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Levan-based nanocarrier system for peptide and protein drug delivery: Optimization and influence of experimental parameters on the nanoparticle characteristics

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ABSTRACT

Microbial levans are biopolymers produced from sucrose-based substrates by a variety of microorganisms. There is very limited information related to the levan-based drug delivery systems. In this study, bovine serum albumin (BSA) encapsulated-levan nanoparticles were prepared using levan produced by a new *Halomonas* sp. Effects of polymer and BSA concentrations and rotating speed on *in vitro* characterization of the nanoparticles were investigated. The size of levan nanoparticles, with the surface charges +4.3 mV to +7.6 mV, changed between 200 nm and 537 nm. The encapsulation capacity of the particles changed between 49.3% and 71.3% depending on the levan concentration used in the formulation. The cumulative *in vitro* release of protein from the particles was shown to be controlled release of BSA. This study affirmed the suitability of levan by *Halomonas* sp. to be used as a nanocarrier system for potential delivery of macromolecular drugs such as peptides and proteins.

1. Introduction

Nanotechnologies have long been used in designing systems for targeting specific cell populations or controlled release of bioactive substances and hence, related research has been focused on the development of nanoparticles for their use in a wide range of applications as delivery vesicles for drugs, DNA, antigens, and protection proteins and enzymes, especially for controlled or sustained drug-delivery systems employing biopolymers (Bowman & Leong, 2006; Luten, Van Nostrum, De Smedt, & Hennink, 2008; Park, Ye, & Park, 2005).

Nanoparticle drug delivery systems are nanometric carriers used to deliver drugs or biomolecules. Generally, nanometric carriers also comprise sub-micro particles with sizes below 1000 nm and they provide prolonged stability for unstable proteins and peptides during storage as well as in biological media (Jung et al., 2000; Pisal, Kosloski, & Balu-Iyer, 2010).

There are significant advantages in utilizing a biopolymer in drug delivery systems when compared with synthetic polymers. Besides their biodegradability and biocompatibility, other advantages include their ease of production from natural sources such as biowaste and use of unsophisticated equipments such as fermenters for their production where conditions can be adjusted to synthesize biopolymers with desired properties. Moreover, with natural systems, higher levels of molecular complexity can be achieved when compared to chemical synthesis (Coviello, Matricardi, & Alhaique, 2006). Changing the biopolymer structure by modifying substitute groups, by changing the physicochemical environmental conditions and/or by molecular manipulation of microorganism metabolism are additional important advantages (Cimini, Restaino, Catapano, De Rosa, & Schiraldi, 2010). Faster biodegradation and higher biocompatibility or their combinations are some of the motives for the increased interest in tailor-made biopolymers with innovative chemical structures. Especially, new drug delivery systems which provide advantageous properties such as drug solubility under physiological conditions, drug encapsulation and yield, bioavailability, sustained and controlled release and reduced drug doses have a significant role in drug carrier systems in last decade (Hasirci et al., 2006; Jayakumar, New, Tokura, & Tamura, 2007).

Biopolymeric drug carriers of nanoscale size range have attracted increasing attention and natural based polysaccharides are widely used in pharmaceutical industry because of their biocompatibility and low toxicity (Heath, Haria, & Alexander, 2007; Lambert, Fattal, & Couvreur, 2001). A number of these polysaccharides are polyelectrolytes and show great potential to interact with therapeutic molecules and therefore are attractive materials as drug delivery vehicles for peptides, proteins and other

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Table 1
Codes, drug encapsulation capacity, mean particle size and zeta potential values of levan nanoparticles.

Codes	Levan concentration (% w/v)	BSA concentration (% w/v)	Rotating speed (rpm)	Particle size (nm ± SD)	Zeta potential (mV ± SD)	Encapsulation capacity (% ± SD)
A1	0.1	0.1	500	435 ± 24.1	4.3 ± 0.1	52.7 ± 1.5
A2	0.5	0.1	500	521 ± 11.0	7.1 ± 0.2	71.3 ± 3.5
B1	0.1	0.1	20.000	200 ± 22.5	4.4 ± 0.2	49.3 ± 3.1
B2	0.5	0.1	20.000	344 ± 36.5	7.3 ± 0.3	69.3 ± 1.5
C1	0.5	0.2	500	537 ± 9.5	7.6 ± 0.2	68.7 ± 1.2

therapeutic agents (Baldwin & Kiick, 2010; Paños, Acosta, & Heras, 2008). Cationic or anionic polysaccharides such as chitosan and alginate (George & Abraham, 2006), dextran (Cheung, Ying, Rauth, Marcon, & Wu, 2005), pullulan (Akiyoshi et al., 1998; Fundueanu, Constantin, & Ascenzi, 2008) and fucoidan (Sezer & Akbuğa, 2006; Sezer et al., 2008) and their modified forms are used for preparation of nano and microparticles.

Levan is a naturally occurring polymer of β-D-fructofuranose with β (2–6) linkages between fructose rings and it shows amphiphilic, strongly adhesive and film-forming properties with good biocompatibility (Kang et al., 2009). In medicine, levan is used as plasma substitute and prolongator of drug activity. Besides its prebiotic and hypocholestrolaemic effects, levan is also shown to exert immunomodulatory, cell-proliferating, skin moisturizing and irritation-alleviating effects and it is also used as a coating material in drug delivery systems (Fett, Wells, Cescutti, & Wijey, 1995; Kang et al., 2009; Kim et al., 2005). These properties distinguishing levan from other polysaccharides have long been the focus of interest however due to its high cost, levan has only been available in small quantities. Recently, a newly isolated halophilic bacterial strain *Halomonas* sp. AAD6 was found to produce high levels of levan with high biocompatibility and hence *Halomonas* sp. was reported as a levan producer microorganism for the first time (Poli et al., 2009).

Due to its amphiphilic nature, levan has been reported to form nanoparticles by self-assembly in water (Renuart & Viney, 2000). Despite a great number of studies on the different properties of the microbial polymer, levan, were done, there is very limited information related to the levan-based drug delivery systems. Considering this fact, the main objective of this study was to elucidate the potential of levan produced by *Halomonas* sp. to be used as a biopolymer in nanoparticle drug delivery systems. For this purpose, levan nanoparticles were prepared with BSA, used as a model protein, and various properties of these particles were analyzed to investigate the effects of polymer and BSA concentrations and rotating speed on particle size, charge, encapsulation capacity and release properties of the nanoparticles.

2. Experimental

2.1. Materials

BSA (fraction V, minimum 96%) was provided by Sigma Chemical Co. (St. Louis, USA), protein molecular weight marker was obtained from Fermentas Chemical Co. (Maryland, USA) and sodium sulphate supplied from Merck Chemical Co. (Darmstad, Germany). All other chemicals were of pharmaceutical or molecular grade.

2.2. Microbial production and purification of levan

Halophilic *Halomonas* sp. AAD6 (JCM15723, DQ131909) bacterial strain was used for the production of levan and bioreactor cultivations were performed in a 1L BIOSTAT Q multi-fermenter with well-controlled environment of pH and temperature. In each run, the working volume was 500 mL, the temperature and pH were kept constant at 37 °C and pH 7, respectively. Aeration was provided at a rate of 0.1 vvm and agitation was set to 200 rpm. The composition of the medium used for the bioreactor cultivations, was (g L^{-1}): 137.2 NaCl; 50 Sucrose; 7 K_2HPO_4; 2 KH_2PO_4; 0.1 $MgSO_4 \cdot 7H_2O$; 1 $(NH_4)_2SO_4$ and 0.5 peptone.

At the stationary phase of growth, cells were harvested by centrifugation at 13,000 × *g* for 20 min and the levan in the supernatant phase was precipitated with an equal volume of ethanol. Alcoholic solutions were kept at −18 °C overnight and then centrifuged at 15,000 × *g* for 30 min. The levan pellet was resuspended in distilled water, dialyzed against distilled water for at least three days and then lyophilized. To remove impurities, levan was further purified by passing through a DEAE-Sepharose CL-6B column. Flow-through fractions containing pure levan were collected and bound impurities were eluted from the column with 1 M NaCl. Levan fractions were tested for total carbohydrate and protein contents and then dried by lyophilization (Poli et al., 2009). Nucleic acid content of the pure samples was analyzed with Quant-iT PicoGreen Kit (Invitrogen, USA) where fluorescence was measured with the LightCycler Instrument (Roche, Mannheim, Germany).

2.3. Preparation of levan nanoparticles

Levan nanoparitcles were prepared by precipitation method (Berthold, Cremer, & Kreuter, 1996). 10 mL aqueous solution of Na_2SO_4 (20% w/v) containing BSA was added dropwise from a distance of 10 cm into 10 mL pure levan solution (pH 3.5) and this suspension was stirred at 500 rpm for 30 min on magnetic stirrer (Ika-Werk, Germany) or 20,000 rpm for 5 min using ultraturax homogenizer (Ika, Euroturrax T20, Germany). Levan nanoparticles were separated by centrifugation at 15,000 rpm (5810R, Eppendorf, Germany), washed three times with distilled water and then freeze-dried (Lyovac GT 2E, Steris, Germany). A number of variables such as polymer, drug concentrations and preparation rotating speed were investigated for the purpose of optimization of the nanoparticle formulations (Table 1).

2.4. Morphological analysis

The prepared levan nanoparticles were mounted on metal grids with double sided adhesive tape, coated with gold to ~500 × 10^{-8} cm in thickness using SC7640 sputter coater (Quorum Technologies, Newhaven, UK) under high vacuum, 0.1 Torr, 1.2 kV, and 50 mA at 25 °C ± 1 °C. The surface morphology of coated samples was examined by scanning electron microscopy (SEM; Jeol JSM-5910 LV, Tokyo, Japan) at 20 kV.

2.5. Size distribution of nanoparticles

Measurements were performed at 25 °C, using a particle size analyzer (Nano-ZS Zen 3600, Malvern Ltd., Malvern, UK) with a laser diode illuminated light scatter sensor. The particle suspensions were sonicated in an ultrasonic bath for 0.5 min prior to analysis and bidistilled water was used as a dilution medium. Analyses were performed in three different batches and the results were expressed as a mean of three measurements.

2.6. Zeta potential

The zeta potential values of nanoparticles were determined in phosphate buffered saline (pH 7.4, BP) after measurement of the electrophoretic mobility at 25 °C using a Zetasizer (Nano-ZS Zen 3600, Malvern Ltd., Malvern, UK). Measurements were carried out in triplicates.

2.7. Determination of entrapment efficiency

The BSA content of the nanoparticles was calculated by difference between the total amount added in the aqueous solution and the amount of free BSA in the crude nanoparticle system. The free protein amount in the supernatant was assayed spectrophotometrically at 595 nm (BioSpec-1601, Shimadzu, Japan) with Bradford's protein assay method (Bradford, 1976). The values are the average of three experiments.

The BSA encapsulation efficiency (EE) was calculated by the following equation:

$$\%EE = \frac{A_{IP} - A_{FP}}{A_{IP}} \times 100 \quad (1)$$

where 'A_{IP}' is amount of the initial BSA used in the preparation of the nanoparticles and the 'A_{FP}' is the amount of free BSA detected in the supernatant after centrifugation of the aqueous dispersion.

2.8. In vitro release study

Protein release from the nanoparticles was determined after incubating 10 mg BSA encapsulated-levan particles in 2 mL phosphate buffered saline (PBS pH 7.4, BP) in a shaker bath at 37 ± 0.1 °C and 100 rpm. Samples were removed at certain time intervals and centrifuged for 10 min at 15,000 rpm. Fresh buffer was added to the pellets after each sampling. The amount of released protein was assayed spectrophotometrically as mentioned above. Each experiment was carried out three times. The *in vitro* release data of the nanoparticles were evaluated kinetically by zero-order and first-order kinetics, Higuchi model and Hixson–Crowell model (Higuchi, 1963; Hixson & Crowell, 1931). The correlation coefficients of the kinetic models were determined using Version 1.0.40 of a software program developed by Ege, Karasulu, Karasulu, and Ertan (2001).

2.9. Polyacrylamide gel electrophoresis (SDS-PAGE)

The structural integrity of BSA after release studies was checked by SDS-PAGE and compared with native BSA. Protein samples were diluted with Tris buffer (pH 6.8) containing 2% SDS. Electrophoresis of samples was performed at a constant voltage of 80 V in Tris/glycine/SDS buffer using a Sigma electrophoresis system. After migration, the gel was stained with coomassie blue in methanol–acetic acid–water solution (2.5:1:6.4) to reveal protein, destained and dried.

2.10. Statistical analysis

All the values are the means of three experiments ±S.D. Statistical data analysis was performed using the one-way analysis of variance with $P < 0.05$ as minimum level of significance.

3. Results and discussion

3.1. Characteristics of levan nanoparticles

Levan is an amphiphilic polymer because of the CH_2 groups in the furanose residues in its structure as shown in Fig. 1. Although the levan backbone is basically flexible, extensive intramolecular hydrogen bonding results in significant conformational rigidity so that each molecule adopts a compact, spherically symmetrical globular conformation in aqueous solution (Kang et al., 2009; Renuart & Viney, 2000).

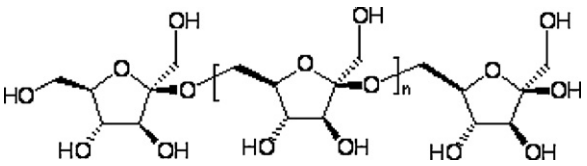

Fig. 1. Chemical structure of levan isolated from *Halomonas* sp.

In order to evaluate the potential use of self assembled levan particles as a nanocarrier system for peptide and protein based drugs, levan nanoparticles were formed where BSA was used as a model protein. BSA is known to be composed of 582 amino acid residues and has an isoelectric point value of 4.7 and therefore, it was expected to have negative net charge under the experimental conditions. The secondary structure of BSA has been suggested to be predominantly composed of α-helices (66%) with a remaining content of β-sheets turn and side chains (34%). Almost all the hydrophobic residues were found inside through and between the helices, while the polar residues were on the outer wall of the structure (Gelamo, Silva, Imasato, & Tabak, 2002).

In this study, a simple salting-out method was used to prepare BSA encapsulated-levan nanoparticles. Morphology and surface characteristics of the particles were investigated by SEM. As shown in Fig. 2, particles were characterized by a relatively smooth and porridge-like surface with few small pores. The sizes of BSA

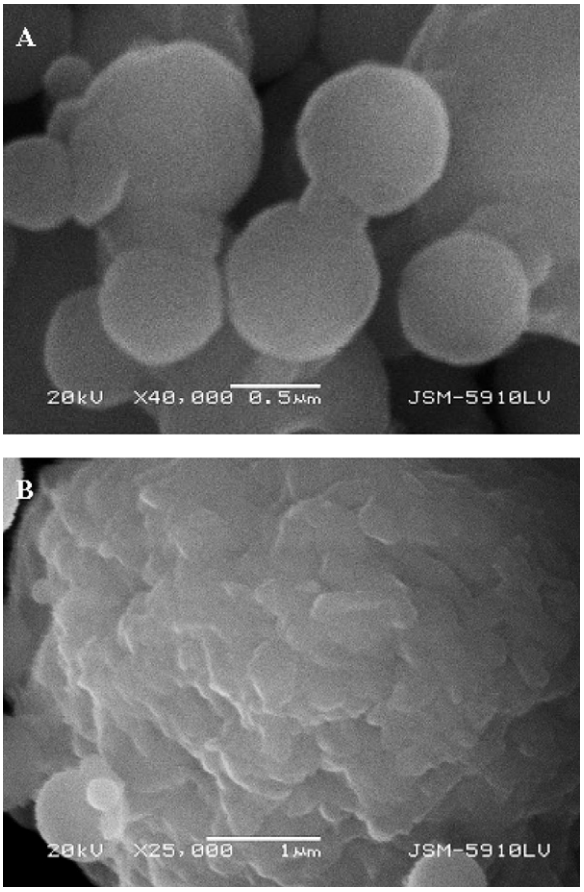

Fig. 2. Scanning electron micrographs showing the external (A) and surface morphology (B) of BSA-encapsulated levan nanoparticles (A2 series).

encapsulated-levan nanoparticles ranged between 200 nm and 537 nm depending on the stirring speed and protein and polymer concentrations (Table 1). Nanoparticles with smallest particle size were obtained at high stirring rates and low polymer amounts in the formulation. Increasing the levan concentration from 0.1% to 0.5% or decreasing the stirring speed from 20,000 rpm to 500 rpm was found to result in larger particles (Table 1). Whereas at low stirring rates, a five-fold increase in levan concentration did only result in 20% increase in particle size, at high stirring speeds, such an increase resulted in a more profound increase in particle size (70%). Some physicochemical properties of the polymer play important roles when the particles begin to shape in the solution such as low hydrodynamic volume and viscosity are known to favor the formation of smaller sized particles. These results show similarity with the previous literature (Jones, Decker, & McClements, 2009; Jung et al., 2000; Sara, Rinehart, & Gadala-Maria, 2006; Sezer, Kazak, Toksoy Öner, & Akbuğa, 2009).

The encapsulated protein amount did not affect the particle size when the BSA amount was increased two-fold in the formulation (Table 1) ($P < 0.05$). The particle size distribution (polydispersity index) of levan nanoparticles was found to be narrow at low polymer concentrations and it widened with increasing polymer concentration (data not given) ($P < 0.05$).

The zeta potential values of levan nanoparticles were between +4.3 mV and +7.6 mV with higher potential values at higher polymer concentrations (Table 1). Positive zeta potential values were recorded for all nanoparticle formulations and the charge value of levan solution was determined as +5.8 mV. This indicated that the outer surface of the nanoparticles consisted of levan only and the polymer encapsulated negatively charged BSA. Most probably, as a result of interaction between negatively charged polar residues of BSA and levan substitute groups, a BSA–levan complex is formed and constituted the hydrophobic core of particles whose outer layer was covered with excess levan. The pure levan samples used in this study were found to be mainly composed of carbohydrates, 0.07% (w/w) protein and 0.08% (w/w) nucleic acid by mass. These trace amounts of proteins could be the reason for the observed weak cationic property as also suggested by Grube, Bekers, Upite, and Kaminska (2002).

As shown in Table 1, protein encapsulation capacity of levan nanoparticles changed between 49.3% and 71.3% and it was observed that the particles containing the highest polymer concentration showed the highest BSA encapsulation (71.3%). The encapsulation capacity increased with increasing levan in the formulations. The lowest protein encapsulation capacity (49.3%) was obtained from nanoparticles prepared with the highest rotating speed and 0.1% polymer concentration. This result was similar with the results of the other biopolymeric nanoparticle systems reported earlier (Berthold et al., 1996; Coppi, Iannuccelli, Leo, Bernabei, & Cameroni, 2002). The spread length of levan chain in solution may vary in correlation with the molecular weight, which may affect protein interaction and encapsulation. The molecular weight of levan polymer used in this study was more than 1,000,000 g mol^{-1} (Poli et al., 2009). The fact that BSA encapsulation efficiency trended up approximately 20% when the levan concentration increased from 0.1% to 0.5% also supports this hypothesis.

Higher concentrations of levan in the formulations resulted in only slightly increased zeta potential of the particles so that BSA entrapment in and adsorption on the particle would have significantly reduced the positive surface charge of the particles. The carboxyl groups on the surface of a large protein molecule may form hydrogen bonds with polymer structure at the spread chain. Electrostatic interactions between BSA and levan could be the reason for the high protein encapsulation during the self assembly of particles (Grenha, Seijo, & Lopez, 2005). On the other hand, the state of complex 3D conformation of the large protein molecules at the given solution pH and ionic strength conditions is also important. In addition, effect of the levan molecular weight on encapsulation is largely the consequence of diverse nature of the interactions between the polymer chain and both positively and negatively charged functional groups as well as the hydrophobic regions of protein molecules (Coppi et al., 2002; George & Abraham, 2006; Jayakumar et al., 2007).

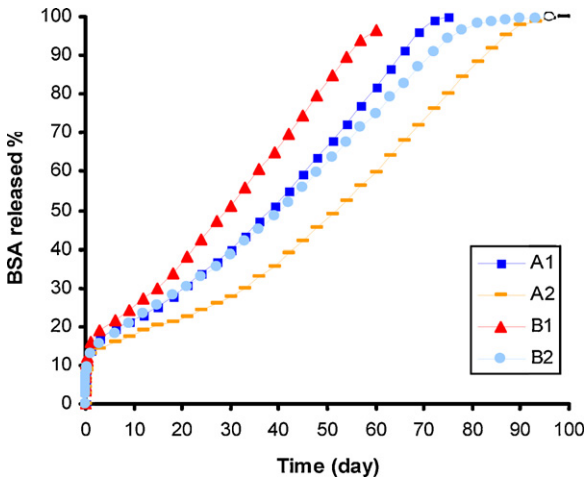

Fig. 3. Effect of polymer concentration on protein release from levan nanoparticles [rotating speeds were 500 rpm (A series) and 20,000 rpm (B series)].

3.2. *In vitro protein release from levan nanoparticles*

The protein release profiles from levan nanoparticles are shown in Figs. 3–5. As seen in Fig. 3, protein release from the nanoparticles with BSA encapsulated at 0.1% concentrations showed the slowest release from the particles with higher concentration of levan (A2 formulation). The mean total amount of BSA released from the nanoparticles was between 10% and 23% within the first 10 days and then the remaining protein was released completely within 60–100 days (Fig. 3). Otherwise, levan concentration was indicated retardant effect for release the protein from the nanoparticles ($P < 0.05$). As given from the release profiles in Fig. 4, protein release from the nanoparticles was affected by the BSA concentration in the formulation so that it slowed down significantly at low BSA concentrations. These results suggest that BSA release behaviour is closely related to initial protein loading capacity.

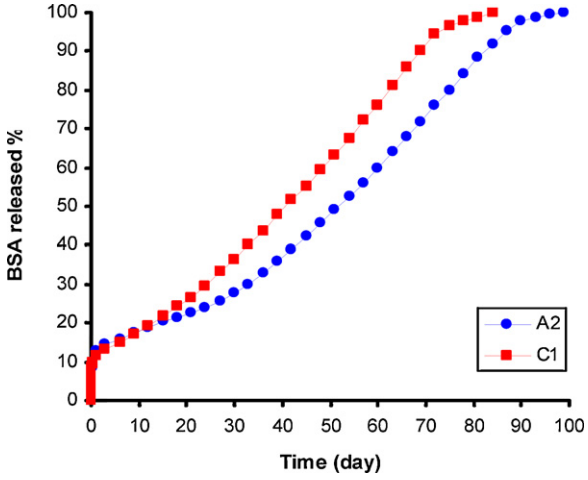

Fig. 4. Effect of BSA concentration on protein release from levan nanoparticles [(BSA concentrations were 0.1% (A2 series) and 0.2% (C1 series)].

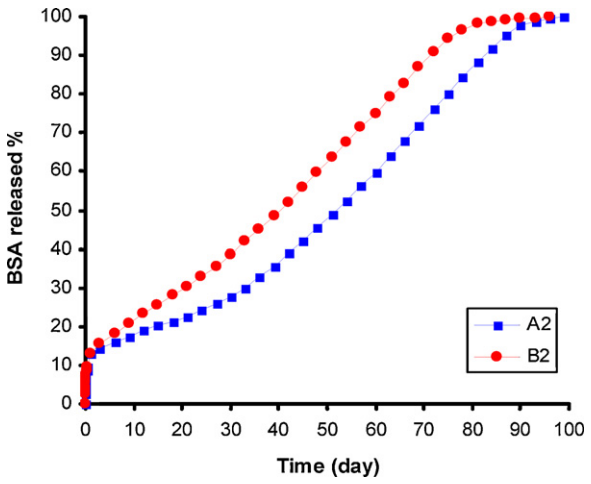

Fig. 5. Effect of rotating speed on protein release from levan nanoparticles [rotating speeds were 500 rpm (A2 series) and 20,000 rpm (B2 series)].

Higher BSA loading caused an enlargement of particle size, thereby increasing the total particle surface area available for burst release from the nanoparticles. In formulations with higher protein loading, BSA binding on polymer molecules could be weaker than at lower protein concentrations where BSA molecules are more likely to form multiple hydrogen bonds with polymer molecules. The other effective parameter on the release behaviour from the levan nanoparticles was rotating speed of the preparation so that the smallest particle size was obtained by the highest rotating speed (Table 1). When the effect of rotating speed on the release rate of BSA from the nanoparticles was analyzed, formulations with smaller particle size were found to show faster release than those with larger sizes (Fig. 5) ($P < 0.05$).

As seen from the release profiles in Figs. 3–5, protein release from the levan nanoparticles showed a bi-phasic behaviour. The first day, 10–20% of the protein released rapidly from the particles. In the second phase, approximately more than 60% of the BSA released from the particles at constant speed and particles degraded after the completion of swelling process. BSA release was faster than the swelling phase ($P < 0.05$). These results are similar with the results of Anal, Stevens, and Remuñán-López (2006) and Liu, Huang, Peng, Ding, and Li (2007).

Protein release kinetics was analyzed by plotting the cumulative release data to time by fitting to an exponential equation of the type (Kulkarni, Soppimath, & Aminabhavi, 1999) as represented below.

(i) Zero-order kinetics (Xu & Sunada, 1995)

$$W = k^1 t \quad (2)$$

(ii) First-order kinetics (Xu & Sunada, 1995)

$$\ln(100 - W) = \ln 100 - k^2 t \quad (3)$$

(iii) Hixon–Crowel's cube-root equation (Otsuka & Matsuda, 1996)

$$(100 - W)\frac{1}{3} = 100\frac{1}{3} - k^3 t \quad (4)$$

(iv) Higuchi's square root of time equation (Higuchi, 1963)

$$W = k^4 t \quad (5)$$

The r^2 value is an empirical parameter characterizing the release mechanism. On the basis of Higuchi's square root of time equation values were seen lower than the zero-order kinetics data and moreover the calculated regression coefficients showed a higher r^2 value with zero-order kinetics (between 0.9805 and 0.9932) (Table 2). The rank, according to the release kinetic values (r^2) of the formulations were: zero-order kinetics > Higuchi > Hixon–Crowel > First-order kinetics. The integrity of the incorporated BSA retained after the encapsulation and release processes was analyzed by SDS-PAGE and as shown in Fig. 6, samples showed no additional band of residue protein at 10th and 70th days.

Table 2
Release kinetic values (r^2) of levan nanoparticles.

Codes	Zero order (r^2)	First order (r^2)	Higuchi (r^2)	Hixon Crowel (r^2)
A1	0.9893	0.7244	0.9090	0.8795
A2	0.9805	0.7122	0.8741	0.8619
B1	0.9932	0.8232	0.9399	0.9321
B2	0.9910	0.7928	0.9392	0.9235
C1	0.9915	0.7774	0.9022	0.8990

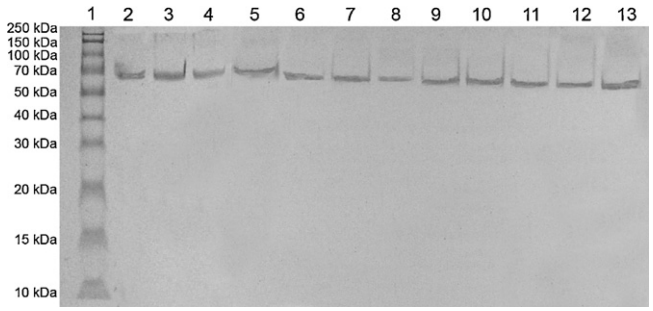

Fig. 6. SDS-PAGE photographs of BSA after released from nanoparticles. Lines 1, protein marker (1 μg/μl); line 2, BSA solution (5 μg/μl); lines 3, 6, 8, 10, 12 are protein released from nanoparticles (A1–C1) after 10 days and lanes 4, 7, 9, 11 and 13 are protein released from nanoparticles (A1–C1) after 70 days.

4. Conclusions

In this study, various biodegradable levan-based nanoparticle systems with different particular size, charge, and release profiles have been investigated in order to assess the suitability of levan from *Halomonas* sp. to be used in drug delivery systems. Characteristics of the BSA loaded nanoparticle systems, such as protein encapsulation capacity, release rate and kinetics were evaluated. The *in vitro* release studies confirmed the prolongation of the protein release time when using higher amounts of polymer in the preparation of levan nanoparticles. Polymer and protein concentrations were found to be effective factors on the nanoparticles properties. The adopted microencapsulation procedure is shown to be simple, fast and with high entrapment efficiency. BSA release kinetics did fit to zero-order release profiles. Thus, levan nanoparticles can be used effectively as drug carriers for peptide and proteins.

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