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Emulsan, a tailorable biopolymer for controlled release

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Abstract

Microsphere hydrogels made with emulsan–alginate were used as carrier for the microencapsulation of blue dextran in order to study the effect of emulsan on the alginate bead stability. Blue dextran release studies indicated an increase of microsphere stability in presence of emulsan, as a coating agent. BSA adsorption by emulsan–alginate microspheres is also enhanced 40% compared to alginate alone. XPS studies confirm the presence of BSA adsorbed on emulsan microsphere surfaces. These results are in agreement with the equilibrium adsorption model of Freundlich. Studies of BSA adsorption using non-equilibrium Lagergren second-order and intraparticle models, are suggesting a complex mechanisms of protein adsorption by chemisorption and intraparticle diffusion.

Also, enzymatic release of BSA from emulsan microspheres containing azo-BSA under physiological conditions is suggests the possibility of using microspheres as a depot for pre-proteins of medical interest.

Keywords: Emulsan; Bioemulsifier; Controlled release; Agriculture; Fermentation

1. Introduction

The world-wide pharmaceutical market was estimated at $602 billion (USD) in 2006 with a constant growth rate higher than 10% per year since 2001. However, each new molecular design usually takes between 10 and 12 years to reach the market with an estimated cost of $800 million. In spite of the higher costs and longer times to market, the number of new molecules in the market has increased from a share of 44% in 1991 to 67% in 2005 (Anonymous, 2006). The improvements of new molecules are based on a combination of innovative chemical structures, relevant therapeutic principles, better pharmacokinetics, and fewer side effects. In particular, new drug delivery systems which improve beneficial properties such as drug solubility under physiological conditions, drug efficiency, bioavailability and concurrently reduce drug doses and side effects will have a significant role in medicine in the coming years. As an example, the US market size for drug delivery alone was estimated at $43.7 billion in 2003, and it is projected to have a constant grow rate of 11.3% yearly to rise up to $74.5 billion by 2008 (Orive et al., 2003). In addition, as more sophisticated and sensitive molecules, including proteins, peptides and oligonucleotides are finding their way to the market; more advanced delivery systems are needed. The therapeutic protein market alone is growing with an annual growth rate of 10.5% since 2003 and it is predicted to be constant through 2010 (Anonymous, 2006). In particular, growth factors and hormones, enzyme therapy replacement, and molecules for immune disease and cancer therapies are becoming a focal point in the pharmacological industry. Non-invasive formulations (oral, transdermal, and inhaled) will likely lead to enhanced efficacy and utilization. It is our plan to use biopolymers such as emulsan and its gel derivatives as drug delivery vehicles.

There are significant advantages to utilizing a biopolymer for drug delivery instead of synthetic polymers. Production does not require costly and sophisticated

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equipment (distillation, hydrogenation process, high heat transfer, high pressures, etc.), special facilities (e.g. large scale organic solvent installations), or expensive waste treatments. Bioprocesses are based on fermentation which can be scaled up and down without major changes of equipment, procedures, or facilities. Biopolymers are environmentally friendly as biosynthesis can be performed using natural sources (e.g. recycling agricultural wastes can be used as main feeding compounds instead of oil and oil derivatives), and importantly all are biodegradable (Panilaitis et al., 2006). Another interesting advantage of biopolymers is the level of molecular complexity achievable with natural systems when compared to chemical synthesis. In addition, the tailorability of the molecular structure of the polymers by modifying molecular weight, regio- and stereo-selective activity, and other functional group chemistries by changing the physicochemical environmental conditions (e.g. media composition, temperature, and pH) and/or by molecular manipulation of microorganism metabolism is a significant advantage (Zhang et al., 1999; Johri et al., 2002).

Emulsan is an extracellular polysaccharide produced during the late exponential and stationary growth phases by *Acinetobacter venetianus* RAG-1. Emulsan molecule is composed of a sugar backbone decorated by fatty acids which in combination confers amphipathic properties to the molecule (Gorkovenko et al., 1999) (Fig. 1). This unique chemical composition can be manipulated in order to generate emulsans with varied chemical and biological characteristics (Zhang et al., 1999; Johri et al., 2002; Panilaitis et al., 2006).

Alginates are linear biopolymers composed of ß-mannuronic acid (M units) and α-guluronic acid (G units) linked by 1–4 bonds, purified from seaweeds and some bacteria. Alginate is one of the most popular hydrogels and has been successfully employed for many applications in the human body, including tissue implants. Several commercial products containing ultrapure alginate (in accordance with GMP/ISO 9000 guidelines) are extensively used as excipients in the pharmaceutical industry (Dornish et al., 2001). Alginate hydrogels are formed by crosslinking in presence of divalent cations (e.g. calcium, zinc), which cooperatively interact with different chains forming ionic bridges between different polymer chains. Gelation and structure of alginate gels are due to the stacking of the G units present in alginate chains, and also to the molecular weight of alginate monomers which can be controlled to some extent (Kikuchi et al., 1999). The major drawbacks of alginate hydrogels are the high hydrophilicity of the polymer, the narrow ranges of degradation rates, and the poor mechanical strength.

It has been postulated that due to the lipophilic nature of cell membranes, it is better in general to interact with more hydrophobic gel surfaces (Norris and Sinko, 1997; Gåserød et al., 1998). Practically, in the development of orally administered drugs, bioadhesion plays an important role in the targeting of gastrointestinal tissue, in which epithelial cells are always covered by protective mucus. A feasible approach is to delay the transit through the gut, increasing the likelihood of contact between the delivery device and the tissue. Several strategies with varied success have been pursued to improve alginate qualities, including chemical modification to chemically link a large range of polymers, such as polyethylene derivatives, chitosans, methylcellulose, and surfactants (Pluronic 80, members of sorbitan series) (Gåserød et al., 1998; Lee et al., 2004). However, chemical modification of polymers is a time-consuming procedure, and requires additional purification and waste treatment steps which are correlated with an increase in production costs.

The aim of the present work is to examine emulsan–alginate hydrogel microspheres for controlled release using Blue dextran as a model to establish the effect of emulsan coating on bead stability. In addition, BSA was used as protein model for adsorption studies by the microspheres, followed by enzymatic release utilizing a serine protease. The studies were complemented by BSA adsorption in equilibrium (Freundlich) and dynamic (Lagergren and interparticle diffusion) models in order to characterize the possible mechanisms of adsorption.

2. Methods

2.1. Materials

Chemical reagents were of analytical grade, and microbiological media were of highest available grade (Aldrich

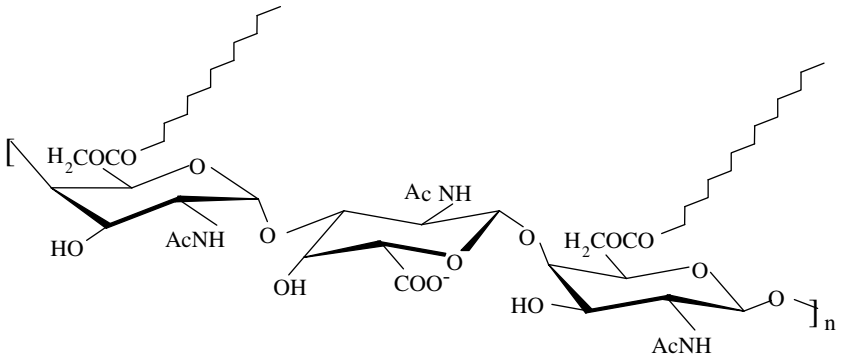

Fig. 1. Scheme of emulsan structure from *A. venetians,* RAG-1.

Milwaukee, WI; Difco, Franklin Lakes, NJ). Low viscosity alginate (average Mn $\sim 1 \times 10^5$ Da), acid blue 2, blue dextran (MW $\sim 2 \times 10^6$ Da), azo-BSA, BSA (fraction V), subtilisin Carlsberg (from *B. licheniformis*) were purchased from Sigma (St. Louis, MO).

2.2. Bacterial cultures and emulsan purification

Emulsan synthesis by *A. venetianus* strain RAG-1 (ATCC 31012) was produced in saline medium supplemented with ethanol, and purified as previously described (Panilaitis et al., 2006).

2.3. Microsphere formation, and release studies

Sodium alginate (2.0%) was dropped into a solution containing 30 mM $CaCl_2$, with or without emulsan (0.15–2.75 mg/ml) under continuous stirring in order to avoid coalescence of gel beads. Microspheres were aged in calcium chloride solution for 48 h, followed by filtration on paper (Whatman #1). Filtered microspheres were kept in solution containing $CaCl_2$ and 10 μM NaN_3 at 5 °C until use.

Polymer release studies were performed loading 1.0 mg/ml blue dextran (BD) or acid blue 2 (AB2) into alginate solutions and stirred until homogeneous dissolution, following by dropping into $CaCl_2$ solution as mentioned before.

In adsorption BSA experiments, typically 500 μg/ml of BSA or azo-BSA were incubated with 200 μg of gel microspheres at 37 °C for 25 minutes. The microspheres were centrifuged, washed with 150 mm NaCl, and stored at 5 °C until use.

Release experiments from the microspheres containing blue dextran or acid blue 2 were incubated in presence of 25 mM buffer phosphate (pH 7.5) in a shaker (3.3 Hz, 37 °C) for specified times. Samples were centrifuged (10,000×*g* for 5 min at 5 °C). The supernatants were collected and measured spectrophotometrically at 607 nm.

Protein release experiments were carried out using vials containing microspheres with adsorbed azo-BSA and incubated with subtilisin for 30 min at 37 °C. Aliquots of 500 μl of supernatants before and after incubations were passed through a 100 kDa cut-off filter (Microcon, Millipore, Billerica, MA), and the filtered products were analyzed for sulfanilic acid and/or BSA release. Spectrophotometric quantification of adsorbed azo-BSA was performed by dissolving the beads for 1 h in presence of 10 mM EDTA and 100 mM phosphate buffer (pH 7.0) at 37 °C. Sulfanilic acid was quantified spectrophotometrically at 334 nm using a calibration curve after precipitating azo-BSA and BSA with 5% trifluoroacetic acid at 0 °C for 15 min, and centrifuging the supernatant at 10,000×*g* for 5 min. BSA was determined by Coomassie Brilliant Blue dye binding assay using a proper standard (Bradford, 1976).

2.4. Characterization of microspheres by optical microscopy (OM) and X-ray photoelectron spectroscopy (XPS)

OM was performed with an Axiovert S-100 inverted microscope (Carl Zeiss, Jena, Germany). A Surface Science Inc. model SSX-100 X-ray photoelectron spectrometer was used to analyze the surface of the lyophilized beads. Survey scans (spot 1000 mm, resolution 4, window 1000 eV) and elemental scans (spot 1000 mm, resolution 4, window 20 eV) were performed using a flood gun (charge neutralizer) setting of 5 eV and nickel wire mesh held over the sample to prevent charging of the sample surface. Nitrogen, and sulfur present on BSA molecules adsorbed on the beads were scanned in high-resolution mode between 389–403 eV, and 159–171 eV, respectively.

2.5. Equilibrium adsorption

The Freundlich adsorption equation can be expressed in a linearized form as (Glasstone, 1946):

$$\log q_e = \log Q_f + (1/n) \cdot \log C_e, \tag{1}$$

where Q_f, and n are the empirical Freundlich parameter and exponent, respectively. If n is higher than 1, this implies that the adsorption is a favorable process, while if n is less than 1 the adsorption is unfavorable.

2.6. Adsorption kinetics

First- and second-order Lagergren, and Interparticle diffusion models were tested (Ho and McKay, 1999). The Lagergren's pseudo-first-order equation is given as

$$\log(q_e - q) = \log q_e - \frac{k_1}{2.303} \cdot t. \tag{2}$$

Second pseudo-order equation can be described by

$$\frac{dq}{df} = k_2 \cdot (q_e - q)^2. \tag{3}$$

Separating variables and integrating Eq. (3) using boundary conditions allows the equation to be expressed as

$$\frac{q_e}{q} \cdot (q_e - q) k_2 \cdot t, \tag{4}$$

where q_e and q are the amount of BSA adsorbed at equilibrium and at t (time) expressed in mg/μg; k_1 and k_2 are the pseudo-first and pseudo-second-order compound adsorption rate constants in min^{-1} and mg/μg min, respectively, and t is time in minutes. Rearranging Eq. (4) to obtain a linear form

$$\frac{t}{q} = \frac{1}{k_2 \cdot q_e^2} + \frac{t}{q_e}, \tag{5}$$

$$k_2^0 = k_2 \cdot q_e^2, \tag{6}$$

where k_2^0 is the defined as the initial adsorption rate expressed in μg/mg min. The slopes and intercepts of plots t/q vs. t were used to estimate the pseudo-order rate

constants and the amount of BSA adsorbed at equilibrium ($q_{e\ calc}$).

The intraparticle diffusion model is described by the equation

$$q = k_1 \cdot t^{(0.50)}, \quad (7)$$

where k_i is the intraparticle diffusion rate constant expressed in μg/mg min (Glasstone, 1946).

3. Results

Based on the extensive work on alginate coacervates reported in the literature in the drug delivery field, alginate microspheres were coated with emulsan in presence of calcium. Chemical analysis of microspheres brings 10.9–12.5% of emulsan per bead by sulfuric acid–phenol assay.

Emulsan–alginate and alginate microspheres were used as a reservoir of blue dextran, a linear α-1,4 glucan covalently coupled with acid blue 2 dye, in order to study polysaccharides release from the microspheres by spectrophotometry. Controls of release from microspheres were performed with acid blue 2. The initial release of the dye, acid blue 2, from both types of microspheres was proportional to the square root of time suggesting a diffusion controlled mechanism. The release rates of acid blue 2 were 12.7 and 7.6 times higher than the release rates of blue dextran in alginate and emulsan microspheres, respectively (Table 1). Also, the release rate of blue dextran in emulsan coated microspheres was about one third of that measured in alginate alone formulations (Table 1). The differences of blue dextran concentrations released from alginate and emulsan microspheres can be described as a shielding effect of the emulsan coating. The blue dextran differential release profile from alginate and emulsan microspheres, respectively showed a typical Gaussian curve indicating the protective effect of emulsan coating alginate hydrogels (Fig. 2). After about one hour of incubation in the shaker, the emulsan coating was apparently eroded from the bead surface and differences of soluble blue dextran release in both types of microspheres went to zero.

In another set of experiments, preliminary kinetic studies of BSA adsorption by alginate and emulsan–alginate microspheres were performed. Emulsan–alginate microspheres showed about 40% more BSA adsorption than alginate alone formulations (Fig. 3). Also, the amount of BSA adsorbed onto the microspheres can be modified by changing the emulsan concentration on the microspheres. An increased BSA adsorption correlates well with the increase of emulsan content in the microspheres (Table 2). Due to its molecular weight (about 67 kDa) BSA cannot diffuse into an alginate gel core, therefore protein adsorption on microspheres surface can be detected by XPS. Analysis of microspheres by XPS confirm the BSA adsorption revealed the presence of one peak at 400 eV which can be assigned

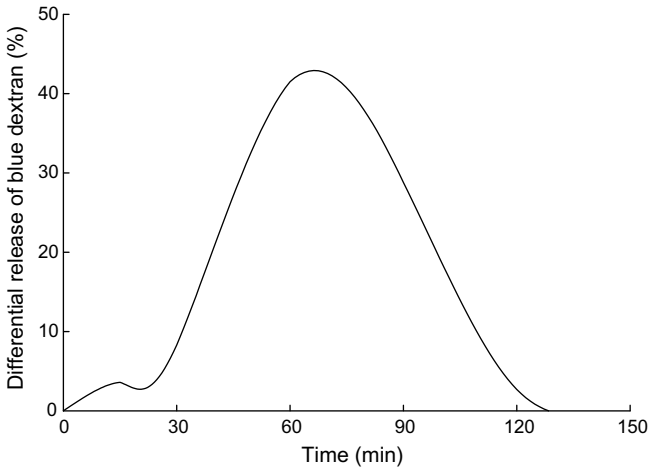

Fig. 2. Differential release of blue dextran between alginate and emulsan microspheres.

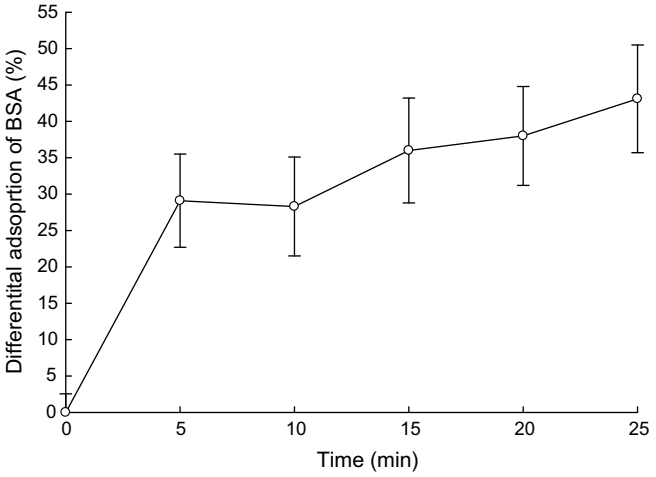

Fig. 3. Differential adsorption of BSA between emulsan and alginate microspheres.

Table 1
Releasing rates of acid blue 2 (AB2), and blue dextran (BD) from alginate and emulsan–alginate microspheres

Microsphere	Molecule	Symbol	Rates (μg/ml min)	Ratios	
Alginate	AB2	α	72.0	$\alpha/\beta = 12.7$	
	BD	β	5.7		$\beta/\delta = 2.9$
Emulsan	AB2	γ	14.0	$\gamma/\delta = 7.6$	
	BD	δ	1.9		

Table 2
Effect of emulsan concentration on BSA adsorption by the microspheres incubated for 30 min at 37 °C

Emulsan (mg/ml)	BSA (%)
0.00 ± 0.00	2.9 ± 0.5
0.16 ± 0.07	27.5 ± 3.6
0.41 ± 0.05	63.0 ± 2.8
0.75 ± 0.09	71.0 ± 1.6
2.75 ± 0.08	80.0 ± 1.3

to nitrogen in emulsan–alginate beads, which was absent in alginate microspheres.

To evaluate BSA adsorption with the beads in equilibrium, the Freundlich model was assessed. The correlation factors for the linear regression of the experimental data using Freundlich model was higher than 0.9, indicating a reasonable fit. Analysis of the Freundlich model n parameter predicts that the adsorption of BSA by the beads is favorable (values between to 1 and 10) (Weber and Morris, 1963). A comparison between the maximum adsorption for the emulsan–alginate versus the alginate beads showed significantly higher adsorption of BSA by the emulsan–alginate system by a factor of about 2.5 of Freundlich isotherm constant (Table 3).

Out of equilibrium, dynamic kinetic models of BSA adsorption by the two types of beads were also investigated using Lagergren and the Intraparticle Diffusion models, and validated by linear regression (Glasstone, 1946; Weber and Morris, 1963). The first-order rate expression of Lagergren does not fit well to the full range of the adsorption data, with correlation coefficients lower than 0.6 in both types of microspheres (data not shown). The low linear correlation coefficient for the Lagergren pseudo-first-order reaction indicates a lack of predictability of the first-order kinetic model, probably because of high kinetic constants. The pseudo-first-order Lagergren model is generally more applicable over initial stages of adsorption, but if the kinetic constants are very high the model does not fit (Ho and McKay, 1999). These results indicate that the adsorption is not pseudo-first-order reaction. However, the Lagergren second-order model fit well to experimental data, which indicates an adsorption rate-controlled mechanism. In this model it is interesting to mention that the equilibrium pseudo-second-order constant of alginate is approximately three times higher than for the emulsan–alginate beads, conversely the q_e is about half that observed with the emulsan–alginate beads, suggesting an energy dependent process (Table 4). Also, the low correlation factor of the intraparticle diffusion kinetic model compared to Lagergren pseudo-second-order model validated this hypothesis. However, both Lagergren models are unable to identify a diffusion mechanism. The diffusion intraparticle model derived from Fick's second law of diffusion indicates that the kinetic constant of intraparticle diffusion for emulsan–alginate beads was 2.2 times higher than for the alginate beads. This result indicates that the mass transfer resistance of emulsan–alginate beads increases because of biopolymer coating.

Table 3
Freundlich adsorption isotherms

Parameter	Microspheres		Ratio (Em/Alg)
	Emulsan	Alginate	
Q_f (μg/cm^3)	0.0518	0.0205	2.52
n	2.63	3.85	–
r^2	0.998	0.94	–

Table 4
Dynamic absorption of BSA using second-order Lagergren and Intraparticle diffusion models

Model	Parameter	Microspheres		Ratio (Em/Alg)
		Emulsan	Alginate	
Lagergren (second-order)	k_2	0.652	2.10	0.3
	$k^o{}_2$	0.0217	0.0132	1.6
	r^2	0.990	0.964	–
	$q_{e\ calc}$	0.1826	0.0791	2.3
Intraparticle diffusion	k_i	0.0128	0.00589	2.2
	r^2	0.96	0.90	–

The kinetic values at initial times and at equilibrium for BSA on emulsan–alginate beads were higher than for the alginate beads. The error calculated for the amount of BSA adsorbed was about 12% and 6% for the emulsan–alginate and alginate beads, respectively. These studies suggest the presence of two different BSA binding sites, one with high affinity and other with low affinity, which are also independent of the adsorbent. A previous study of the interaction between free fatty acids and BSA reported two binding sites with different affinities (Richieri et al., 1993). In the case of the interaction of BSA with free fatty acids, the higher association binding constants were found with palmitic, oleic, linoleic, linolenic and arachidonic acids (Richieri et al., 1993), which are the major fatty acids of emulsan (Panilaitis et al., 2006).

Considering the high BSA binding capability of emulsan–alginate microspheres, another important factor to be addressed is the possibility of delivering a pre-protein, without biological activity, which can be activated via physiological enzymatic mechanisms. Subtilisin, a microbial protease, which belongs to the same group as the mammalian intestinal proteases, was selected as enzyme model. In both types of microspheres in where azo-BSA was adsorbed, sulfanilic acid was released under physiological conditions by subtilisin with a first rate kinetic until 30 min. Sulfanilic releasing rates of azo-BSA microspheres by subtilisin showed were 2.71×10^{-3} mg/ml min and 9.64×10^{-3} mg/ml min for alginate and emulsan beads, respectively.

4. Conclusions

Bearing in mind the blue dextran release studies, alginate microspheres coated with emulsan posses enhanced stability compared to alginate beads alone. The results suggested that the release of loaded molecules can be manipulated in certain range by using different emulsan coating concentrations. In addition, protein adsorption, using BSA model, was enhanced by the presence of emulsan in the microspheres. The enzymatic release study of azo-BSA from the microspheres surface using subtilisin showed that the protein adsorbed in the bead surface is accessible under physiological conditions to be cleaved. This result suggests the possibility of using pre-proteins without biological activity, which normally are under strict physiological regulations

(e.g. hormones, proliferative factors, etc.) and could be adsorbed on emulsan surfaces to be activated by physiological mechanisms.

The correlation factors of non-equilibrium adsorption models tested, Lagergren second-order and intraparticle, are very close and they suggest that the adsorption of BSA by the microspheres is a complex mechanism, probably based on the combination of chemisorption and intraparticle diffusion.

The two forms of molecule delivery reported in the present work have the potential of combining a pre-protein, without biological activity, loaded inside the particle that could be activated by an enzyme adsorbed in the microsphere surface during the process of release. So, our results suggest that emulsan microspheres are excellent candidates for delivery of sensitive molecules, like proteins, and could be useful in sophisticated models in where sustained release is required and concentration levels in the body of biological active molecules should be regulated under physiological conditions.

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