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Chapter 2

Emulsan–Alginate Microspheres as a New Vehicle for Protein Delivery

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A solution containing emulsan, a lipoheteropolysaccharide, and calcium was used to produce emulsan-alginate microspheres (EAMs). Optical, scanning electron microscopy and EDX (Energy Dispersive X-ray) analysis of the microspheres suggested different morphologies and compositions, respectively, when compared with microspheres prepared only from alginate. The EAMs were twice as stable in phosphate solution compared to alginate alone when assessed with blue dextran encapsulation. The EAMs were able to adsorb about twice the amount of BSA (Bovine Serum Albumin) compared to alginate alone. When azo-BSA was adsorbed on the emulsan-alginate microspheres, protein release could be triggered with enzymes. BSA released from the EAMs retained about of 78% of the α-helix structure.

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In recent years with the emergence of the fields of biotechnology and biomedicine there has been growing interest in the development of systems able to transport, capture or deliver molecules. Biocompatible gels have become an important topic for encapsulation of living cells, drug delivery systems and implants (*1*). Alginate, a linear polysaccharide of ß-D-mannuronic (M) and α-L-guluronic (G) acids found in algae and some bacteria, can be gelled by calcium and others bivalent cations. Alginate gels are considered safe and currently used in many biotechnology applications (*1*). However alginate gels are unstable in the presence of cation chelating agents such as phosphate and/or competing non-gelling cations such as sodium or potassium which are present in biological fluids (*2*).

In order to prevent alginate microsphere swelling, polymers like chitosan, poly-L-lysine, polyacrylates and others have been added to stabilize alginate gels by crosslinking or co-gelation (*3,4*). Chitosan, a cationic copolymer of N-acetylglucosamine and glucosamine, is water soluble and biodegradable polymer often used in the pharmaceutical industry as an excipient because of is biocompatibility and lack of toxicity in mammals (*1*). However, the degree of acetylation of chitosan, which correlates with its biological and chemical properties, depends on the chemical treatment of chitin by alkaline N-deacetylation. In addition, sometimes extra chemical steps are required in order to improve the mucoadhesive properties of chitosan for drug delivery purposes (*5*). Also, the major source of chitin is the exoskeleton of crustaceans, which can lead to problems in terms of contamination from heavy metals, a serious disadvantage for human consumption (*1*), along with the variability in source supply of the chitin, impacting molecular weight and degree of deacetylation.

Emulsan, a polysaccharide of ~1x106 Da., is produced by *Acinetobacter venetianus* RAG-1 and composed by D-galactosamine, D-galactosaminuronic acid, and 2,4-diamino-6-deoxy-D-glucosamine linked with fatty acids by N- and O-acyl bonds to the sugar backbone with interesting biological and chemical properties (*6*). Studies in our laboratory have shown that both physiological control of the biosynthesis process or genetic manipulation of the organism changes the structure and thus function of this amphiphilic polymer can be altered toward specific goals (*7, 8*). Interestingly, crude emulsan secreted by the bacterium harbors up to 23% by weight of adsorbed protein in the native state (*7*), a property that could be useful for protein delivery. The disadvantage of the emulsan is that the polymer does not easily form stabilized gel systems.

In order to study the ability of emulsan as protein delivery the polymer was combined with alginate in gel form. Bovine serum albumin (BSA) was selected for adsorption studies because it is well characterized and responsible for 99% of free fatty acid transport in cows, with equilibrium constants on the order of $10_7 M_{-1}$ (*9*).

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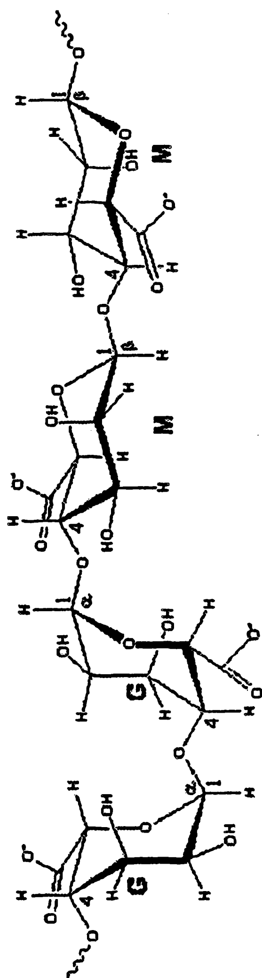

Figure 1. Chemical structure of alginate. G and M are β-D-mannuronic and α-L-guluronic acids respcetively.

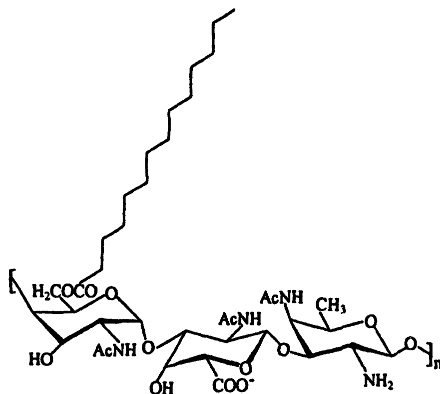

Figure 2. Chemical structure of emulsan.

The aim of the present work was to characterize a new biogel system based on the combination of emulsan-alginate for protein delivery. Analysis of biogel surfaces was conducted using optical microscopy, scanning electron microscopy and energy dispersive X-ray mapping, and the vesicles were assessed for gel integrity and chemical and biocatalyzed protein release. The BSA before and after release was compared for structural features by circular dichroism. The results suggest these hybrid systems offer important options in the adsorption and delivery of proteins, mimicking the native function of emulsan as a carrier of large amounts of protein in the environment,

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Experimental methods

Materials

Chemical reagents were of analytical grade and microbiological media were of highest available grade (Aldrich Milwaukee, WI; Difco, Franklin Lakes, NJ). Low viscosity alginate (average M_n~ $1x10_5$ Da) , blue dextran ($2x10_6$ Da), acid blue 2, BSA (fraction V), azo BSA, subtilisin Carlsberg (from *B. licheniformis*), and *C. rugosa* lipase were purchased from Sigma (St. Louis, MO).

Bacterial cultures and emulsan purification

Emulsan (average M_n $1x10_6$ Da) synthesis by *A. venetianus* strain RAG-1 (ATCC 31012) was produced in saline medium supplemented with ethanol, and purified according to our previously reported procedures (*8*).

Optical Microscopy (OM) and Scanning Electron Microscopy (SEM).

OM was performed with an Axiovert S-100 inverted microscope (Carl Zeiss, Jena, Germany). SEM was performed with a Leo 982 (Noran, Germany) using lyophilized microsphere samples with no previous treatment.

Microsphere formation.

Sodium alginate (1.5 to 5.0 %) was dropped into a solution containing 20 to 100 mM CaCl2, with or without emulsan (0.22 to 3.0 mg/ml) under continuous stirring in order to avoid coalescence of gel beads. Fresh microspheres were incubated in the respective calcium chloride solutions for 48 hours in a rocker at 25 rpm at 5°C for aging, followed by filtration on paper (Whatman #1). Filtered microspheres were kept in solution containing CaCl2 and 10 µM NaN3 at 5°C until use. For polymer release studies, 1.0 mg/ml blue dextran or acid blue 2 were loaded into 2.0% alginate solution and stirred until total dissolution, followed the gelation procedure mentioned above. For protein experiments, typically 500 µg/ml of BSA or azo-BSA were incubated with 200 µg of gel microspheres at 37°C for one hour. The microspheres were centrifuged, washed with 150 mm NaCl, and stored at 5°C until use.

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Energy Dispersive X-ray mapping (EDX).

Microspheres were mounted on an environmental scanning electron microscope (ESEM) plate without any previous treatment for EDX-ray analysis consisting in Falcon System running Genesis 1.1 software and a super ultra thin window). The X-ray spectra were obtained in ESEM mode with a 20 kV accelerating voltage and a 35-degree take-off angle. Elemental micro-probe and elemental distribution mapping techniques were used for analyzing the elemental composition. The spectra were collected for 100 seconds with a dead time between 20-30%. Spectra major peaks were identified by X-ray energies characteristic of each element.

Circular dichroism (CD)

The CD spectra were recorded on a spectropolarimeter (Jasco J-710, Tokyo, Japan) with a 0.1 mm cell path length at 25°C. Scans were obtained in a 200 to 250 nm with 1-nm bandwidth and 4-seconds integration time. Four scans were accumulated for each sample at a scan rate of 100 nm min-1. The α-helix BSA

content was estimated considering a molecular weight of 66,700 Da, and 582 amino acid residues with equations described elsewhere (*10*).

Release studies

The release of blue dextran from the microspheres was studied in 25 mM buffer phosphate solution (pH 7.5) incubated in a rotatory shaker at 200 rpm and 37°C. Biocatalytic release of azo-BSA and sulfanilic acid from the microspheres was performed with subtilisin or lipase in 25 mM Tris-ClH buffer solution (pH=7.8) incubated at 200 rpm and 37°C. Azo-BSA and sulfanilic acid were determined spectrophotometrically at 334 nm. The presence of sulfanilic acid in the supernatant was assayed after precipitation of azo-BSA with 5% TCA for 15 minutes at 0°C, followed by centrifugation (10,000 xg, 20 minutes at 4°C). Controls without enzymes, and with protease previously inhibited with 1.0 mM diisopropyl fluorophosphate, or thermal inactivate lipase (heated at 100°C) were included. Chemical release of BSA from the microspheres was performed by incubating the microspheres in a buffer phosphate (100 mM, pH=7.4) until total microsphere disintegration. For CD analysis of BSA, samples were filtered through 100 kDa. MWCO devices (Centricon, Millipore, Billerica, MA, USA).

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Results and discussion

Gelation and characterization of alginate microspheres mixed with other polymers are well described previously in the literature (*1,2*). However, no references for the interaction of emulsan and other polymers were found. Comparative optical microscopy of alginate and emulsan-alginate microspheres showed different surface morphologies (Figure 3). The results were confirmed by SEM (Figure 4).

Alginate gel microspheres showed smooth surfaces compared to the emulsan-alginate microspheres suggesting surface interactions due to the presence of the emulsan likely influence the localized calcium interactions in gel formation. Also, the presence of fatty acid acyl residues on the emulsan main chain, and the lack of these side chains on alginate likely contribute to the observed differences in surface homology. Concentrations ranging from 20 to 100 mM of calcium chloride combined with 1.5% to 5.0% alginate which modify the gelation rate did not change the morphology of the emulsan-alginate microspheres observed by microscopy (data not shown). Another advantage of the system is that size of the microspheres can be modified by changing the speed of the pump, which is able to produce homogenous sizes between 300 μm to 2.5 mm diameter.

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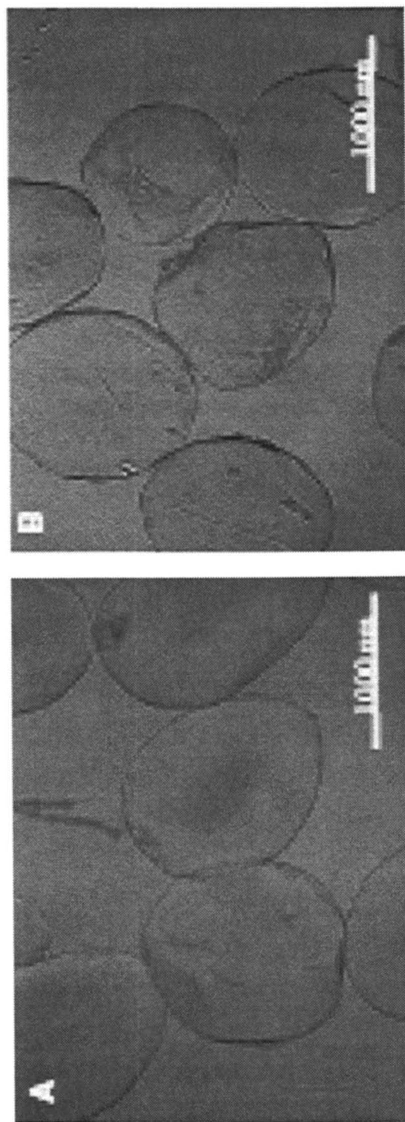

Figure 3. Optical microscopy (16x) of microspheres: A, alginate, and B emulsan-alginate.

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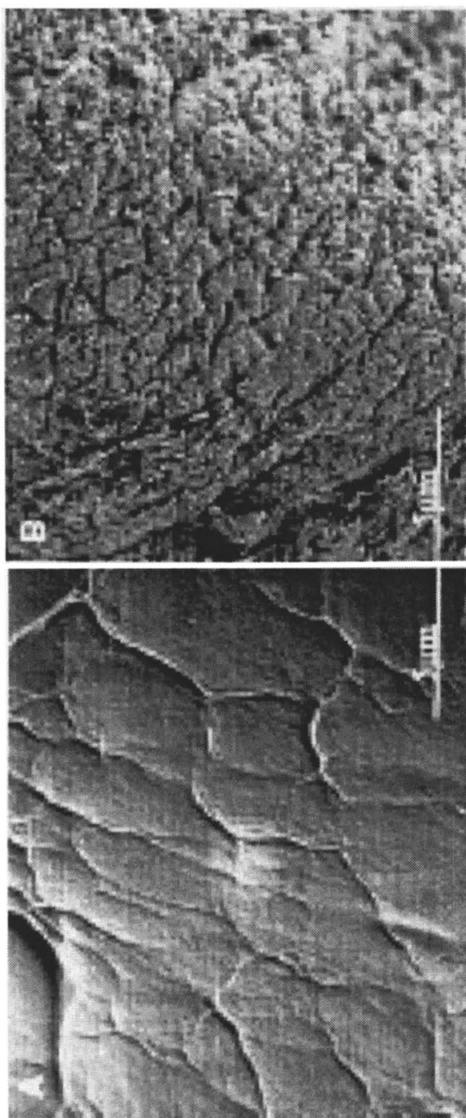

Figure 4. SEM (x 5,000) of alginates (A), and emulsan-alginate microspheres (B).

Alginate crosslinking and gelation are provided by the exchange of monovalent ions, like sodium, from α-L-guluronic acid residues with divalent cations, such as calcium and magnesium (*1*). Alginate crosslinking is produced when two adjacent α-L-guluronic acid residues interact cooperatively forming a binding cage for polyvalent cations. If 20 or more monomer units from different chains interact a gel network is formed. The gelation and strength can be correlated with the presence of α-L-guluronic (G) blocks in the alginate chain. The increase of the length of G block content implies more calcium crosslinking which has direct relevance to the functionality, stability and structure of alginate gels (*2*). Interferences that reduce the complexation between divalent ions and α-L-guluronic residues therefore also decrease the strength and stability of the gel. If emulsan interferes within calcium-alginate crosslinking of the microspheres and consequently with gel formation, emulsan-alginate microspheres would have a weaker structure and disintegrate more easily in phosphate solutions compared to alginate microspheres without emulsan. Conversely, if emulsan forms a coating on the alginate microspheres, perhaps due to the differences in chain chemistry and thus solubility, the time release of tracer molecules retained or adsorbed in the microsphere core will be delayed compared to alginate alone microspheres. In order to test this hypothesis, blue dextran or acid blue 2 (dye covalently attached to dextran) were loaded into 2.0 % alginate solutions and dropped into the calcium solution for gelation. Typically 30 mM was used, and the time release for these compounds was studied in terms of release from both alginate and emulsan-alginate microspheres. Acid blue 2 was totally release in less than 15 minutes without any differences in the time release profiles in both types of microspheres (data not shown). At pH 7.5 the 2 x106 Da MW blue dextran released from alginate microspheres showed a hyperbolic profile and the process was completed in 1 hour. The blue dextran release profile from emulsan-alginate microspheres was sigmoid and the process was completed in about 2 hours (Figure 5). The time-release for dextran was strongly influenced by the disintegration of the microspheres which increased with pH (data not shown). These results suggest that there was no interference from emulsan in the alginate gelling process and probably little diffusion of the emulsan to the inside the alginate gel core. This interpretation is also supported by previous data showing that 1.5 to 3.0% alginate gels had 170 to 147 Å pore sizes (*11*), with an approximately exclusion pore size of 21-25 kDa. (*12*). Considering that alginate gelation proceed by diffusion of calcium ions and crosslinking from the microsphere surface to the microsphere core, and the emulsan is 1x106 Da, which is about 40 times larger than the exclusion pore size, diffusion of significant amounts of emulsan to the inside the gel core is not likely. On the contrary, the presence of emulsan apparently reinforced the microsphere structure probably because of the hydrophobic nature conferred by the fatty acid side chains.

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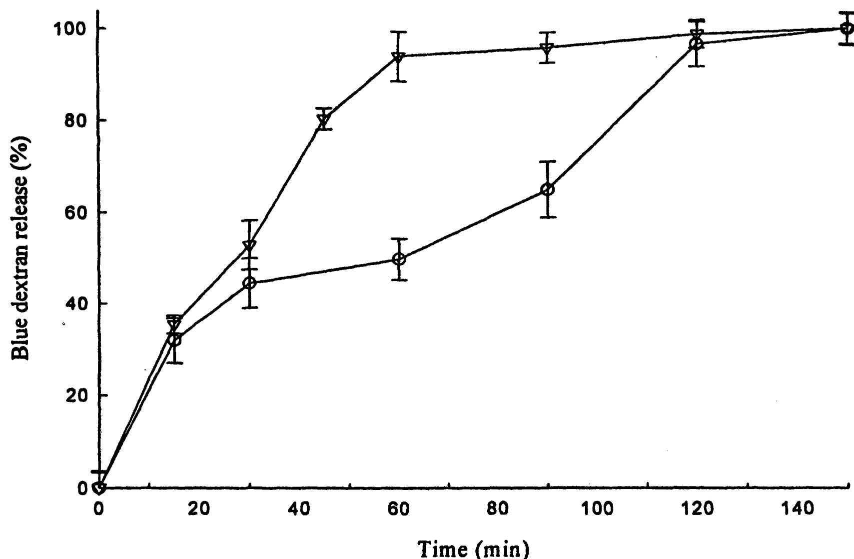

Figure 5. Release of blue dextran from Alginate (∇), and emulsan-alginate microspheres (O) incubated at 200 rpm at 37°C.

EDX analysis of both microspheres (alginate and emulsan-alginate) revealed the presence of four major elements: carbon, oxygen, chloride and calcium (Figure 6). Semiquantitative chemical analysis of both types of microspheres provided different oxygen/carbon ratios of 1.09 and 1.20 for emulsan-alginate and alginate microspheres, respectively. An approximately 10% difference between calcium: carbon ratios for both types of microspheres was found with values of 0.43 and 0.34 for emulsan-alginate and alginate respectively. Chloride was constant in both types of microspheres, suggesting the enhanced amount of calcium in emulsan-alginate microspheres was because of the presence of emulsan since calcium chloride is the only source of both ions.

In order to determine the effect of emulsan on BSA adsorption by the microspheres, solutions containing different amounts of emulsan were used to coat alginate microspheres followed by BSA adsorption. The results in Figure 7 showed that the increase of emulsan concentration in the microspheres was proportional to the increase in BSA adsorption by the microspheres. The BSA adsorption kinetics on the emulsan-alginate microspheres was more than twice that for BSA adsorption to the alginate microspheres at 37°C (Figure 8). However, saturation levels of BSA in the alginate microspheres was reached in about 10 minutes, while in the emulsan-alginate microspheres this process required about 20 minutes.

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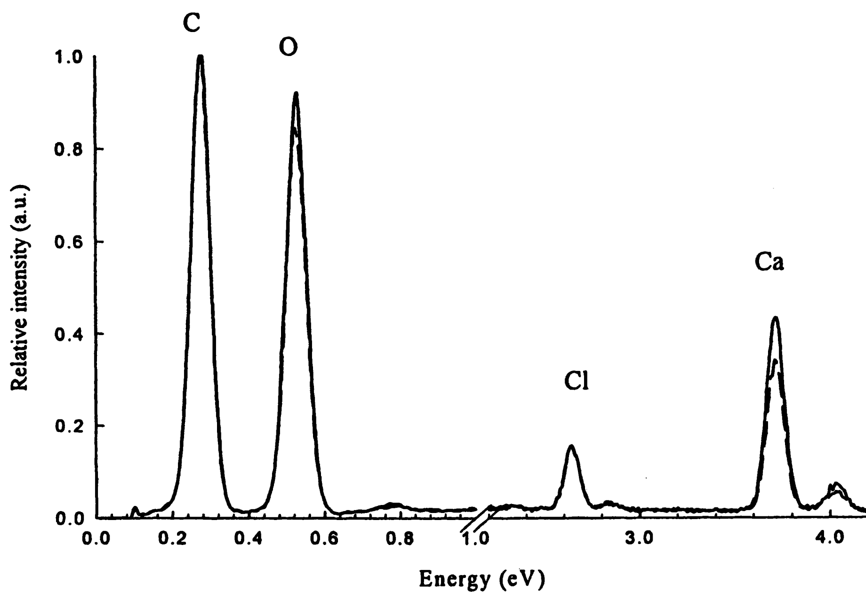

Figure 6. EDX spectra of alginate (dashed line), and emulsan-alginate microspheres (continuous line).

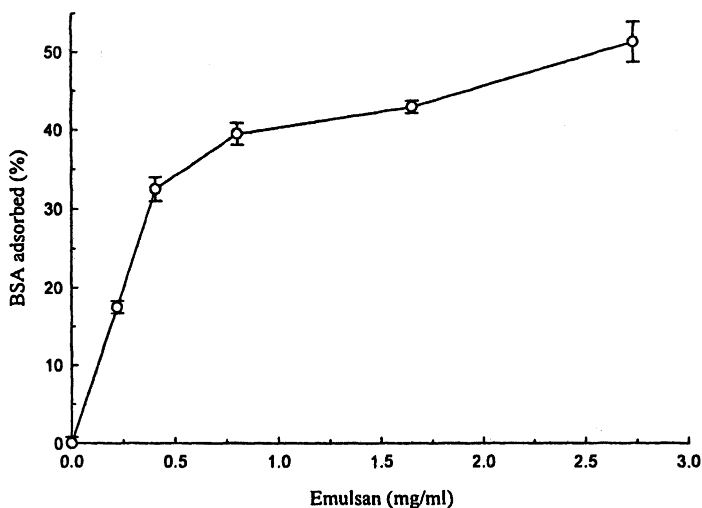

Figure 7. Effect of emulsan concentration on BSA adsorption by emulsan-alginate microspheres incubated at 37°C for 25 minutes.

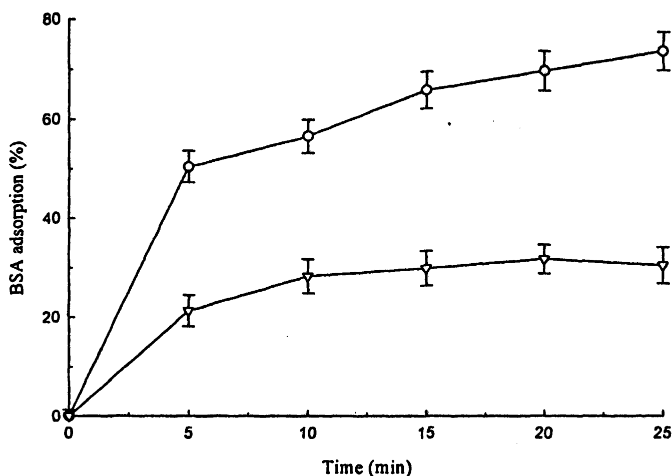

Figure 8. Adsorption of BSA by alginate (∇) and emulsan-alginate microspheres (○).

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Based on the results of blue dextran release from emulsan-alginate microspheres using calcium complexing agents such as phosphate, and taking in account the potential use of these systems in oral drug delivery, enzyme triggered release methods from these systems was explored. Two scenarios were considered, the use of pre-proteins adsorbed in the microspheres with subsequent cleavage by a protease (e.g. insulin), which would trigger biological activity. A scheme of this model is displayed in **Figure 9**. BSA covalently linked to sulfanilic acid, azo-BSA, was used as a model substrate for the catalysis and adsorbed by the emulsan-alginate microspheres. Studies of sulfanilic acid release by biocatalysis from the emulsan-alginate microspheres was performed with subtilisin. Subtilisin, a common serine protease belonging to the same family as trypsin and chymotrypsin, which are most abundant proteases present in mammalian intestine.

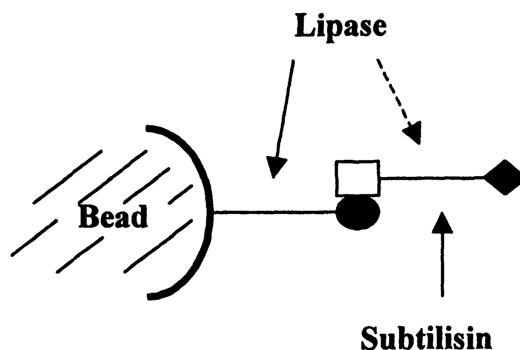

Figure 9. Diagram of enzymatic release of azo-BSA from emulsan-alginate microspheres. Symbols: □—◆, azo-BSA; —●, fatty acid; □, BSA; and ◆ sulfanilic acid respectively.

The azo-BSA release from the microspheres is induced by the hydrolysis of the fatty acid ester side chains on the emulsan. *Candida rugosa* lipase was used to follow the release of azo-BSA by specific enzymatic cleavage of the fatty acids, and the nonspecific enzymatic cleavage of azo-BSA into sulfanilic acid. The results are displayed in **Table I**.

In enzymatic cleavage from the microspheres either with subtilisin or lipase, the release of the substrate was greater than 60%. Impressively, the release of azo-BSA from the microspheres catalyzed by lipase was greater than 90%. However, nonspecific hydrolysis of azo-BSA by lipase was detected, probably attributed to the similarities between the ester and amide bonds. Also, both reactions were performed in closed systems, contrary to the biological

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environment in the digestive track which is an open and dynamic system where the presence of end-products from biocatalysis affects reaction kinetic as well as the conversion of substrate and side products.

Table I. Enzymatic release of azo-BSA from emulsan-alginate microspheres incubated 20 minutes at 37°C.

Treatment	*Percentage of total release*	
	Azo-BSA	*Sulfanilic acid*
Subtilisin	0	62.9 ± 2.3
Lipase	82.6± 3.1	15.2 ± 1.2
No enzyme	0	0

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Another question to be addressed is the status of the protein structure after release from the microspheres. Secondary structure can provide a qualitative estimate of function and reflects to some extent biological activity. Protein inactivation by unfolding is one of the major obstacles in drug delivery. For this purpose, analysis of BSA structure by circular dichroism before and after the release from the microspheres was assessed. After release from the emulsan-alginate microspheres, BSA showed some loss in helix content based on the decrease of negative elipcticity at 222 nm typical of α-helix (Figure 10).

In the experimental conditions utilized, native BSA dissolved in phosphate buffer at pH 7.4 had 56.0±1.4% α-helix content and 43.9±3.2% for the desorbed-filtered BSA without any other excipient. In previous work with poly(lactic-co-glycolic acid) microspheres the BSA α-helix content was 21% without any excipient and the α-helix value reported was 39.0% in the presence of additives (*13*). These values are between 27 to 60 % loss of the total α-helix content for PLGA microspheres compared to roughly 22% α-helix loss using the emulsan-alginate microspheres.

Conclusions

Microscopy studies revealed the presence of emulsan likely on the surface of the emulsan-alginate microspheres. The presence of emulsan on the microsphere surface increased the stability of the gels in comparison to alginate alone based on the release of blue dextran, the amount of protein adsorbed and the delayed time of about two fold for the release of the carbohydrate to

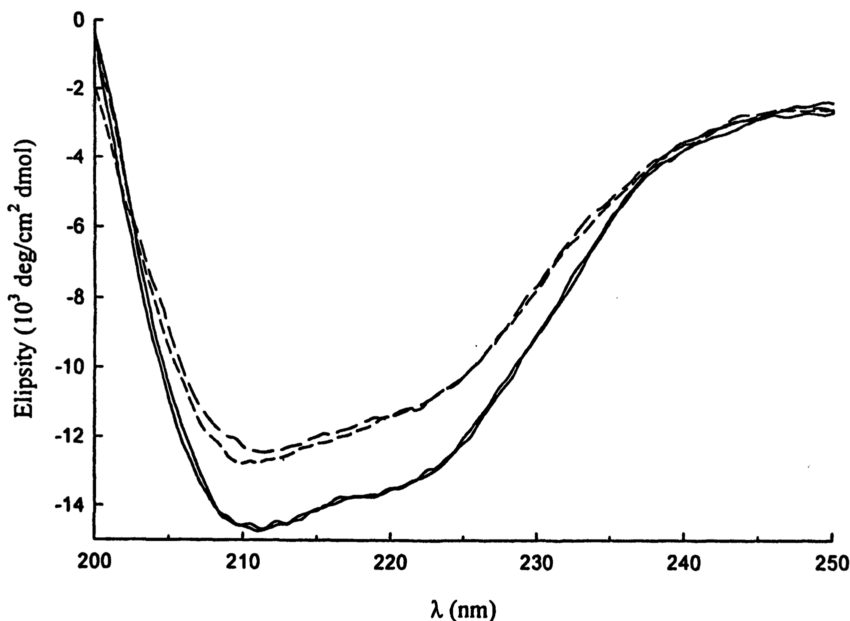

Figure 10. Circular dichroism spectra of BSA after adsorption (continuous line), and before release (dashed line) from emulsan-alginate microspheres.

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solution. This study also demonstrated that triggered release of adsorbed protein from emulsan-alginate microspheres was feasible and the protein adsorbed and then released retained its native state. The higher preservation of BSA structure after adsorption and desorption from emulsan-alginate microspheres compared to PLGA microspheres suggests a potential use of these systems in protein drug delivery. Also, the option to tailor emulsan hydrophobicity and molecular weight for the delivery of specific proteins with different structures and complexities suggests new possibilities for the field of controlled release.

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