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Emulsan–Alginate Beads for Protein Adsorption

Guillermo R. Castro [a,b,c], **Jingsong Chen** [a,b], **Bruce Panilaitis** [a,b] **and David L. Kaplan** [a,b,*]

[a] Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA

[b] Bioengineering & Biotechnology Center, Tufts University, 4 Colby Street, Medford, MA 02155, USA

[c] Applied Biotechnology Institute (CINDEFI), INIFTA — School of Sciences, Universidad Nacional de La Plata, Calle 50 y 115 (B1900AJL), La Plata, Buenos Aires, Argentina

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Abstract

Emulsan–alginate beads were prepared and challenged using bovine serum albumin (BSA) to assess adsorption in comparison to alginate beads. BSA binding to the emulsan–alginate beads was improved over the alginate bead controls and protein adsorption was less sensitive to changes in ionic strength. BSA adsorption between pH 8.5 and 5.3 in alginate beads was 2–3-times lower compared to the emulsan–alginate beads in the same pH range. BSA adsorption and kinetic constants were at least 2-times higher for the emulsan–alginate beads compared to the alginate controls based on the Langmuir adsorption model. To further explore the utility of these novel emulsan–alginate bead systems, complex cell-free supernatants from some pathogenic microorganisms were exposed to the emulsan–alginate beads and increased protein adsorption was found when compared to controls. These trends were also confirmed with α-hemolysin toxicity studies. The data suggest that the protein-binding capacity of emulsan–alginate beads exceeds alginate controls, attributable to the unique binding features of emulsan.

Keywords

Adsorption, alginate, emulsan, hydrogel, biopolymer

1. Introduction

Protein adsorption has found extensive scientific and technological utility in many areas of science and engineering. For example, in downstream processing of many industrial processes, including purification of recombinant proteins and antibodies, it is critical to remove toxins or other factors present in cellular extracts [1]. Ther-

* To whom correspondence should be addressed. Tel.: (1-617) 627-3251; Fax: (1-617) 627-3231; e-mail: david.kaplan@tufts.edu

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apeutically, protein adsorption is studied for extracorporeal treatment of patients to remove serum proteins to improve hemodynamics and to restore leukocyte responsiveness in septic shock [2]. In human and animal nutrition, sequestering protein toxins in food processing is critical to health and safety [1]. In recent years, the development of systems able to transport, capture or deliver biologically active molecules has accelerated. For example, biocompatible hydrogels have become an active area of research for encapsulation of living cells, drug delivery and implants [3].

Alginate is a linear polysaccharide of β-D-mannuronic and α-L-guluronic acids, which can form hydrogels in the presence of calcium and other bivalent cations. Alginate gels are considered safe and currently used in many biotechnology applications [3]. However, alginate gels are unstable in presence of cation chelating agents such as citrate, lactate, phosphate, or tartrate and/or competing cations such as sodium or potassium that are commonly present in biological fluids [4]. In order to prevent bead swelling, polymers such as chitosan, poly(L-lysine), polyacrylates, and others have been added to stabilize calcium–alginate gels [5]. Recently, several studies reported the use of chitosan in alginate gels. Chitosan, a cationic co-polymer of N-acetylglucosamine and glucosamine, is a water-soluble and biodegradable polymer often used in pharmaceutical industries as an excipient, because of its biocompatibility [3]. 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, the major source of chitin is the exoskeleton of crustaceans, and when this source is combined with variable storage and treatment of the material prior to processing, variable material properties are often an issue [3].

Emulsan is an amphipathic exo-lipoheteropolysaccharide of approx. 1000 kDa produced by *Acinetobacter venetianus* strain RAG-1. This polymer is released into the medium in large amounts during stationary growth phase. The main chain consists of three amino sugars: D-galactosamine, D-galactosamine uronic acid and 2,4-diamino-6-deoxy-D-glucosamine, and the amphipathic properties of the polymer are conferred by fatty acid side-chains appended *via* N- and O-acyl bonds to the sugar backbone [6]. In our recent studies we have demonstrated that emulsans provide unique and important attributes in terms of macrophage activation responses related to pro-inflammatory cytokines and as delivery agents for vaccines [7]. Furthermore, we have recently shown that both physiological and genetic manipulation of the biosynthetic pathway can be used to regulate the structure and function of these complex polymers for particular properties [8, 9]. Specific properties that can be controlled include solution behavior, such as emulsifying and surface tension features [10], and biological functions such as cell activation [7]. These types of structure–function controls depend on the nature of the fatty acids present on the polysaccharide backbone and their degree of substitution. Of specific interest relevant to the present study was the observation that when emulsan is produced and secreted by the bacterium it carries up to 23% by weight of adsorbed proteins [8, 11]. The proteins are non-covalently bound to emulsan and hot

phenol extraction or proteolytic digestion are required to remove these "contaminants" in order to purify the emulsan for subsequent analytical characterization or for solution and cellular studies. This same native capacity to adsorb proteins was considered a potential benefit that has been explored in the present study.

Some microbial proteins generated by Gram-positive microorganisms, such as *Actinobacillus* spp., *Bacillus* spp. and *Staphylococcus* species, and by Gram-negative bacteria, such as *Escherichia* and *Salmonella* species, can be considered toxic [12]. *Bacillus subtilis*, a generally non-pathogenic microorganism which is considered as GRAS (Generally Regarded As Safe), is an important producer of extracellular enzymes, but some of them are virulence factors (e.g., some proteases, esterases and lipases). *Bacillus cereus* produces a strong extracellular food-poisoning multi-component protein toxin with the same type of multimeric structure as *B. anthracis* toxin [13]. *Staphylococus* species produce a large amount of exoproteins, some of which are cytotoxic, including lipases, collagenases and pyrogenic toxins [13]. In particular, *Actinobacillus suis* and *Staphylococcus aureus* are described as good producers of hemolysins, a group of toxins with the ability to lyse red blood cells [13]. Gram-negative *E. coli* and *Salmonella* species generate enterotoxins and cause food poisoning. It is very well recognized that effect of toxins on cells is mediated by adsorption to cell lipid surface described as the first stage of intracellular translocation [13].

Based on the history of use of alginates in gels [3], and the innate ability of emulsans to adsorb proteins, the potential for combining these two polymers into protein adsorption beads was studied in the present work. The hypothesis was that the combined features of both polymers would provide enhanced protein adsorption and in the long run improved options for controlled release and cell activation through the use of the two polymers in gel form. In order to characterize the protein adsorption process, Bovine serum albumin (BSA) was selected as the model protein for the present studies because it is well characterized and it is responsible for 99% of free fatty acid transport in mammals, with average equilibrium constants of approx. 10^7 M^{-1} [14]. Comparative analysis of the beads was investigated using Environmental Scanning Electron Microscopy (ESEM). Effects of temperature, pH and ionic strength on BSA adsorption were determined both on the emulsan–alginate systems and a control system consisting of alginate alone. In addition, the classical isotherm BSA adsorption model of Langmuir was tested. Aside from the studies with BSA, the adsorption of extracellular extracts from pathogenic microorganisms and specifically α-hemolysin were also evaluated with the beads as an additional measure of function for these new systems.

2. Materials and Methods

2.1. Materials

Chemicals and solvents were analytical grade and other reagents and microbiological media were of highest available grade obtained from Aldrich (St. Louis, MO,

USA) or Difco (Franklin Lakes, NJ, USA) and used as provided. Low viscosity alginate (A2158), α-hemolysin from *S. aureus* (H 9395) and BSA (fraction V, A3059) were purchased from Sigma (St. Louis, MO, USA).

2.2. Microbial Cultures and Emulsan Purification

A. venetianus strain RAG-1 (ATCC 31012) was maintained on Luria Bertrani (LB) agar slants covered with LB medium to prevent cell dehydration at 5°C. Ethanol saline (ES) medium and culture conditions for emulsan production and purification were described previously [9]. Fatty acids covalently coupled to the polysaccharide backbone were removed with the hot alkaline methanol procedure previously described [9]. *Actinobacillus suis* van Dorsesen and Jaartveld (ATCC 15557, α-hemolysin producer), *Bacillus subtilis* BGSC 1A1, *Bacillus cereus* BGSC 6A5 (ATCC 14579), *Escherichia coli* BLR (Novagen, Madison, WI, USA), *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 8096, α-hemolysin producer), *Staphylococcus epidermidis* (ATCC 12228) and *Salmonella typhimiurum* TA98 (Xenometrix, San Diego, CA, USA) were cultivated in 250 cm^3 flasks containing 100 cm^3 of LB or nutrient or tripticase soy media and broth (Difco) at 30 or 37°C for 24–48 h following ATCC protocols (http://www.atcc.org).

2.3. Bead Formation

Alginate sodium salt (2 g) was dissolved in 100 ml distilled water or in 50 mM Tris-HCl (pH 7.5) buffer. Alginate solution (2.0 %) was pumped into an aqueous solution of $CaCl_2$ (25.0 mM) with or without emulsan (3.0 mg/cm^3) in 25 mM Tris-HCl (pH 7.5) or sodium acetate (pH 5.0) buffers at 0.30 to 1.0 cm^3/min under continuous stirring. Fresh beads were incubated in the calcium solutions for 1–48 h in a shaker at 50 rpm and at 5°C, followed by filtration on paper (Whatman #1). Filtered beads were kept at 5°C in 70% ethanol until use. Emulsan content in the beads was quantified by a phenol sulfuric acid technique [15].

2.4. Characterization of Beads Using Environmental Scanning Electron Microscopy (ESEM)

Bead samples were mounted on a microscope plate without any previous treatment for ESEM analysis (FEI, Quanta 200 Scanning Electronic Microscope equipped with a Falcon System running Genesis 1.1 software and a super ultra thin window (SUTW)). Microscopy chamber was saturated with water, and the pressure was kept in 4.3 to 6.75 Torr range to avoid extensive sample dehydration.

2.5. Protein Adsorption

Alginate and emulsan–alginate beads (50.0–300.0 $\pm$ 5.0 mg) were placed in 1.5 ml tubes and filled with 1.0 ml of a protein solution. The tubes were incubated for 10–120 min at 24 to 37°C, followed by centrifugation at 10 000 $\times$ g for 2 min at room temperature. Aliquots of 0.50 ml of supernatant were filtered through a 100 kDa MWCO ultrafiltration device (Microcon, Millipore, Billerica, MA, USA)

and the BSA content quantified using Coomassie Brilliant Blue reagent calibrated with BSA, Fraction V, as standard [16]. Kinetics of BSA adsorption were determined by incubation for 10–60 min at 30°C in buffer containing 154 mM NaCl, 25 mM acetate buffer (pH 5.0) with 50 μg/ml BSA, and incubated for 10–60 min at 30°C. Assays to determine the effects of ionic strength, temperature and pH on the kinetics of BSA adsorption were performed using NaCl concentrations from 10.0 mM to 1.0 M (30°C, pH 5.0), temperatures from 24 to 37°C (100 mM NaCl, pH 5.0) and pH from 3.3 to 8.5 (100 mM NaCl at 30°C). Adsorption was determined with solutions containing 20.0–45.0 μg/ml of BSA using 25 mM sodium acetate (pH 3.3–5.6), or Bis-Tris (pH 5.8–7.2), or Tris-HCl (7.5–8.50), or Bis-Tris-Propane (7.0–9.5) buffers.

2.6. Langmuir Adsorption Model

The linear representation of the Langmuir isotherm can be expressed by the equation [17]:

$$\frac{1}{q_e} = \frac{1}{Q_m} + \frac{1}{Q_m \cdot K_L}\frac{1}{C_e}, \tag{1}$$

where Q_m and K_L are the Langmuir constants, and C_e the equilibrium concentration of BSA left in the aqueous solution expressed in μg/cm^3. Also, the Langmuir equation can be expressed in dimensionless form using the equilibrium parameter (R_L), defined as:

$$R_L = \frac{1}{1 + K_L \cdot C_o}, \tag{2}$$

where K_L is the Langmuir constant and C_o is the highest initial solute concentration tested. The R_L values indicate the type of isotherm: irreversible if R_L is equal to zero, favorable if R_L is higher than 0 but less than 1, or unfavorable if R_L is higher than 1.

Langmuir experiments were carried out in 25 mM sodium acetate buffer (pH 5.0), 160 mM NaCl and 50 μg/ml BSA solution at 34°C.

2.7. Adsorption and Evaluation of Toxins

As above for BSA adsorption, the alginate and emulsan–alginate beads (50.0–300.0 ± 5.0 mg) were placed in 1.5 ml tubes and filled with 1.0 ml solution. The tubes were incubated 10–120 min at 24–37°C, followed by centrifugation at 10 000 × g for 2 min at room temperature. Aliquots of 0.5 ml of the supernatant were filtered through a 100 kDa MWCO ultrafiltration device (Microcon, Millipore, Billerica, MA, USA), and then assayed for total protein content. Hemolytic activity was estimated on 8% washed rat erythrocytes in 145 mM NaCl, 50 mM Tris-HCl (pH 7.7) with appropriate volume of sample. The reaction mixtures were placed at 37°C for 1 h. Unlysed erythrocytes were removed by centrifugation for 1 min at 5000 × g (4°C). The release of hemoglobin in the samples and controls was measured in a spectrophotometer at 405 nm, and compared with a calibration curve of

commercial pure α-hemolysin. Hemolysin activity was assayed in cell-free supernatant microbial cultures of *A. suis* and *S. aureus* subsp. *aureus* Rosenbach filtered through 0.22 μm devices before and after incubation with the beads as previously described.

2.8. Cytotoxicity

Human bone marrow stem cells (hMSCs) were isolated, culture expanded and stored as described previously [18]. Briefly, human unprocessed whole bone marrow aspirates were obtained from donors <25 years of age (Clonetic-Poietics, Walkersville, MD, USA), resuspended in Dulbecco Modified Eagle Medium (DMEM) supplement with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 40 U/cm^3 penicillin and 40 mg/dm^3 streptomycin (P/S) and 1 ng/cm^3 basic fibroblast growth factor (bFGF) and plated at 8 μl aspirate/cm^2 in tissue-culture polystyrene; non-adherent hematopoietic cells were removed with the culture medium during medium exchange after 4 days. Culture medium was changed twice per week thereafter. Primary hMSCs were detached at approx. 80% confluence using 0.25% trypsin-1.0 mM EDTA and replated at 5000 cells/cm^2. Passage 1 (P_1) hMSCs near confluence were trypsinized and subsequently frozen in 8% dimethylsulfoxide (DMSO)/10% FBS/DMEM for future use. P_1 hMSCs were defrosted, replated at 5000 cells/cm^2 (P_2) and trypsinized near confluence. P_3 hMSCs were plated onto 6-well plates at a density of 5000/cm^2, cultured at 37°C/5% CO_2 for 6 days to reach confluence. The emulsan–alginate or alginate beads were sterilized by incubation in 70% alcohol solution for 2 h at room temperature, and then extensively rinsed with sterile phosphate buffer solution (PBS). After this sterilization process, beads were co-cultivated with the hMSCs. Cell morphology images were taken at 1, 12, 24 and 72 h after cultivation with a phase-contrast microscope (Axiovert S-100 inverted microscope, Carl Zeiss, Jena, Germany). After 72 h of cultivation, cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) staining ($n = 4$). Cells were incubated in MTT solution (0.5 mg/cm^3, 37°C/5% CO_2) for 2 h. The intense red colored formazan derivative formed was dissolved and the absorbance was measured with a microplate spectrophotometer (Spectra Max 250, Molecular Devices, Sunny Valley, CA, USA) at 570 nm.

2.9. Statistics

Two or three independent experiments were conducted with a minimum of duplicates ($n = 2$) or triplicates ($n = 3$) for each data point. Data for these measurements were analyzed using a Student's t-test. Statistically significant values were defined as $P < 0.05$. Data in the graphs represent the mean ± standard deviation.

3. Results and Discussion

ESEM imaging of the beads in a water-saturated chamber showed significantly different surface morphology between the alginate and emulsan–alginate beads

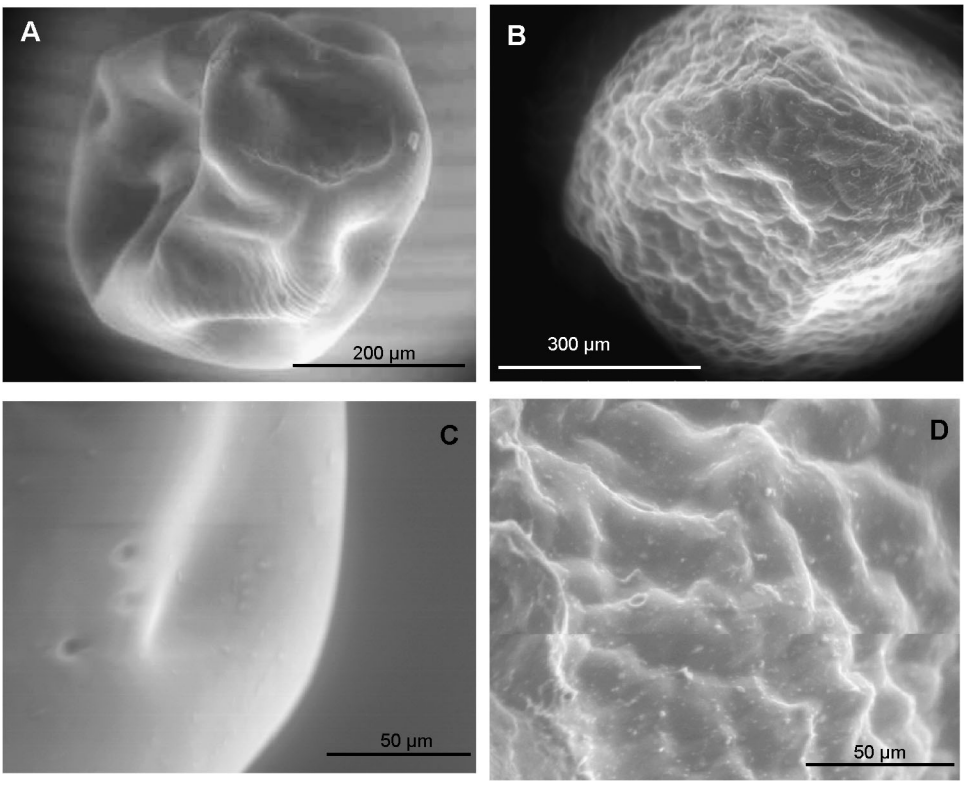


Figure 1. Environmental scanning electron microscopy images of alginate (A, C) and emulsan–alginate beads (B, D).

(Fig. 1). The alginate beads displayed a smooth surface, in contrast to the irregular and mottled surface on the emulsan–alginate beads. The presence of the fatty-acid acyl chains on the emulsan main chain and the lack of these pendent groups on alginate is a likely reason for the differences in bead morphology. Average diameters of both types of beads were 450 ± 50 µm, with an average bead weight of 5.5 ± 1.0 µg. Carbohydrate analysis of the beads containing emulsan resulted in $10.5 \pm 1.2\%$ emulsan per bead.

Experiments of protein adsorption by alginate and emulsan–alginate beads were conducted using BSA. After protein incubation with the beads, residual BSA in the supernatant was determined by Coomassie blue. Isothermal kinetic analysis of BSA adsorption with the two types of beads at 25°C is shown in Fig. 2. The adsorption of BSA occurred in two steps with both types of beads; rapid adsorption involving 51.8 and 26.4% of total protein for the emulsan–alginate and alginate beads, respectively, in 10 min. However, 1-h incubations were required to reach equilibrium adsorption, reflecting a slower second phase. While different amounts of BSA were adsorbed with the two types of beads in the first 10 min, adsorption from 10 to 60 min increased similarly by 47.3 and 48.9% for the emulsan–alginate

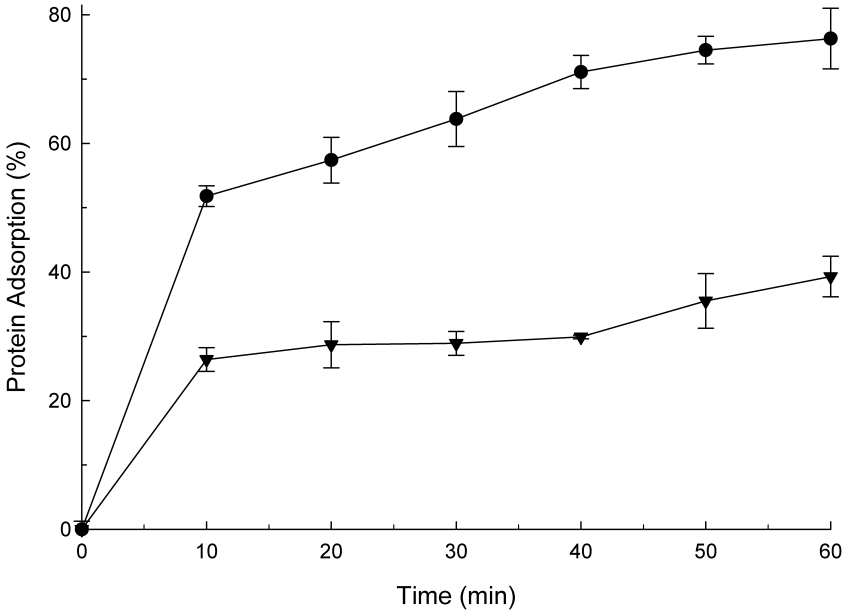


Figure 2. Kinetics of BSA adsorption by emulsan–alginate (●) and alginate (▼) beads.

Table 1.
Comparison of BSA adsorption by synthetic and inorganic materials with emulsan-coated alginate beads

Maximum adsorption (μg BSA/mg support)	Support	References
16.8–78.7	CB-6AB, CB-4AS	[19]
16.8–84.3	Silica	[20]
64.5–146.3	PNIPA	[21]
90.0	Colloidal Al_2O_3	[22]
51.8–149.7	Emulsan–alginate beads	Present work
200.0 (approx.)	PHEMA	[23]
200.0 (approx.)	Cu-CTS-SiO_2	[24]
250.0	Fe_3O_4 magnetic particles	[25]

Abbreviations: CB-4AB, Cibacron-blue-modified 4% agarose gel; CB-6AS, Cibacron-blue-modified 6% agarose gel; PNIPA, poly(*N*-isopropylacrylamide); PHEMA, poly(2-hydroxymethyl methacrylate); Cu-CTS-SiO_2, copper–chitosan–silica gel.

and alginate beads, respectively. Total BSA adsorption at equilibrium was 73.6 and 30.5% for emulsan–alginate and alginate beads, respectively. In previous work, BSA was used as a model protein to study adsorption in many physical supports ranging from inorganic materials to synthetic polymers (Table 1). The results with emulsan–alginate beads reported here indicate similar values to inorganic materials and synthetic polymers (Table 1). Among the comparative advantages of using

biopolymers to inorganic materials for protein adsorption is the selectivity of the polymer matrix, which can be easily manipulated by common tailoring procedures like biotransformations and/or different microbial feeding strategies. In contrast, synthetic polymers require multi-step procedures for synthesis and polymer purification which are expensive and time-consuming. Also, the degradation products of synthetic polymers sometimes are not environmentally/biologically friendly.

The effect of temperature, pH and salt on BSA adsorption by the beads is displayed in Fig. 3. A pH increase from 4 to 5 resulted in a dramatic reduction of about 60% in BSA adsorption by alginate beads. In contrast, BSA adsorption by emulsan–alginate beads remained constant in the same pH range but showed less of a decrease, about 25% of the BSA adsorption between pH 5.3–5.5. BSA adsorption with the emulsan–alginate beads was about 10-times more resistant to the changes in pH compared to the alginate samples. A possible explanation is the presence of charge complexes through the interaction of free amino groups of emulsan with protein carboxylic groups, which could reduce the impact of pH changes on protein adsorption. Also, the decrease of BSA adsorption with increase in pH can be attributed to an increase in electrostatic repulsion between the ionized carboxylic acid groups present in the emulsan beads and BSA, which has an isoelectric point of 5.15. A similar pH-dependence associated with ionization of emulsan carboxylated groups was reported for the emulsifying activity [10].

The influence of ionic strength from 1.0 to 1000 mM of NaCl on BSA adsorption is shown in Fig. 4B. BSA adsorption increased from 70 to 100% as the ionic strength increased with the emulsan–alginate beads, and from 13 to 68% with alginate beads. Also, the differences of BSA adsorption by emulsan–alginate and alginate beads decreased as the ionic strength increased, a finding that suggests different mechanisms of protein interaction with the polymer matrices at different ionic strengths. Ionic strength can influence adsorption mediated via conformational changes of BSA upon binding to the surface [14], but also it could affect the bead structure by competition of sodium with calcium, which can cause gel disruption [26].

An increase of temperature between 24–37°C induced $>40\%$ increase in adsorption by the emulsan–alginate and alginate beads, and the difference was almost constant over the range of temperatures studied. The increase in adsorption could be attributed to an increase in chain dynamics, exposing more binding sites to the solution. Before and after the BSA adsorption equilibrium, the amount of BSA determined in the supernatant decreased with increase of temperature (data not shown), indicating a kinetically controlled mechanism. In addition, secondary adsorption related to BSA–BSA interactions and cooperative effects, rather than BSA–bead interactions, motivated by changes of BSA topology associated with binding [14], may also play a role.

Based on the experimental results shown in Fig. 3, the combination of different experimental conditions such as pH, ionic strength and temperature on emulsan–

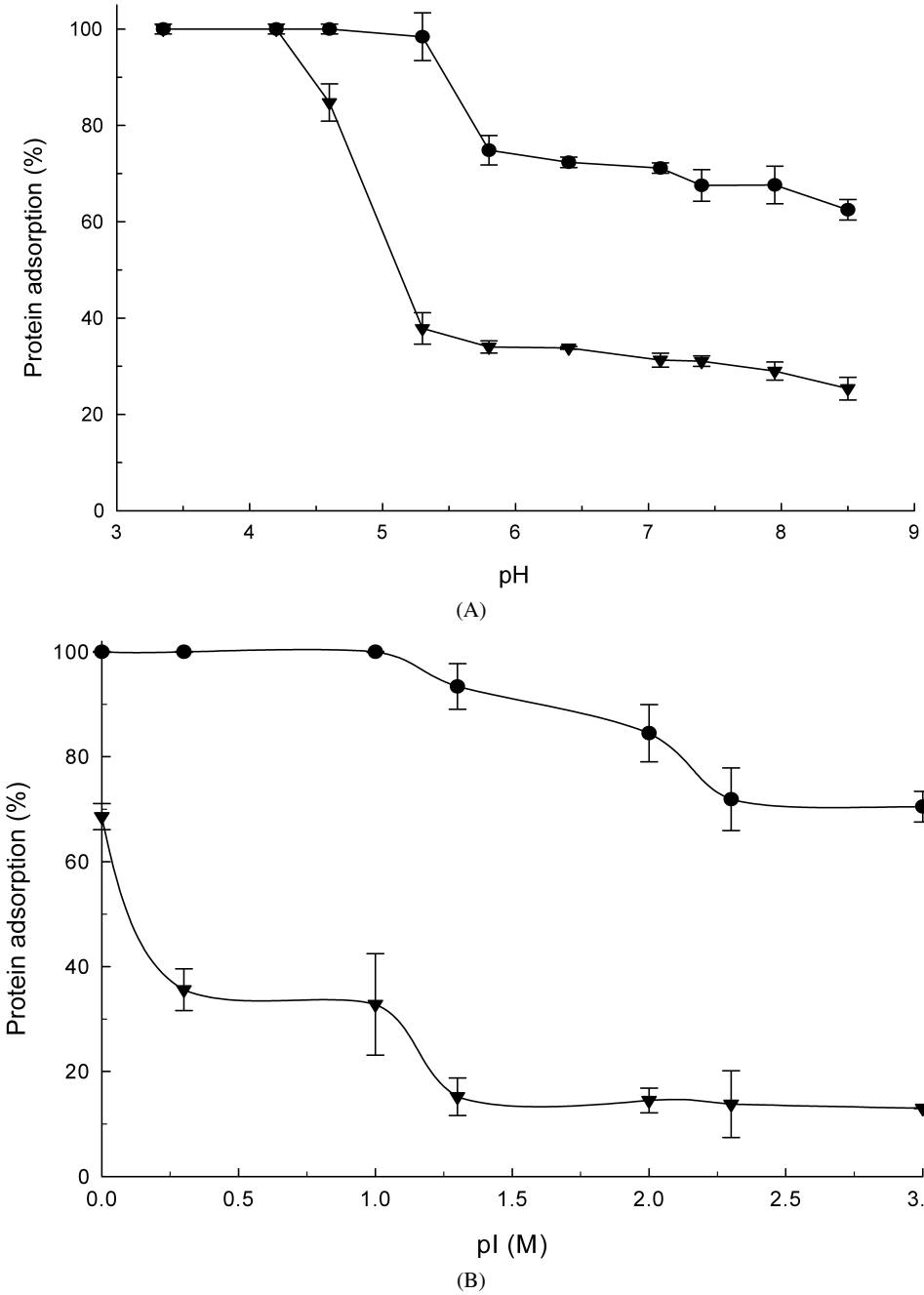
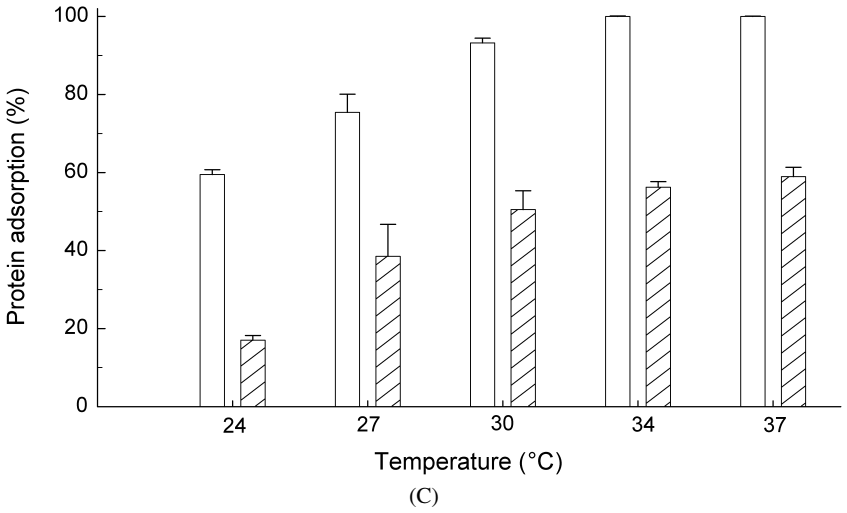


Figure 3. Effect of pH (A), ionic strength (B) and temperature (C) on BSA adsorption by emulsan-coated alginate (●, open bars) and alginate (▼, hatched bars) beads.



(C)

Figure 3. (Continued.)

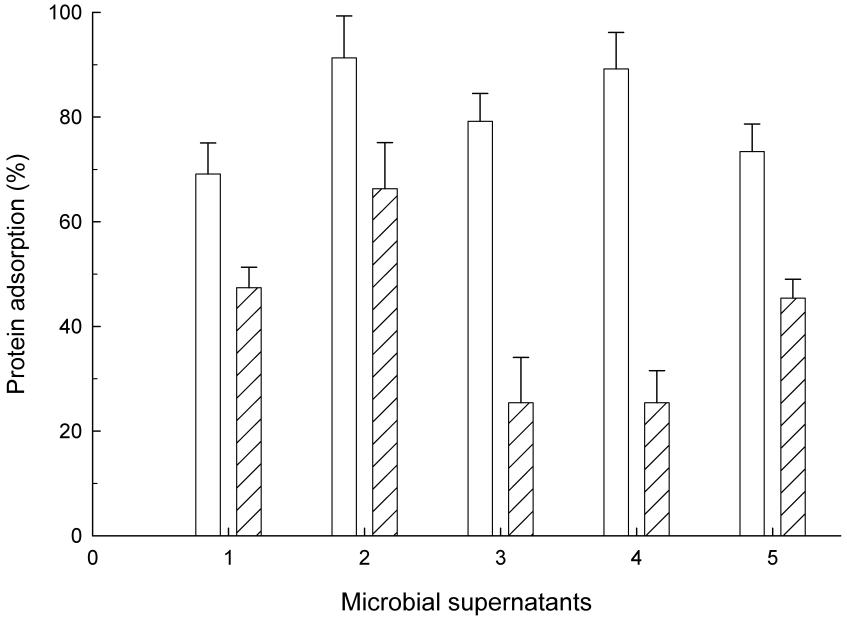


Figure 4. Relative protein adsorption of microbial cell-free supernatants to emulsan–alginate (open bars) and alginate (hatched bars) beads. Microbial cultures: *Bacillus subtilis* (1), *Bacillus cereus* (2), *Escherichia coli* (3), *Staphylococcus epidermidis* (4) and *Salmonella typhimurium* (5).

alginate beads allowed for the selective adsorption proteins on the bead surface based on the different properties (e.g., isoelectric point, hydrophobicity).

In order to evaluate protein adsorption with the beads in equilibrium using the BSA model, the Langmuir model was assessed (Table 2). The correlation factor for

Table 2.
Langmuir adsorption isotherm

Parameter	Microspheres		Em/Alg ratio
	Emulsan	Alginate	
Q_m (μg/cm^3)	0.24	0.07	3.08
K_L (cm^3/mg)	0.15	0.07	2.17
r^2	0.98	0.97	–
R_L	0.06	0.12	–

the linear regression of the experimental data using the Langmuir model was >0.9, indicating a reasonable fit. Therefore, BSA adsorption from aqueous solution follows monolayer adsorption [17]. This result suggests that the protein adsorption in the gel bead system does not proceed by the interaction of two BSA molecules, one adsorbed on the microsphere surface, while the other BSA molecule is in solution as hypothesized [14]. Analysis of R_L, a dimensionless Langmuir equilibrium parameter, predicts that the adsorption of BSA by the beads is favorable; the value is between 0 and 1. A comparison between the maximum adsorption for the emulsan–alginate and alginate beads showed significantly higher adsorption of BSA by the emulsan–alginate system by a factor of about 2.5, based on comparisons of the Langmuir isotherm constants (Table 2).

Once the conditions and possible adsorption mechanism for BSA were established, potential applications for more complex protein mixtures, microbial supernatants, were tested. To study the capability of emulsan–alginate beads to sequester potentially toxic proteins from cultures of microorganisms, cell-free supernatants of seven selected potentially toxin-producing microorganisms were analyzed in combination with the beads. Alginate and emulsan–alginate beads were used for the adsorption experiments based on the data with BSA. Emulsan–alginate beads bound more than 70% of total protein in all the cell extracts tested (Fig. 4), and exhibited higher binding capacity then the alginate beads, about 38% higher on average. The adsorption of extracts by emulsan-coated alginate beads of *S. epidermidis, E. coli* and *B. cereus* was between 80 and 90%. This is of particular interest as *B. cereus* is closely related to *B. anthracis* species [12]. In contrast, protein adsorption of extracellular extracts of *S. epidermidis* and *E. coli* were less than 25% of the alginate beads. In the case of *S. typhimurium*, the emulsan–alginate beads had an approx. 30% higher binding capacity than the alginate beads. The crucial role of fatty acids on the emulsan in this adsorption process was demonstrated when the fatty acids were stripped off of the emulsan polysaccharide backbone, resulting in similar adsorption capacities for *E. coli* and *B. subtilis* extracellular extracts between the alginate and emulsan (without fatty acids)–alginate beads (data not shown). Therefore, by changing the fatty acid composition of emulsan on the bead

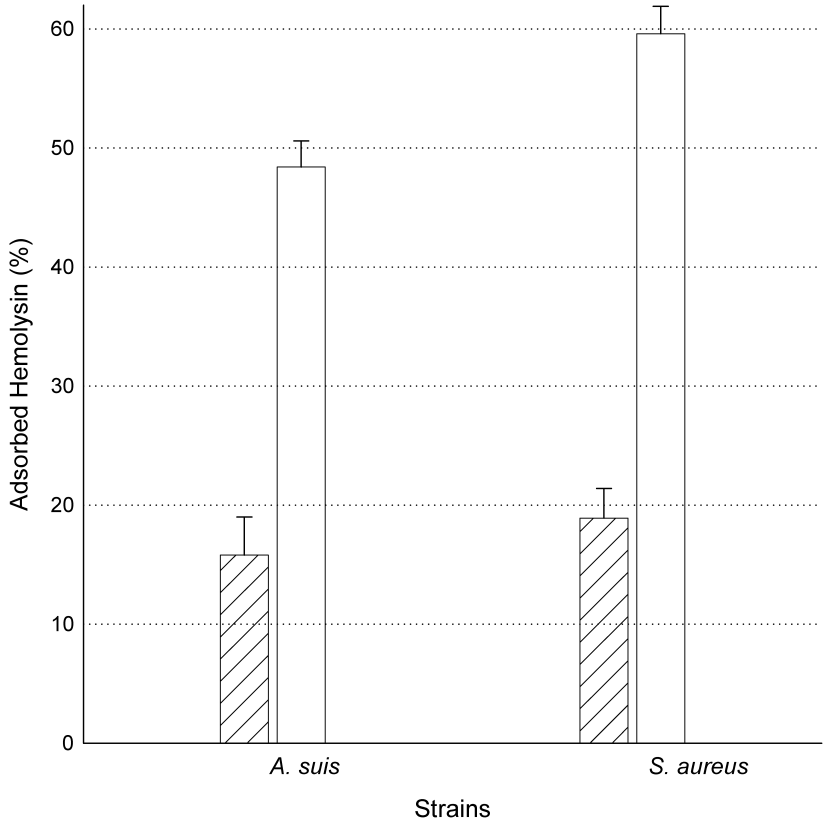


Figure 5. Percentage of hemolysin produced by *A. suis* and *S. aureus* adsorbed by alginate (hatched bars) and emulsan (open bars) microspheres.

surface, protein adsorption can be changed, resulting in an enhanced or decreased adsorption of desired proteins.

Additionally, α-hemolysin present in the supernatant of *A. suis* and *S. aureus* was determined before and after incubation with the beads, and correlated with an α-hemolysin standard. Less than 20% of α-hemolysin was adsorbed by alginate beads in both extracellular cell free extracts, while about 50% was sequestered by the emulsan–alginate beads (Fig. 5). α-Hemolysin poseses a 9-amino-acid sequence tandemly repeated many times in the molecule is a calcium dependent motif which is essential for hemolytic activity and close to the C-terminus of the protein [13]. This domain could be responsible for binding to the alginate. The increase of binding in the emulsan–alginate beads could be attributed to the contribution of the rim domain [27], a hydrophobic domain of the α-hemolysin monomer responsible for anchoring the protein to the membrane surface. This domain could bind to the fatty acids present in the emulsan–alginate beads. Based on our experimental results, a controlled balance of polar and hydrophobic spots in the emulsan molecule will allow enhanced α-hemolysin adsorption. The removal of α-hemolysin from flu-

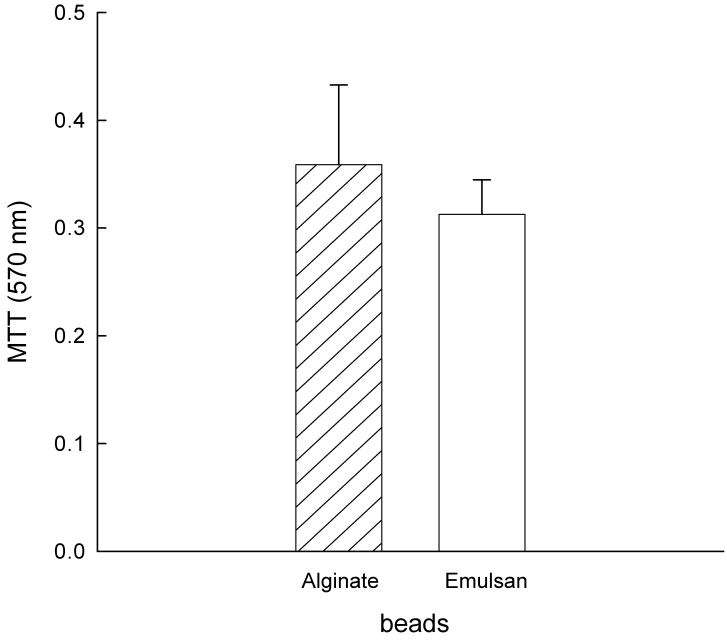


Figure 6. MTT analysis of bone marrow stromal cells incubated with emulsan-coated (hatched bars) and alginate beads (open bars) for 72 h.

ids is particularly relevant in patients with severe staphylococcal infections where α-hemolysisn plays a central role in pulmonary infections, hemostatic disturbances and thrombocytopenia, among other pathologies.

In vitro cell-culture experiments were performed to evaluate potential cytotoxicity of the emulsan–alginate beads [18]. Cell controls were performed in the presence of buffer, and a non-toxic biomaterial alginate bead was chosen as the control [3]. Morphological study of hMSCs cultivated in buffer and with both types of beads showed no toxicity (vacuolization, growth inhibition) (Figs 6 and 7). The MTT assay was performed at day 3 and showed that there was no difference in viability of the cells in the tests and controls. These data indicate that the emulsan–alginate beads are non-toxic biomaterials and could be a useful tool for biomedical applications.

4. Conclusions

The present work demonstrates that emulsan–alginate beads display different morphological, structural and adsorption properties compared to alginate (control) beads. High adsorption of BSA, as well as mixed extracellular microbial proteins by the emulsan–alginate beads offers new possibilities for the use of these beads due to the carrying capacity of these systems, as well as the unique structural tailorability and biological interactions of the emulsan polymers. Potential applications for these systems may include controlled release with high ligand binding capacity which

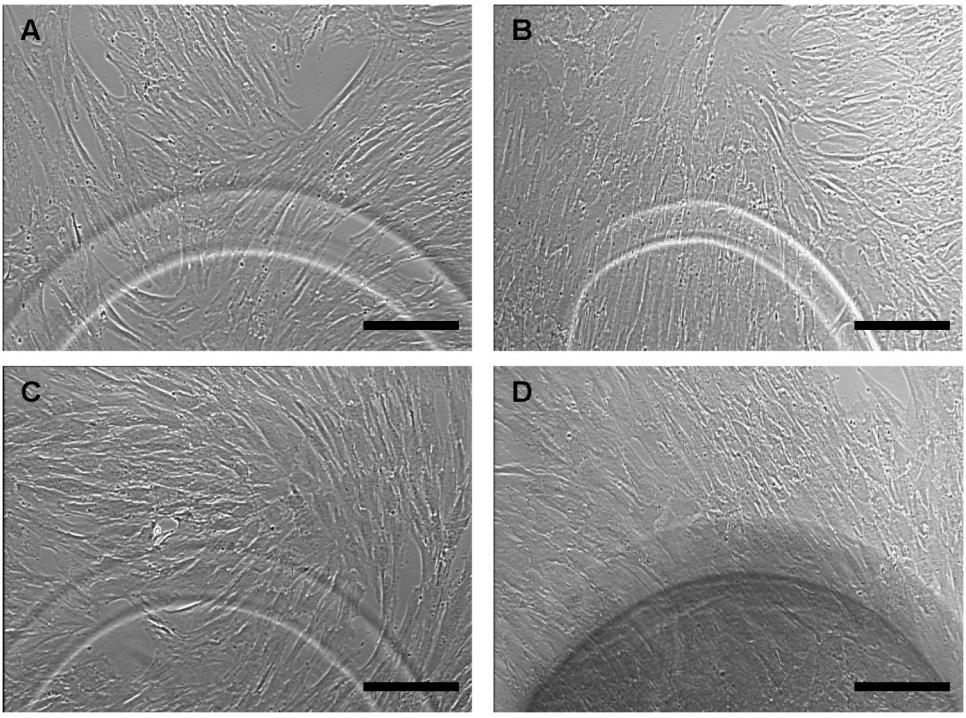


Figure 7. Photomicrographs of bone marrow stromal cells incubated with emulsan beads for (A) 5 min and (B) 72 h or with alginate beads for (C) 5 min and (D) 72 h. Bars = 100 μm.

would allow for decreased dosage to compensate for low solubility and stability of several important pharmaceutical compounds. The results with emulsan–alginate beads showed BSA adsorption similar to chemically synthesized polymers like Cibacron-blue-modified agarose and polyacrylamide gels (Table 1), but with the advantages of no chemical reactions needed, no toxic intermediates present that would otherwise require special precautions during purification steps, and biodegradability/biocompatibility. Alternatively, the use of biocompatible filter-like devices to reduce protein and/or toxin content in foods and/or biological systems would be a potentially useful option to explore based on the results shown.

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