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Full Article

Design and evaluate alginate nanoparticles as a protein delivery system

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ABSTRACT

In recent years, encapsulation of drugs and antigens in hydrogels, specifically in calcium alginate particles, is an interesting and practical technique that was developed widespread. It is well known that alginate solution, under proper conditions, can form suitable nanoparticles as a promising carrier system, for vaccine delivery. The aim of this study was to synthesis alginate nanoparticles as protein carrier and to evaluate the influence of various factors on nanoparticles properties. Alginate nanoparticles were prepared by ionic gelation method. Briefly, various concentrations of $CaCl_2$ were added to different concentrations of sodium alginate dropwisly by homogenizing magnetically at 1300 rpm. The effects of homogenization time and (-) rate were investigated on nanoparticle feature. Nanoparticles were characterized for their morphology and size distribution. Evaluation of loading capacity and loading efficiency of nanoparticles were performed by using various concentration of BSA. The concentration of 0.3%w/v sodium alginate and 0.1%w/v $CaCl_2$ solution, homogenization time 45 min and homogenization rate 1300 rpm were observed as suitable condition - to prepare optimized nanoparticles. It can be concluded that the properties of nanoparticles are strongly dependent on the physicochemical conditions. The optimum concentrations of alginate and $CaCl_2$and appropriate condition led to forming desirable nanoparticles that can be used as carrier for drug and vaccine delivery.

Keywords: Alginate nanoparticles, $CaCl_2$ cross linking agent, ionic gelation, sustain release, vaccine delivery

INTRODUCTION

Over the last decades, using of nanoparticles (NPs) has been increased to prepare different medical applications, for instance in vaccine and gene delivery and controlled release of drugs. Consequently, several techniques have been developed for preparing these particles by using various biodegradable and biocompatible polymers (Catarina Pinto *et al* 2006), such as polysaccharides, lipids and synthetic polymers to increase therapeutic benefit with minimizing side effects (Jahanshahi *et al* 2004, Moghimi *et al* 2001, Zhang *et al* 2001). Among polymers, alginate due to its unique properties have been used as a carrier for variety of biological agents such as genes (Jiang *et al* 2007), antigens and drugs that protect them during the transit

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in the human body (Chretien & Chaumeil 2005). Sodium alginate Microcapsules and poly-L-lysine effectively protect living endocrine tissues from immune rejection after transplantation (Leblond *et al* 1999, Lanza *et al* 1999, De Vos 1997). Alginate NPs increase the metabolic stability of bound antisense oligonucleotides and protect them from degradation in bovine serum and to modify its bio-distribution after intravenous administration (Vauthier *et al* 1998, Aynie *et al* 1999, Lambert *et al* 2001).

Alginate, a water soluble salt of alginic acid, extracted from marine brown algae. It is natural, anionic polysaccharide consisting of a chain of (1–4)-linked β-D-mannuronic acid and α-L-guluronic acid in different arrangements of residues. Alginate is biocompatible, biodegradable and mucoadhesive polymer that don't produce toxicity in the body (Kim & lee 1992, Kurt & Taylor 2009). Alginate particles can be easily prepare by inducing gelation with calcium ions and other divalent cations (Bowersock *et al* 1996, Rajaonarivony *et al* 1993) extrusion (Murata *et al* 1993, Lee & min 1996, Thu *et al* 1996), emulsification (Wan *et al* 1992, Chan *et al* 1997, Gürsoy *et al* 1998, Rebeiro *et al* 1999) and ionic gelation (Rajaonarivony *et al* 1993, Sarmento *et al* 2005). Ionic gelation is one the main method of incorporating polymers to prepare hydrogels. Hydrogels are polymeric networks that due to preparation and processing condition were form in different diameters (beads, microparticles and nanoparticles), which entrapping different amount of drug and biomolecules. This method offers some advantages such as biocompatibility of NPs, simplicity in preparation and low cost (Christenson *et al* 1993). The gelling properties of particles strongly depend to some factors such as physical condition, monomeric composition of polymer, molecular weight and concentration of alginate polymer and cross-linking agent (Ouwerx *et al* 1998, VeIings *et al* 1995). Mayur and coworkers studied the influence of various parameters such as sodium alginate concentration, calcium chloride concentration, and hardening time on papain entrapped in alginate beads, stability improvement and site-specific delivery (Mayur *et al* 2004). One of the parameters that should be considered for preparing micro and nanoparticles is the size of particles. It is well known that the size of the particles should be proportionate with loaded protein and administration way, for example nasal (Rezaei Mokarram & Alonso 2006), oral (Prego *et al* 2005, Qurrat-ul *et al* 2003) and subcutaneous (Kohli & Alpar 2004). The aim of the present study was to formulate alginate NPs as protein delivery system and evaluate the influence of various concentrations of sodium alginate, calcium chloride and some physical conditions such as homogenization time and rate on NPs physicochemical characteristics.

MATERIALS AND METHODS

Materials. Low molecular weight sodium alginate (medium viscosity, 3500 cps for a 2% W/V solution), Poly -L- Lysine (30000-70000Mw) were purchased from Sigma - Aldrich (USA), calcium chloride dihydrate was purchased from Merck, Bovine serum albumin (BSA), Coomassie Brilliant Blue G-250, Methanol 95% and phosphoric acid 85% (w/v) were purchased from Merck, Aqueous solutions were prepared with double distilled water. The all of other materials used in this study were analytical grade.

Preparation of alginate nanoparticles. Alginate NPs were prepared by ionic gelation technique . In order to manufacture of NPs, $CaCl_2$ solution was added to sodium alginate solution dropwisly under homogenization rate 1300 rpm at room temperature. Then, the homogenization was continued for 45 min. Subsequently, the suspension was centrifuged at 11200 rpm, 30 min and the supernatant was discarded. Pellet of NPs was freeze-dried. In order to find suitable concentrations for NPs formation the effects of various concentrations of $CaCl_2$ (0.025, 0.05, 0.075, 0.1, 0.15% w/v) and sodium alginate (0.07, 0.15, 0.3, 0.5, 0.75% w/v) on nanoparticles properties were studied. After finding suitable concentration, effect of different homogenization rate (600, 800, 1000, 1100, 1300 rpm) and homogenization time from 15–60 min on particle

characteristics were investigated. Finally after preparing optimized NPs, loading capacity (LC) and loading efficiency (LE) in NPs were performed by using bovine serum albumin (BSA) as a protein model. For evaluation one factor only its own parameter changed and other factors remained constant.

Loading BSA to alginate nanoparticles. Calcium chloride aqueous solution 10 ml (0.1% w/v) was added to 30 ml of sodium alginate solution (0.3% w/v) containing various concentrations of BSA (0.3, 0.7, 1, 1.3, 1.7, 2 mg/ml) under homogenization rate 1300 rpm at room temperature and then 0.5ml of poly- L- Lysine was dropwisly added to the suspension. Subsequently, the suspension of NPs was homogenized for 45 min at room temperature.

BSA Loading Efficiency and Loading Capacity. LE and LC of BSA in NPs were detected indirectly by determining free BSA in the supernatant. For this purpose, NPs suspension was centrifuged at 11200 rpm for 30 min. The amount of BSA in the supernatant was estimated by Bradford protein assay (Bradford 1976). The supernatant of blank NPs was adopted as the blank to correct the absorbance reading value of the BSA-loaded NPs. The concentration of BSA in the supernatant was calculated using the standard curve prepared at same time. The LE and LC values were calculated according to the following equations:

$$LE(\%) = \frac{Total\ amount\ of\ protein - Free\ protein}{Total\ amount\ of\ protein} \times 100$$

$$LC(\%) = \frac{Total\ amount\ of\ protein - FRee\ protein}{Dried\ wieght\ \ nanoparticles} \times 100$$

Morphological characterization, size and surface charge. The morphological characteristics, surface appearance and size distribution [polydispersity Index (pdI)] of NPs were examined by scanning electron microscopy (SEM) (INCN Wave oxford) and Dynamic light scattering (DLS), respectively. The particle size distribution and zeta potential of NPs were evaluated by a dynamic light scattering technique with a Zetasizer (Nano-ZS red badge, Malvern Instrument, UK; zen 3600 Laser Particle Size Analyzer). Zeta potential determinations were based on electrophoretic mobility of the NPs in aqueous suspensions.

Fourier transform infra-red (FTIR) measurements. The interactions between sodium alginate and $CaCl_2$ in blank NPs and calcium alginate and BSA in BSA - loaded NPs, samples were evaluated by Fourier transform–infrared spectroscopy (FT-IR; Jasco FTIR-410; Jasco, Colchester, United Kingdom) at room temperature. NPs were lyophilized, gently mixed with KBr powder and compressed in to the pellets and samples were measured.

In vitro release of BSA. Invitro release behavior of BSA from alginate NPs was determined as follows. The distinctive amount of NPs was divided in several test tubes (1mg in each tube). Subsequently, 1 ml of phosphate buffer saline (PBS, pH7.4) was added to each test tube. Then, the tubes were placed in shaker-incubator at 37 °C. At scheduled time intervals, samples were taken and centrifuged at 13000 rpm for 20 min. The amount of BSA in the supernatant was determined by Bradford.

RESULTS

Ionic gelation method result in formation of calcium alginate complexes and interaction between the calcium ions and the glucuronic sequences of alginate polymer redounded to formation egg-box structures (Grant *et al* 1973). This process has several significant aspects: less toxic reagents, simplification of the procedure that does not require specialized equipments, simple optimization to improve yield and entrapment efficiency (Christenson 1993).

Effects of physicochemical factors on NPs preparation. Alginate NPs were prepared by using 30 ml of various concentrations of alginate solution. The effect of $CaCl_2$ concentration on NPs formation was studied by fixing the alginate solution at 0.3% w/v. The optimum condition for preparation of suitable NPs were observed in 0.3% w/v of sodium alginate , 0.1% w/v of $CaCl_2$, 45 min (homogenization time)and1300 rpm (homogenization rate) (Table 1).

Table1. Effect of physicochemical factors on nanoparticle formation.

physicochemical factors		Yield of nanoparticles (mg)	Nanoparticle formation
Alginate concentration[a] (%w/v)	0.07	21.9±0.4	S[d]
	0.15	36.4 ±3.1	S
	0.3	73±2.1	SN[d]
	0.5	120.2±0.2	G[d]
	0.75	8.4±0.36	G
CaCl2 concentration[b](%w/v)	0.025	1.73±0.254	S
	0.05	5.23 ±0.2	S
	0.075	72±0.45	S
	0.1	73.2±2.1	SN
	0.15	87.6±0.2	MG[d]
Homogenization time[c](min)	0	71.33±1.52	MG
	15	73.2±0.6	MG
	30	71.9±0.85	NS[d]
	45	72±2.1	SN
	60	72.16±0.76	SN
Homogenization speed[c](rpm)	600	90.16±0.76	G
	800	85.66±1.6	G
	1000	67.5±0.5	MG
	1100	70.30±0.5	NS
	1300	73±2.1	SN

[a]$CaCl_2$ concentration 0.1%w/v
[b] sodium alginate concentration 0.3%w/v
[c]$CaCl_2$ concentration 0.1%w/v;sodium alginate concentration 0.3%w/v
[d]G, continuous gel; SN,suitable nanoparticles; S, solution; MG, microgel gel;NS,non spherical

Effect of BSA concentration on LE and LC. Evaluation of NPs efficiency for encapsulation of protein was performed with BSA as a protein model**.** For this purpose, BSA was added to sodium alginate solution and subsequently, BSA-loaded NPs were prepared. As shown in Figure 1, by increasing the BSA concentration, the LE and LC increased. Results indicated that with 2 mg/ml BSA concentration led to maximum and considerable LE (76.3%) and LC (73.5%).

Nanoparticles characteristics. The results indicated that the optimized blank NPs have a particle size distribution <100 nm with pdI=0.46 (Table 2). The zeta potential of blank NPs was -46.7. The BSA-loaded NPs exhibited relatively narrow particle size distribution by relatively low pdI value (0.36) and zeta potential of BSA loaded NPs in comparison with blank NPs decreased (-24) (Table 2). SEM images shown in figure 2 indicated that BSA loaded NPs are spherical without any aggregates. The FTIR spectra of pure alginate, blank NPs and the BSA- loaded NPs are shown in Figures 4 and 5, respectively.

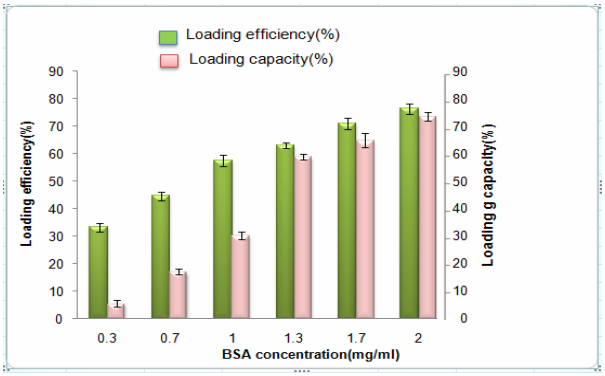

Figure 1. Effects of different BSA concentrations on loading efficiency (LE) and loading capacity (LC) of sodium alginate nanoparticles.

In vitro release of BSA. The release profile of BSA from alginate NPs was carried out by using phosphate buffer (PBS, pH7.4) at 37 °C. The release behavior is shown in figure 3, the slow release of BSA from NPs occurred in the first 8 h and then followed next 120 hours with constant rate and approximately 60% of BSA released within 3 days.

Table 2. Polydispersity index and Zeta (ζ) potential of Blank and BSA-loaded alginate nanoparticles.

Nanoparticles	average (nm) mean±S.D.	Polydispersity, mean±S.D.	Zeta potential (mV), mean±S.D.
Optimized blank nanoparticles	80±0.01	0.46±1.5	-6.7±4.9
BSA-loaded- nanoparticles	50±0.01	0.36±0.8	-24±3.7

DISCUSSION

The results indicated that use of sodium alginate with the concentrations higher than 0.3% w/v leads in formation of macroscopic gels (Table 1). It seems that the alginate concentration higher than 0.3% w/v is due

to high viscosity and lack of enough shear stress redounded to formation of gels. In contrast, the samples with concentrations of higher than 0.1% w/v $CaCl_2$ induced microscopic gel aggregates. It is suggested that calcium divalent cations allowing the formation of microdomains with high concentrations of alginate instead of an infinite network of gels.

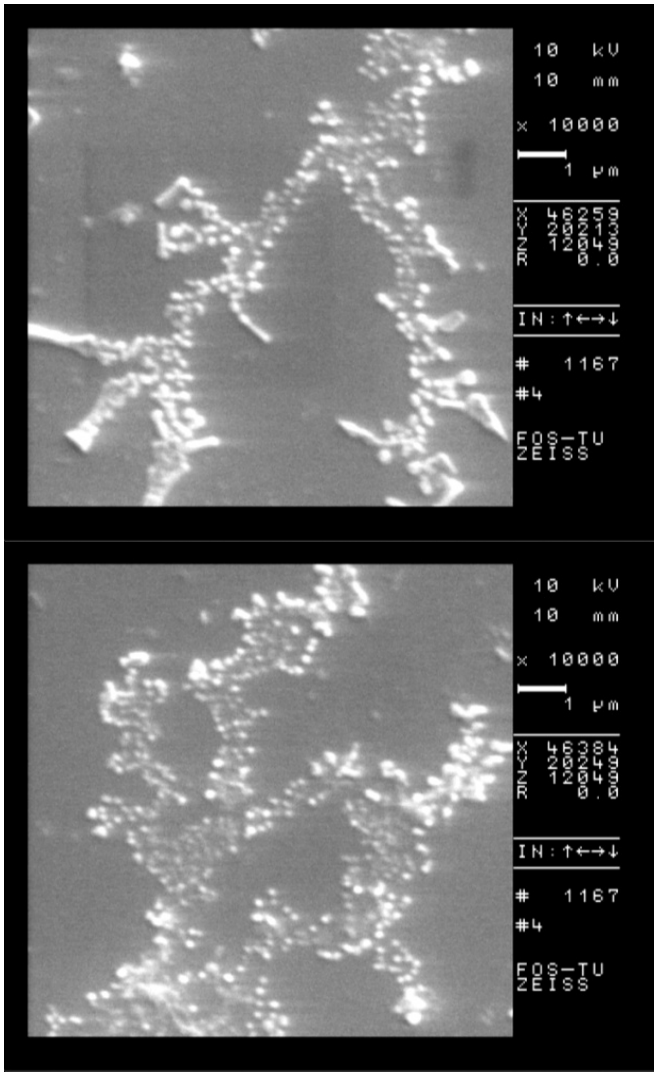

Figure 2. SEM images of BSA loaded alginate nanoparticles (sodium alginate concentration 0.3, CaCl2 0.1%w/v and BSA

In other respects, this effect is presumably due to increasing the number of Ca^{2+} per unit of solution volume, equilibrium between Ca^{2+} and alginate binding sites and consequently formation of NPs disordered. Our observations are in agreement with Ganeshchandra et al 2007 (Ganeshchandra *et al* 2007) and in contrast

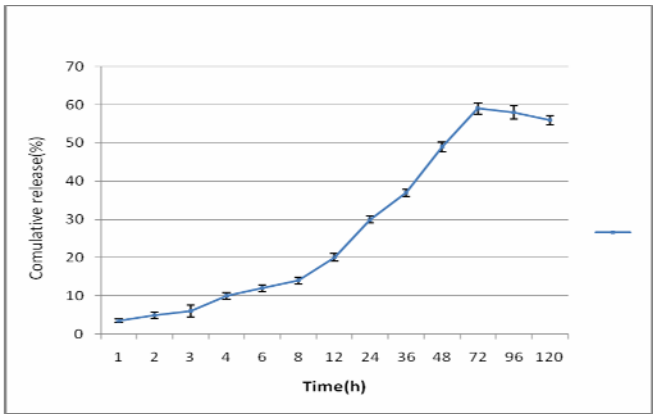

Figure 3. In vitro release profile of BSA from alginate nanoparticles (sodium alginate concentration 0.3%w/v, CaCl2 0.1%w/v and BSA 2mg/ml).

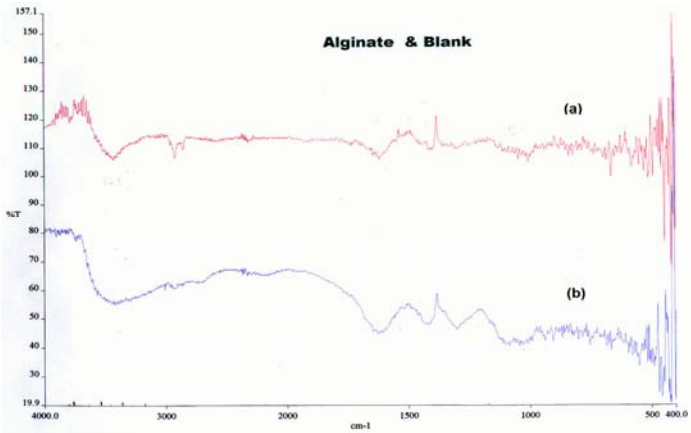

Figure 4. FTIR spectra of alginate powder (a) and blank alginate nanoparticles (sodium alginate concentration 0.3%w/v, CaCl2 0.1%w/v) (b).

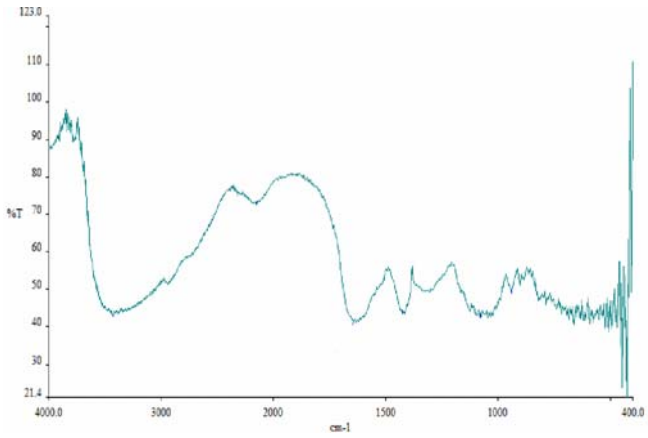

Figure 5. FTIR spectrum of BSA-loaded nanoparticles.

with Rezaei Mokarram et al, who reported the microspheres prepared with a concentration 1 %w/v

produce regular and homogenous microspheres, but at lower or higher than 1 %w/v leads to irregular and agglomerated microspheres. In case of $CaCl_2$ in lower concentrations than 0.1% w/v, the numbers of binding sites on alginate were more than the number of Ca^{2+} ions. In this condition, the Ca^{2+} could not induce desirable NPs with good density. The present study showed that with increasing the homogenization time from 15 min to 60 min, the particle size were decreased. However, after 45 min, no significant change in particle size were observed. In similar study, Ferreiro et al. showed that increase in homogenization time decreased particle size of oligonucleotide loaded alginate microparticles (Ferreiro *et al* 2002). In the otherhand by increasing the homogenization rate leads to decreasing in particle size and redounded to good spherical shape (Table 1). The results of this work are in agreement with other studies (Haznedar & Dortunç 2004). Ganeshchandra et al. showed that by increasing homogenization rate from 500 rpm to 3000 rpm, the particle size of NPs decreased gradually (Ganeshchandra *et al* 2007). Our study revealed that by increasing the BSA concentration, the LE and LC were increased. It can be explained that, encapsulation of proteins within NPs depends on unsaturated sites to formation of hydrogen and electrostatic bounds on alginate chains. It is obvious that BSA interacts with hydroxyl groups of alginate chains via electrostatic and hydrogen bounds. By increasing the BSA concentration to 2 mg/ml, free binding sites within polymer chains saturated with BSA molecules. Our results are confirmed by Ahmad et al 2006 (Ahmad *et al* 2006) and Sangeetha et al 2007 (Sangeetha *et al* 2007). BSA loaded NPs showed pdI=0.36, which were smaller than blank NPs and Zeta potential also decreased from -46.7 to -24 mv. It can be explained after the addition of poly-L-lysine, the electrostatic interaction between the positively charged $-NH_3^+$ of poly-L-lysine and negatively charged $-COO^-$ of alginate resulted to smaller size and lower negative charge. The SEM photograph of BSA loaded NPs showed that NPs were spherical and discrete without any aggregation (figure 2). On the basis of the FTIR spectra, a slight difference in the width and frequency of the peaks can be observed between BSA loded and unloaded nanoparticles. In three spectra the strong and broad peaks in the 3400–3300 cm^{-1} ranges correspond to O–H stretching and intermolecular hydrogen bonding. Around the wave numbers of 1700–1400 cm^{-1}, observed peaks belong to the C=O stretching (amide). Our results showed that, carboxyl peaks near 1613 cm^{-1} (symmetric $-COO^-$ stretching vibration) and 1415 cm^{-1} (asymmetric COO^- stretching vibration) are broad after interaction with BSA. The unload and loaded NPs spectra were compared, the results showed that the changes in the amino, carboxyl and amide groups peaks, which revealed an ionic interaction between the carbonyl groups of alginate and the amino groups of BSA. In our study we observed the carboxylate groups' peaks are present at the wave number area of 1000–1400 cm^{-1}. The similar observations were reported previously by Mitrevej et al (Mitrevej *et al* 2001). The in vitro release profile of BSA loaded NPS shown the long release time and slow release rate of BSA. It seems that the BSA macromolecules were bind on to NPs by strong interactions, and the presence of poly -L-lysine layer could increase the stability of NPs and slow down the diffusion of BSA from the NPs. Subsequently, due to degradation and erosion of matrix, the BSA released from the alginate NPs in an extended profile. The results were obtained in the present study confirmed by Anal and coworker in 2003 (Anal *et al* 2003). There are few reports on BSA and DT release profiles by coppi (2002) and Rezaei Mokarram (2008), which their observation are not agree with our study. In these studies, the release was a biphasic linear profile, the first phase was very fast, which followed by a slow release. It is suggested that this difference could be due to the use of different process for preparing of particles (Coppi *et al* 2001, Rezaei Mokarram & Alonso 2008). It can be concluded that the properties of NPs are strongly depending on both calcium chloride and sodium alginate concentrations. The gelification step of the

alginate by calcium is a critical factor for the preparation of the suitable NPs. The optimum concentrations of alginate and calcium led to formation of the desirable NPs and consequently high LE and LC (over 70%) and sustained release profile. It is suggested that these alginate NPs probably could be used as carrier for drug and vaccine delivery.

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