties of alginate matrices. A promising alternative was proposed by Zhang et al. (2010). They incorporated of carbon nanotube in alginate matrix which leaded to improvement of encapsulation efficiency, mechanical stability and decreased the drug leakage. In addition, several researchers have reported that the combination of alginate with starch leads to higher chemical and mechanical stability, greater encapsulation efficiency, and a sustained release of entrapped materials (Chan et al., 2011; Fontes, Araujo Calado, Rossi, & Miguez da Rocha-Leao, 2013; Lopez Cordoba et al., 2013; Martin et al., 2013).

To the best our knowledge, no reports are available that characterize the use of alginate-resistant starch microparticles for nisin encapsulation. Thus, regarding to the advantages of the alginate–starch microparticles and their suitability as a delivery system, this study was designed to examine the physicochemical properties of the alginate-resistant starch microparticles as potential carriers for nisin and to compare these properties to alginate microparticles. The effects of initial nisin concentration on loading capacity (LC), encapsulation efficiency (EE), mean particle size, of both microparticles were investigated. Moreover, the compatibility of nisin entrapped into microparticles was evaluated through FT-IR, XRD and DSC analysis. In addition, in vitro release behaviors were studied to determine the feasibility of using nisin-loaded Alg-RS microparticles as a food preservative system.

2. Materials and methods

2.1. Materials

Nisin Z® was purchased from Handary (Handary, SA, Brussels, Belgium). According to the manufacturer, the formulation contains 2.5% pure nisin. Sodium alginate (from Laminaria hyperborean, molecular mass of 1.97×10^5 Da, mannuronate/guluronate ratio = 0.6) was from BDH (Poole, UK). Hi-maize® 260 resistant starch was supplied by National Starch and Food Innovation (Bridgewater, NJ, USA). Brain heart infusion (BHI) was bought from Merck Co. (Darmstadt, Germany). Sunflower oil with no added antioxidants was supplied by Nina (Tehran, Iran) and was stored at −18 °C until analysis. Calcium chloride, Tween 80 and Span 80 were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

2.2. Preparation of nisin-loaded microparticles

Nisin-loaded microparticles were prepared by a w/o-emulsion external cross-linking procedure (Moebus, Siepmann, & Bodmeier, 2012) with slight modifications. Sunflower oil was used as organic phase. Briefly, an alginate solution (1% (w/v)) was prepared in distilled water overnight under magnetic stirring. Nisin was added to alginate solution to reach a 4:1, 2:1 and 1:1 nisin:alginate weight ratio (25, 50 and 100% w/w, respectively). The mixture was stirred for another 30 min. Then a w/o emulsion was prepared by blending 20 ml of this mixture with 30 ml sunflower oil (containing 1% v/v span 80 and 1% w/v Tween 80) using ultra-Turrax (IKA T25-Digital Ultra Turrax, Staufen, Germany) at a speed 10,000 rpm for 3 min. Emulsification process was done in ice bath to prevent of increasing temperature during high speed homogenization. Microparticles were generated by adding 8 g of calcium chloride solution (25%, w/w) dropwise (over about 4 min) to w/o emulsion (50 ml) under magnetic stirring; the mixture was then stirred for another 20 min. The microparticles were recovered by centrifugation at 2500 rpm for 5 min, followed by vacuum filtration and several washes with distilled water containing 0.1% v/v Tween 80. Finally, they were frozen at −80 °C for 2 h and lyophilized with a freeze drier for 14 h (ALPHA 2-4; Christ, Harz, Germany).

Nisin loaded Alg-RS microparticles were obtained by adding 2 g resistant starch to 100 ml nisin–alginate solutions as described previously.

2.3. Nisin encapsulation efficiency (EE) and loading capacity (LC)

The content of nisin-loaded in microparticles was determined by suspending a known weight of each sample in sodium citrate solution (1%, w/v) and stirred at 37 °C and 180 rpm until complete dissolution of microparticles (Alipour et al., 2010). Nisin extraction was accomplished by adding 0.02 M hydrochloric acid followed by centrifugation at 5000 rpm for 10 min. The concentration of nisin in the supernatant was determined using agar diffusion method and HPLC analysis.

Triplicate tests were performed for each sample. EE and LC were determined using the following equations:

$$\mathrm{EE}(\%) = \frac{\text{Total amount of loaded nisin}}{\text{Initial amount of nisin}} \times 100 \quad (1)$$

$$\mathrm{LC}(\%) = \frac{\text{Total amount of loaded nisin}}{\text{Weight of microparticles after freeze drying}} \times 100 \quad (2)$$

In fact, the encapsulation efficiency describes how much portion of the initial nisin is present in the microparticles and the loading capacity indicates the percentage of the microparticles occupies by the loaded nisin.

2.4. Analysis of nisin by high performance liquid chromatography (HPLC)

HPLC analysis was performed using a Cecil CE-4900 and HPLC (Cambridge, England) equipped as follows: two CE-4100 pumps, multiple solvent delivery unit, vacuum degasser, mixing chamber, six-port valve (Rheodyne, USA), CE-4200 UV–vis detector (Cambridge, England). Twenty microliters of each sample was loaded onto the column (C18, 250 mm × 4 I.D., 5 μm). Samples were eluted with water/0.1% TFA (eluent A) and acetonitrile/0.1% TFA (eluent B). Elution conditions are expressed as proportion of solvent A: 0–100 min, 100%; 10–40 min, 100–50%; 40–50 min, 50–0%, 50–55 min, 0%, 55–60 min 0–100%, 60–65 min 100%, flow rate: 1 ml/min. The column temperature was set at 30 °C and absorbance was monitored at 215 nm. The bacteriocin concentration was calculated using a standard curve, obtained from standard solutions of nisin.

2.5. Agar diffusion assay

The agar diffusion technique is based on the measurement of the inhibition zone produced by microorganisms that are susceptible to nisin. Nisin activity was determined using the food pathogen *Listeria monocytogenes* ATCC 19117 as a test microorganism according to a modified method (Jin, Liu, Zhang, & Hicks, 2009). *L. monocytogenes* was grown in BHI broth at 37 °C for 24 h before the test. One hundred microliter of an overnight broth culture containing 10^7–10^8 CFU/ml of the test organism were inoculated on the BHI

plates and then three wells of 6 mm diameter were sunk into the agar.

To generate a nisin standard curve, standard nisin solutions (0.01–2.5 mg/ml) were prepared in sterile 0.02 N HCl. Twenty microliters of nisin standard solutions were loaded into individual wells. The plates were incubated at 30 °C for 24 h. The diameter of inhibition zone was then measured. The log nisin concentrations were plotted against inhibition zone diameters to construct a nisin standard curve ($y = 8.626x - 20.38$ $R^2 = 0.971$).

For determination of unknown nisin concentrations in this study, samples were loaded into wells and were incubated in the same conditions as the standard solutions. The average of six inhibition-zone diameters for each sample was used to calculate of nisin concentrations using calibration curve.

2.6. Scanning electron microscopy (SEM)

The morphology of the microparticles was examined using a scanning electron microscope (Oxford Instruments INCA Penta FET-X3). The samples were mounted to the specimen holder with a double-sided adhesive tape and vacuum coated with gold. SEM photographs were taken at the required magnification at room temperature and examined using an acceleration voltage of 25 kV.

2.7. Fourier transform-infrared (FT-IR) analyses

The FTIR measurements were performed for structural characterization of microparticles. The dry samples were mixed with KBr and pressed into pellets. FT-IR spectra of samples were obtained from wave number 400–4000 cm^{-1} using a FT-IR spectrophotometer (Perkin Elmer Spectrum RX I, USA). For each spectrum, 16 scans at a resolution of 4 cm^{-1} were obtained.

2.8. X-ray diffraction (XRD) analyses

The X-ray diffraction patterns of samples were obtained using an X-ray diffractometer (Bruker D8 Advanced) with Cu Kα radiation ($\lambda = 1.54178$ Å). The diffraction data was recorded in the range of 4°–70° (2θ) with a step interval of 0.02°. The samples were studied at ambient temperature.

2.9. Differential scanning colorimetery (DSC) analyses

The DSC thermograms of the samples were registered using a Shimadzu DSC-60 differential scanning calorimeter (Shimadzu, Kyoto, Japan). Samples (6–10 mg) were sealed in aluminium pans and were heated from 25 to 300 °C at a scanning rate of 10 °C/min under a nitrogen atmosphere (flow rate, 30 ml/min).

2.10. Particle-size analyses

Particle size and size distributions of microparticles were measured by laser diffraction using a Cilas 1090 particle-size analyzer (Orleans, France) equipped with a 5 mW He/Ne (635 nm) laser beam. Analyses were carried out using aqueous dispersions of unloaded and loaded microparticles in triplicate. Data are reported as the mean diameter and polydispersity expressed by span factor. Span is a measure of the distribution width of particle size in dispersion, which was calculated using the following equation (Elversson, Millqvist-Fureby, Alderborn, & Elofsson, 2003):

$$\text{Span} = \frac{D_{0.9} - D_{0.1}}{D_{0.5}} \quad (3)$$

where $D_{0.1}$, $D_{0.5}$ and $D_{0.9}$ are size of particles below which 10%, 50% and 90% of the samples lies respectively.

2.11. In vitro release studies

In order to perform release experiments a known amount of nisin loaded microparticles was suspended in 25 ml distilled water as release medium under room temperature and static condition. At different time intervals, 1 ml of the samples was withdrawn and replaced with fresh medium. The aliquots were centrifuged at 5000 rpm for 10 min and supernatant was taken for determination of nisin activity by agar diffusion test. All release studies were run in triplicate and mean values of cumulative release (%) were plotted against time in hours.

To describe the release mechanism of nisin from polymeric matrix, the cumulative release data have been fitted to a well-known Korsmeyer and Peppas semi-empirical model (Ritger & Peppas, 1987):

$$\frac{m_t}{m_\infty} = k \cdot t^n \quad (4)$$

where m_t/m_∞ is the fractional release of nisin at time t, k is a constant which indicate the characteristics of the macromolecular network system; and n is the release exponent characterizing the nisin release mechanism.

Values for n lower than 0.5 indicate a simple Fickian diffusion. Values higher than 0.5 are related to non-Fickian release: in other words, active agent release occurred through diffusion in the hydrated matrix and polymer relaxation (Paula, Sombra, Cavalcante, Abreu, & de Paula, 2011).

2.12. Statistical analysis

Data analysis was performed using SPSS statistical software version 16 (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) followed by the Duncan's multiple range procedure was used to identify any significant differences between the samples at a level of 95% of confidence ($\alpha = 0.05$).

3. Results and discussion

3.1. Encapsulation efficiency (EE), loading capacity (LC) and particle size

In this study nisin-loaded Alg and Alg-RS microparticles were produced using a process consisting of emulsification and subsequent external ionic gelation. As proposed by Soppimath, Aminabhavi, Kulkarni, and Rudzinski (2001) loading of drugs into the biodegradable polymeric particles was achieved by either incorporating the drugs at the time of particles production or adsorbing drug on the surface of microparticles after their formulation. In this study, it seems that little amount of loaded nisin were incorporated into the both outer matrix and the surface of microparticles, though a large amount of the nisin was entrapped into the core. Nisin incorporation in microparticles is probably due to interaction between anionic groups of polymeric matrix and cationic groups of nisin. Thus both entrapment and adsorption phenomena can be considered as the mechanism of nisin encapsulation.

Table 1 shows the encapsulation efficiency (EE), loading capacity (LC) and particle size of Alg and Alg-RS microparticles. The effect of nisin:alginate weight ratio (25–100% w/w) on EE and LC of microparticles was determined by HPLC analyses. Similar results were obtained with the agar diffusion assay (data not shown). EE of Alg and Alg-RS microparticles decreased on increasing nisin loadings from $54.58 \pm 1.42\%$ to $48.33 \pm 1.62\%$ and $59.77 \pm 2.26\%$ to $50.28 \pm 1.53\%$, respectively. This observation might be explained due to the saturation of nisin into microparticles. In fact, by increasing initial nisin content, the size of microparticles enhanced that this was associated with reduced number of microparticles. Thus

Table 1
Encapsulation efficiency (EE %), loading capacity (LC %) and particle size parameters of microparticles.[a]

Microparticles	Nisin:alginate ratio (%w/w)	EE (%)	LC (%)	Particle size parameters	
				Mean diameter (μm)	Span
Alg_0	0	0.00a	0.00a	47.52 ± 1.07a	1.52
Alg_1	25	54.58 ± 1.42b	16.15 ± 0.69b	53.44 ± 1.50b	1.81
Alg_2	50	50.46 ± 2.02c	18.00 ± 0.90c	73.02 ± 1.00c	1.88
Alg_3	100	48.33 ± 1.62c	21.15 ± 0.60d	90.99 ± 0.57d	2.23
Alg-RS_0	0	0.00a	0.00a	58.85 ± 0.76e	1.92
Alg-RS_1	25	59.77 ± 2.26d	19.06 ± 0.31c	78.24 ± 1.06f	2.44
Alg-RS_2	50	55.30 ± 1.37b	23.06 ± 0.59e	91.47 ± 1.00d	2.98
Alg-RS_3	100	50.28 ± 1.53c	27.08 ± 1.01f	110.51 ± 1.25g	3.46

Values within each column with different letters are significantly different ($P < 0.05$).
[a] Data reported are average values ± standard deviations.

the ratio of surface to volume decreased and limited surface for nisin adsorption caused a reduction in EE. However, these finding were in contrast with those obtained by Laridi et al. (2003), who reported that the amount of encapsulated nisin in liposome increased with increasing of initial content of nisin. These authors stated that lipid membrane would not be a limiting factor for nisin adsorption onto liposome. So this discrepancy could be due to different types of wall material.

The results showed that the addition of resistant starch at 2% in alginate matrix significantly increased the EE of microparticles ($P < 0.05$). The higher EE in the case of Alg-RS microparticles was interpreted to be due the effect of starch in the stabilization of the alginate matrix (Tal, van Rijn, & Nussinovitch, 2000). This apparent improvement in entrapment efficiency of Alginate microparticles with the addition of starch was also observed in previous works for other entrapped materials (Lopez Cordoba et al., 2013; Rassis, Saguy, & Nussinovitch, 2002; Sultana et al., 2000).

The percentage of LC was evaluated for calculating the amount of microparticles necessary to reach a determined nisin level in a food formulation. The values of LC were 16.15 ± 0.69 to 21.15 ± 0.60% and 19.06 ± 0.31 to 27.08 ± 1.01% for Alg and Alg-resistant starch microparticles, respectively. The %LC values showed a significant ($P < 0.05$) increasing trend with increasing amounts of initial nisin content. Furthermore addition of resistant starch was found to improve LC. Higher percentage of LC for Alg-RS microparticles is in agreement with the previous encapsulation efficiency results. A similar finding was reported by Lopez Cordoba et al. (2013), who obtained a higher LC of yerba mate antioxidants in alginate–starch hydrogel beads than in alginate beads.

Microparticles obtained from alginate showed mean particle size values ranging from 47.52 ± 1.07 to 90.99 ± 0.57 μm and SPAN factor 1.52–2.23. As can be seen, addition of resistant starch filler resulted in a slight increase in particle size and polydispersity. The increasing size of alginate microparticles by the addition of starch filler has also been observed by other authors (Martin et al., 2013; Zou et al., 2011). On the other hand, initial nisin concentration influenced the mean particle size and polydispersity of both formulation ($P < 0.05$). As expected, by increasing nisin:alginate weight ratio from 25% to 100%, the mean diameter of Alg and Alg-RS microparticles increased from 47.52 ± 1.07 to 90.99 ± 0.57 and 59.77 ± 2.26 to 50.28 ± 1.53 respectively. The larger size of capsules containing higher amount of nisin is due to loading of nisin into microparticles. In addition, the increase in particle size with increasing nisin content can be corresponded to the increase in viscosity caused by the total solid mass in the emulsion that led to formation of larger droplets and consequently larger microparticles (Xu & Hanna, 2006). This result corresponded to previous literatures studies describing nisin loaded in poly-L-lactide nanoparticles and solid lipid nanoparticles, respectively (Prombutara, Kulwatthanasal, Supaka, Sramala, & Chareonpornwattana, 2012; Salmaso et al., 2004).

3.2. Morphology

The morphology of microparticles was studied by SEM as shown in Fig. 1. Nisin-loaded Alg microparticles showed some cracks on the microparticle surface (Fig. 1a). Incorporation of resistant starch in alginate matrix resulted in more packed and spherical structure with no cracking apparent on the surface of microparticles (Fig. 1b). This is probably related to homogeneously distributed starch which can occupy the interstitial spaces in the matrix leading to reinforcement of alginate microparticle structure and prevented cracking and controlled shrinkage (Chan et al., 2011; López-Córdoba, Deladino, & Martino, 2014). A similar finding was observed by Chan et al. (2011), who stated that starch was able to act as a structural support to control the size of shrinkage and to maintain the spherical shape of dried beads.

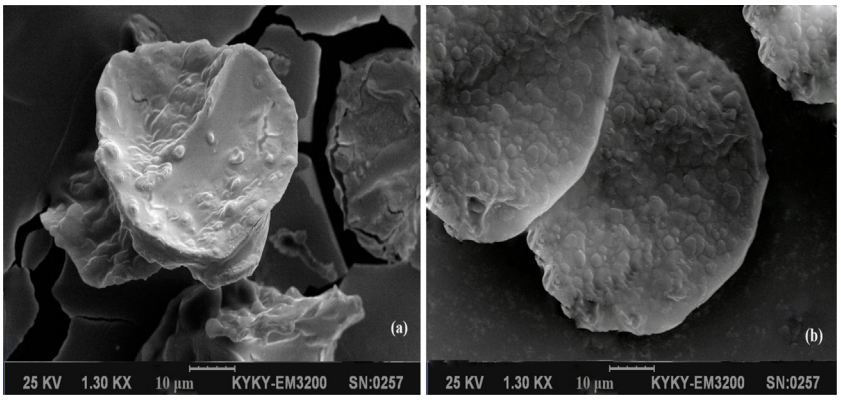

Fig. 1. Scanning electron micrographs of nisin loaded alginate (a) and alginate-resistant starch microparticles (b).

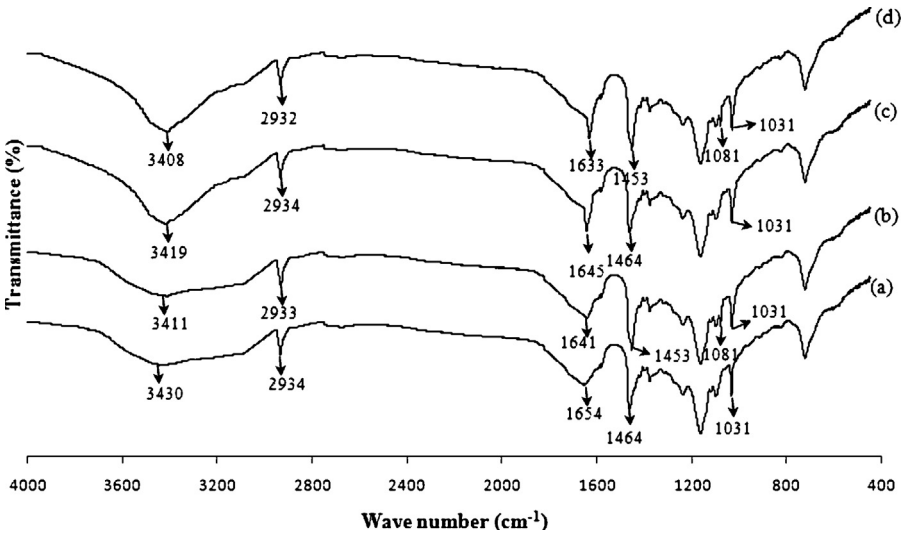

Fig. 2. FTIR spectra of Alg microparticles (a), Alg-RS microparticles (b), nisin-loaded Alg microparticles (c) and nisin-loaded Alg-RS microparticles (d).

3.3. FTIR analyses

FTIR has been used to study the interactions between polymers and nisin. Fig. 2 shows FTIR spectra of Alg microparticles, Alg-RS microparticles, nisin-loaded Alg microparticles and nisin-loaded Alg-RS microparticles. The FT-IR spectrum of alginate microparticles showed characteristic absorption bands at 3430 cm^{-1} (O—H stretching vibrations), 2934 cm^{-1} (C—H stretching vibrations), 1654 cm^{-1} (COO^- asymmetric stretching vibrations), 1464 cm^{-1} (COO^- symmetric stretching vibrations) and 1034 cm^{-1} (C—O—C stretching vibration). The addition of resistant starch to alginate formulation caused a decrease in the wave number COO^- from 1654 and 1464 cm^{-1} to 1641 and 1453 cm^{-1}, respectively. At the same time, the peak of hydroxyl shifted from 3430 to 3411 cm^{-1}. These changes are consistent with an increase in the hydrogen bonding of the particles. Similar observations have been reported (Wang, Hu, Du, & Kennedy, 2010). In nisin-loaded microparticles, the OH stretching peak exhibited a greater intensity and a narrower peak than that of the unloaded microparticles because of incorporation of nisin inside the microparticles. In addition, the peaks around 1645 cm^{-1} and 1633 cm^{-1} in nisin-loaded Alg and Alg-RS microparticles, respectively were sharper and shifted to lower wave numbers. This change was considered to be additional evidence in support of the presence of nisin inside of the microparticles. The results are in agreement with the findings regarding the loading of nisin into chitosan/alginate nanoparticles, which have been reported by Zohri et al. (2010). Since, the pK_a value of alginate is 3.5 (Mi, Sung, & Shyu, 2002) and isoelectric point of nisin is 8.8 (Xiao et al., 2011), thus at neutral pH during microparticles preparation, alginate and nisin are negatively and positively charged, respectively. An electrostatic interaction between the positively charged bacteriocin and polyanionic alginate could be responsible for these changes.

3.4. DSC studies

Fig. 3 displays DSC curves sodium alginate powder, calcium Alg microprticles, Alg-RS microparticles, nisin-loaded Alg microparticles and nisin-loaded Alg-RS microparticles. DSC curve of sodium alginate presented an endothermic peak around 76 °C, could be attributed to the dehydration process whereas the exothermic peak at 248 °C resulted from degradation of biopolymer due to depolymerization reactions (Mimmo, Marzadori, Montecchio, & Gessa, 2005). However the exothermic peak of sodium alginate was absent in the calcium Alg microparticles. Thus, no decomposition event was detected up to 300 °C due presumably to the formation of the structure of "egg box" with calcium ions (Lopez Cordoba et al., 2013). In Alg-RS microparticles the endothermic peak shifted to higher temperature than Alg microparticles. This peak can be also attributed to loss of water due to hydrophilic groups of blends biopolymers. Thermal behavior of microparticles was not influenced by the nisin incorporation. In addition no new peak was observed in nisin-loaded microparticles, which was interpreted to be evidence of nisin incorporation into microparticles.

3.5. XRD analyses

The X-ray diffractograms of individual polymers, unloaded Alg-RS microparticles and nisin loaded Alg-RS microparticles are shown in Fig. 4. As observed, alginate showed two crystalline peaks at $2\theta = 13.3°$ and 21.2°. Resistant starch had intense peaks between 2θ of 11° and 22° due to its close molecular packing and regular crystallization (Wang et al., 2010). Unloaded Alg-RS microparticles showed a wide broad peak in X-ray diffractogram corresponding to the amorphous state. These results are in line with findings reported by Wang et al. (2010) and Fontes et al. (2013) who stated that strong interactions like hydrogen bonds and ionic interactions are taking place between alginate and resistant starch and these interactions are responsible for the miscibility of the two polymers. These interactions destroyed the close packing of the polymer molecules that is considered to be required for the

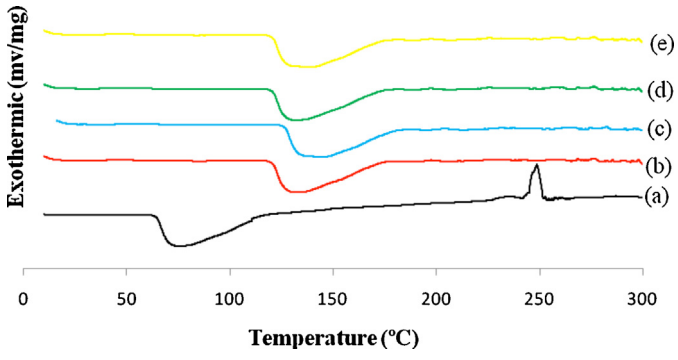

Fig. 3. DSC curves of sodium alginate powder (a), calcium Alg microprticles (b), Alg-RS microparticles (c), nisin-loaded Alg microparticles (d) and nisin-loaded Alg-RS microparticles (e).

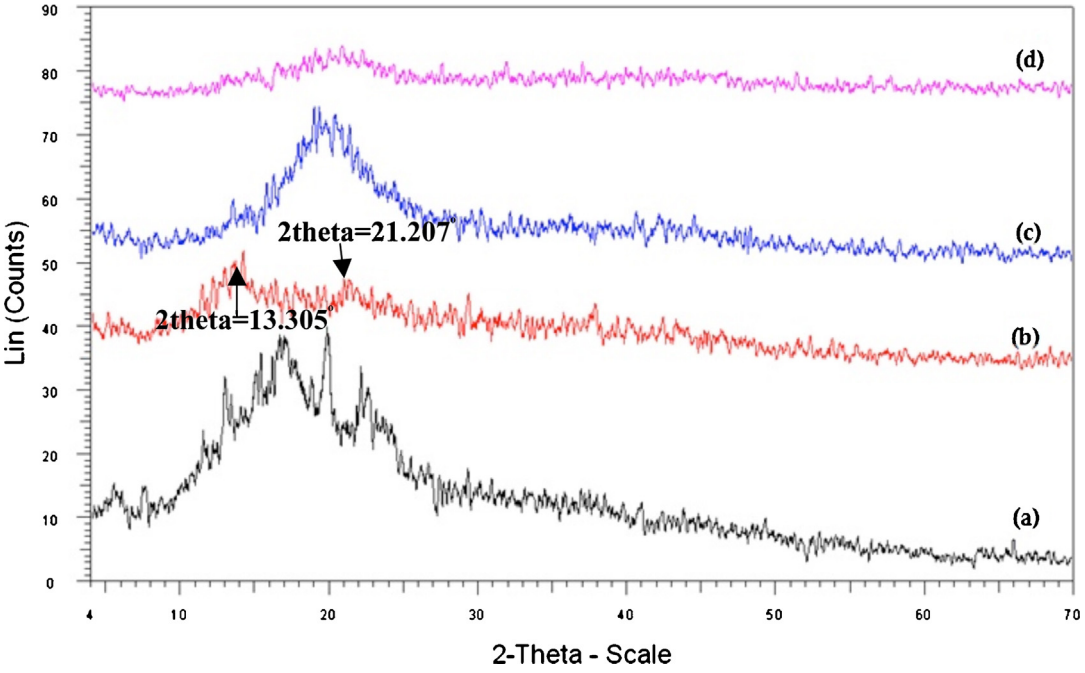

Fig. 4. The X-ray diffractograms of resistant starch (a), alginate (b), unloaded Alg-RS microparticles (c), and nisin loaded Alg-RS microparticles (d).

formation of regular crystallites. The results also are consistent with the FTIR findings. On the other hand, by the incorporation of nisin into microparticles, the broad peak was broader and amorphous behavior was more evident. Similar observation was found by (Bastarrachea et al., 2010) for biodegradable poly butylene adipate-*co*-terephthalate (PBAT) films incorporating nisin. In PBAT films, crystalline peak intensity decreased by increasing nisin content (from 1000 to 5000 IU/cm^2). As the PBAT films containing 5000 IU/cm^2 nisin showed an amorphous behavior. These authors represented that nisin as a foreign substance is able to prevent crystal growth.

3.6. Release studies

In vitro release studies were carried out in order to determine differences in the release profile of microparticles. It was apparent (Fig. 5) that the nisin release from all formulations occurs in two stages. First, a rapid release phase, which corresponds to nisin being physically entrapped in the surface of microparticles, In addition, the fast initial release could be due to the possible uptake of water into the microparticles, and volume expansion of the matrix that occurs because of the water uptake (Naraharisetti, Ning Lew, Fu, Lee, & Wang, 2005). In the second phase, the nisin gradually released, until its content was constant (66–76% in Alg microparticles and 61–70% in Alg-RS microparticles). This second stage can be attributed to the polymer degradation or diffusion of the bacteriocin from the inside of microparticles to the outside. The releasing of nisin depended on the initial bacteriocin content. When the nisin:alginate weight ratio increased from 25% to 100%, the amount of released nisin was found to decrease as shown in Fig. 5a and b. The results also reveal that lower initial level of nisin, gives higher fractional release. This could be mainly attributed to particle size effect. Generally smaller microparticles release encapsulated nisin faster and over shorter time periods due to greater surface to volume ratios. This correlation between particles size and release rate of active compound has been reported previously (Chen & Subirade, 2007; Hosseini et al., 2013). In fact, for smaller microparticles, the release of nisin by diffusion is easier, and for large microparticles, nisin comes out when polymeric matrix starts to degrade.

In addition, lower rate releases were found in Alg-RS microparticles (Fig. 5b). The time required to reach the 50% of initial nisin in the medium was used to evaluate the release rate from microparticles. The values of T_{50} for Alg and Alg-RS microparticles prepared using initial nisin to alginate weight ratio of 25% w/w (Alg_1 and $Alg\text{-}Rs_1$, respectively) were approximately 96 and 168 h, respectively. This higher value for Alg-RS microparticles was attributed to the presence of resistant starch in alginate matrix that retarded the release of nisin. Lopez Cordoba et al. (2013) also reported a delaying yerba mate antioxidants release from alginate hydrogels by using starch filler. The observations indicated that the release of nisin from Alg-RS microparticles occurred in a controlled and sustained manner. This long lasting antimicrobial activity can be useful to improve shelf life of food products.

In order to understand the mechanism of nisin release, the cumulative release data were fitted according to Korsmeyer and Peppas semi-empirical model. Values of n and K, as well as the release kinetics correlation coefficient (R^2) are shown in Table 2. As observed, all of formulations had the diffusion release exponent (n) lower than 0.5 (0.34–0.43). The model achieved good correlations with experimental data ($R^2 > 96\%$). These results demonstrate that the release of nisin from microparticles followed Fickian diffusion, where the release is controlled by diffusional behavior.

Table 2
Kinetic parameters obtained from release curve (n and K) for nisin-loaded Alg and Alg-RS-microparticles.

Microparticles	Kinetic parameters		
	n	K (s^{-n})	R^2
Alg_1	0.341	1.020	0.964
Alg_2	0.386	0.833	0.980
Alg_3	0.411	0.741	0.984
$Alg\text{-}RS_1$	0.399	0.807	0.983
$Alg\text{-}RS_2$	0.419	0.693	0.977
$Alg\text{-}RS_3$	0.432	0.630	0.976

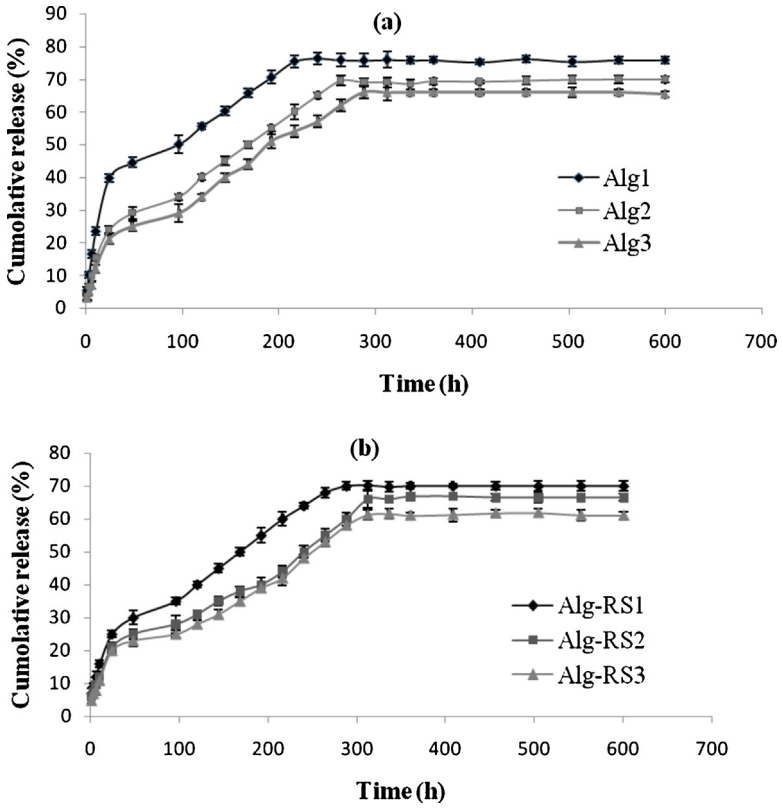

Fig. 5. In vitro release profile nisin from Alg microparticles (a) and Alg-RS microparticles prepared using different weight ratio of nisin to alginate (25, 50 and 100% w/w). Error bars represent the SD ($n = 3$).

4. Conclusion

The bacteriocin nisin was encapsulated in Alg and Alg-RS microparticles at different concentrations. Properties of the microparticles were affected by initial nisin content and starch addition. In both microparticles LC% and particle size increased with increasing nisin content, while EE% declined. Presence of resistant starch in alginate formulation also resulted in a higher percentage of EE, LC and increase in particle size. Results confirmed successful nisin loading in microparticles. The release of nisin occurred through Fickian diffusion. Higher release rates were observed in microparticles with lower amounts of nisin. In addition, blending of resistant starch and alginate had a marked effect on slowing the release of nisin. These results demonstrate that the structural of kinetic properties alginate and resistant starch microparticles are suitable as a controlled release carrier for the food grade peptide, nisin.

Acknowledgments

This research was supported by a grant from the National Nutrition and Food Technology Research Institute of Iran. Furthermore, this article was a part of PhD dissertation at Shahid Beheshti University of Medical Sciences, Tehran, Iran.

References

Alipour, S., Montaseri, H., & Tafaghodi, M. (2010). Preparation and characterization of biodegradable paclitaxel loaded alginate microparticles for pulmonary delivery. *Colloids and Surfaces B: Biointerfaces*, *81*(2), 521–529.

Bastarrachea, L., Dhawan, S., Sablani, S. S., Mah, J. H., Kang, D. H., Zhang, J., et al. (2010). Biodegradable Poly (butylene adipate-co-terephthalate) films incorporated with nisin: Characterization and effectiveness against *Listeria innocua*. *Journal of Food Science*, *75*(4), 215–224.

Benech, R. O., Kheadr, E. E., Laridi, R., Lacroix, C., & Fliss, I. (2002). Inhibition of *Listeria innocua* in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Applied and Environmental Microbiology*, *68*(8), 3683–3690.

Bhatti, M., Veeramachaneni, A., & Shelef, L. A. (2004). Factors affecting the antilisterial effects of nisin in milk. *International Journal of Food Microbiology*, *97*(2), 215–219.

Chan, E. S., Wong, S. L., Lee, P. P., Lee, J. S., Ti, T. B., Zhang, Z., et al. (2011). Effects of starch filler on the physical properties of lyophilized calcium-alginate beads and the viability of encapsulated cells. *Carbohydrate Polymers*, *83*(1), 225–232.

Chen, L., & Subirade, M. (2007). Effect of preparation conditions on the nutrient release properties of alginate – Whey protein granular microspheres. *European Journal of Pharmaceutics and Biopharmaceutics*, *65*(3), 354–362.

El Jastimi, R., Edwards, K., & Lafleur, M. (1999). Characterization of permeability and morphological perturbations induced by nisin on phosphatidylcholine membranes. *Biophysical Journal*, *77*(2), 842–852.

Elversson, J., Millqvist-Fureby, A., Alderborn, G., & Elofsson, U. (2003). Droplet and particle size relationship and shell thickness of inhalable lactose particles during spray drying. *Journal of Pharmaceutical Sciences*, *92*(4), 900–910.

Ercolini, D., Ferrocino, I., La Storia, A., Mauriello, G., Gigli, S., Masi, P., et al. (2010). Development of spoilage microbiota in beef stored in nisin activated packaging. *Food Microbiology*, *27*(1), 137–143.

Fontes, G. C., Araujo Calado, V. M., Rossi, A. M., & Miguez da Rocha-Leao, M. H. (2013). Characterization of antibiotic-loaded alginate–osa starch microbeads produced by ionotropic pregelation. *BioMed Research International*, *2013*, 11 pp., Article ID 472626.

Hosseini, S. M., Hosseini, H., Mohammadifar, M. A., Mortazavian, A. M., Mohammadi, A., Khosravi-Darani, K., et al. (2013). Incorporation of essential oil in alginate microparticles by multiple emulsion/ionic gelation process. *International Journal of Biological Macromolecules*, *62*, 582–588.

Jin, T., Liu, L., Zhang, H., & Hicks, K. (2009). Antimicrobial activity of nisin incorporated in pectin and polylactic acid composite films against *Listeria monocytogenes*. *International Journal of Food Science & Technology*, *44*(2), 322–329.

Kaygusuz, H., & Erim, F. B. (2013). Alginate/BSA/montmorillonite composites with enhanced protein entrapment and controlled release efficiency. *Reactive and Functional Polymers*, *73*(11), 1420–1425.

Kopermsub, P., Mayen, V., & Warin, C. (2011). Potential use of niosomes for encapsulation of nisin and EDTA and their antibacterial activity enhancement. *Food Research International*, *44*(2), 605–612.

Laridi, R., Kheadr, E. E., Benech, R. O., Vuillemard, J. C., Lacroix, C., & Fliss, I. (2003). Liposome encapsulated nisin Z: Optimization, stability and release during milk fermentation. *International Dairy Journal*, *13*(4), 325–336.

Lin, N., Huang, J., Chang, P. R., Feng, L., & Yu, J. (2011). Effect of polysaccharide nanocrystals on structure, properties, and drug release kinetics of alginate-based microspheres. *Colloids and Surfaces B: Biointerfaces*, *85*(2), 270–279.

López-Córdoba, A., Deladino, L., & Martino, M. (2014). Release of yerba mate antioxidants from corn starch–alginate capsules as affected by structure. *Carbohydrate Polymers*, *99*(0), 150–157.

Lopez Cordoba, A., Deladino, L., & Martino, M. (2013). Effect of starch filler on calcium-alginate hydrogels loaded with yerba mate antioxidants. *Carbohydrate Polymers*, *95*(1), 315–323.

Martin, M. J., Lara-Villoslada, F., Ruiz, M. A., & Morales, M. E. (2013). Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum CECT5716*. *LWT – Food Science and Technology*, *53*(2), 480–486.

Martinsen, A., Storro, I., & Skjakbraek, G. (1992). Alginate as immobilization material. 3. Diffusional properties. *Biotechnology and Bioengineering*, *39*(2), 186–194.

Mi, F.-L., Sung, H.-W., & Shyu, S.-S. (2002). Drug release from chitosan–alginate complex beads reinforced by a naturally occurring cross-linking agent. *Carbohydrate Polymers*, *48*(1), 61–72.

Mimmo, T., Marzadori, C., Montecchio, D., & Gessa, C. (2005). Characterisation of Ca- and Al-pectate gels by thermal analysis and FT-IR spectroscopy. *Carbohydrate Research*, *340*(16), 2510–2519.

Moebus, K., Siepmann, J., & Bodmeier, R. (2012). Novel preparation techniques for alginate–poloxamer microparticles controlling protein release on mucosal surfaces. *European Journal of Pharmaceutical Sciences*, *45*(3), 358–366.

Mulders, J. W. M., Boerrigter, I. J., Rollema, H. S., Siezen, R. J., & Devos, W. M. (1991). Identification and characterization of the lantibiotic nisin-Z, a natural nisin variant. *European Journal of Biochemistry*, *201*(3), 581–584.

Naraharisetti, P. K., Ning Lew, M. D., Fu, Y.-C., Lee, D.-J., & Wang, C.-H. (2005). Gentamicin-loaded discs and microspheres and their modifications: Characterization and in vitro release. *Journal of Controlled Release*, *102*(2), 345–359.

Paula, H. C. B., Sombra, F. M., Cavalcante, R. d. F., Abreu, F. O. M. S., & de Paula, R. C. M. (2011). Preparation and characterization of chitosan/cashew gum beads loaded with *Lippia sidoides* essential oil. *Materials Science & Engineering C: Materials for Biological Applications*, *31*(2), 173–178.

Prombutara, P., Kulwatthanasal, Y., Supaka, N., Sramala, I., & Chareonpornwattana, S. (2012). Production of nisin-loaded solid lipid nanoparticles for sustained antimicrobial activity. *Food Control*, *24*(1–2), 184–190.

Rassis, D. K., Saguy, I. S., & Nussinovitch, A. (2002). Collapse, shrinkage and structural changes in dried alginate gels containing fillers. *Food Hydrocolloids*, *16*(2), 139–151.

Ritger, P. L., & Peppas, N. A. (1987). A simple equation for description of solute release II. Fickian and anomalous release from swellable devices. *Journal of Controlled Release*, *5*(1), 37–42.

Salmaso, S., Elvassore, N., Bertucco, A., Lante, A., & Caliceti, P. (2004). Nisin-loaded poly-L-lactide nano-particles produced by CO_2 anti-solvent precipitation for sustained antimicrobial activity. *International Journal of Pharmaceutics*, *287*(1–2), 163–173.

Silva Malheiros, P. D., Serafini Micheletto, Y. M., Pesce da Silveira, N., & Brandelli, A. (2010). Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin. *Food Research International*, *43*(4), 1198–1203.

Soppimath, K. S., Aminabhavi, T. M., Kulkarni, A. R., & Rudzinski, W. E. (2001). Biodegradable polymeric nanoparticles as drug delivery devices. *Journal of Controlled Release*, *70*(1–2), 1–20.

Sultana, K., Godward, G., Reynolds, N., Arumugaswamy, R., Peiris, P., & Kailasapathy, K. (2000). Encapsulation of probiotic bacteria with alginate–starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. *International Journal of Food Microbiology*, *62*(1–2), 47–55.

Tal, Y., van Rijn, J., & Nussinovitch, A. (2000). Starch as a filler, matrix enhancer and a carbon source in freeze-dried denitrifying alginate beads. In N. Katsuyoshi (Ed.), *Hydrocolloids* (pp. 347–354). Amsterdam: Elsevier Science.

Wang, Q., Hu, X., Du, Y., & Kennedy, J. F. (2010). Alginate/starch blend fibers and their properties for drug controlled release. *Carbohydrate Polymers*, *82*(3), 842–847.

Xiao, D., Davidson, P. M., & Zhong, Q. (2011). Release and antilisterial properties of nisin from zein capsules spray-dried at different temperatures. *LWT – Food Science and Technology*, *44*(10), 1977–1985.

Xu, Y., & Hanna, M. A. (2006). Electrospray encapsulation of water-soluble protein with polylactide: Effects of formulations on morphology, encapsulation efficiency and release profile of particles. *International Journal of Pharmaceutics*, *320*(1–2), 30–36.

Zhang, X., Hui, Z., Wan, D., Huang, H., Huang, J., Yuan, H., et al. (2010). Alginate microsphere filled with carbon nanotube as drug carrier. *International Journal of Biological Macromolecules*, *47*(3), 389–395.

Zohri, M., Alavidjeh, M. S., Haririan, I., Ardestani, M. S., Ebrahimi, S. E. S., Sani, H. T., et al. (2010). 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. *Probiotics and Antimicrobial Proteins*, *2*(4), 258–266.

Zou, Q., Zhao, J., Liu, X., Tian, F., Zhang, H. p., Zhang, H., et al. (2011). Microencapsulation of *Bifidobacterium bifidum* F-35 in reinforced alginate microspheres prepared by emulsification/internal gelation. *International Journal of Food Science & Technology*, *46*(8), 1672–1678.