Encapsulation Efficiency and Release Behaviors of Bovine Serum Albumin Loaded in Alginate Microspheres Prepared by Spraying

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ABSTRACT: Spraying and spraying with an electrostatic field (SEF) were employed to prepare alginate microspheres for delivering proteins, especially for intestinal digestive enzymes and cytokines. The encapsulation efficiency (EE) of a model protein [bovine serum albumin (BSA)] at a pH value lower than the isoelectric point was 20% higher than that at a natural pH. Moreover, for the microspheres prepared by SEF, EE improved significantly with increasing electric voltage. The interactions between BSA and the alginate microspheres were identified with Fourier transform infrared spectroscopy. The release profiles *in vitro* showed a controlled and pH-responsive release manner for the encapsulated BSA. A first-order release equation was postulated and modified to describe the release kinetics with an obviously initial burst release related to the eroded porous matrix. The equation fit the release data well when the pH value and composition of the release media were changed. The analysis of the release kinetics indicated that the drug release rate was in an inverse ratio to the diameter of the microspheres. Increasing the gas flow rate or electric voltage decreased both the mean diameter and size distribution of the microspheres significantly and enhanced the release rate of loaded drugs from alginate microspheres. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis analysis revealed that BSA kept its structural integrity during the encapsulation and release process. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 2488–2497, 2008

Key words: drug delivery systems; microencapsulation; simulations

INTRODUCTION

Along with the recent advances of recombinant DNA techniques, the production of therapeutically active peptides and proteins in large quantities has become feasible. However, the therapeutic applications of these proteins are limited because of their short half-life *in vivo* and quick acid-catalyzed or proteolytic degradation in the gastrointestinal tract if they are given orally.[1,2] A potential solution to these problems is the use of microencapsulation to delivery protein drugs in a convenient and controlled manner. Alginate, a natural polysaccharide extracted from brown algae, is nontoxic and biocompatible.[3] Alginate microspheres used as a protein delivery system seem to be very attractive because of their resistance to the acidic environment of the stomach, their easy gel formation, and their availability.[2,4]

Alginate microspheres can be prepared with a variety of commonly used techniques, such as dripping,[5,6] emulsification,[7] and spraying.[8–10] The advantage of spraying over other coating methods is that it can be used to produce microspheres less than 150 μm in diameter on a large scale.[2] However, the method of spraying in common use still presents a polydispersity of obtained microspheres. Additionally, the major disadvantages of calcium alginate microspheres prepared by conventional spraying are a low drug encapsulation efficiency (EE) and a rapid release of the loaded drugs. The loading efficiency of water-soluble drugs, in particular, is much lower than that of water-insoluble drugs, and this is due to leakage of the drugs from alginate microspheres with large gel porosity.[11] The complex drug release mechanism from a swellable alginate matrix results in the uncontrolled and quick release of loaded drugs within a few minutes in simulated intestinal fluid (SIF).[8,10] In most cases, this method is employed to encapsulate micromolecular drugs that are insensitive to shear and heat because of the potential destructive effect of the air shear force. Moreover, the influence of spraying parameters on drug release behavior and on activities of encapsulated drugs, especially macromolecular proteins, has rarely been reported in previous studies.

Extensive experimental studies have shown that when a high electrostatic field is appended to a dripping setup, the polydisperse coefficient and mean diameter of the polymer droplets gradually decrease with increasing applied potential (U).[12–14] To our

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knowledge, there has been no attempt to combine an electrostatic field with a spraying setup. Here we investigated the characteristics and drug release behaviors of alginate microspheres achieved by spraying with an electrostatic field (SEF).

This study focused on the production of alginate microspheres with a high drug EE and controlled release behavior via a spraying method with simple processes. The effects of the preparation conditions on the protein retention and release behaviors of encapsulated bovine serum albumin (BSA) were investigated. The release kinetics equation was modified in light of the analysis of release profiles with an obviously initial burst release phase. Moreover, to decrease the size distribution and improve the protein retention of alginate microspheres, the SEF method was first employed, and the influence of high voltage on BSA release behaviors was also examined.

EXPERIMENTAL

Materials

A low-viscosity sodium alginate obtained from Beijing Xudong Chemical Reagent Co. (Beijing, China) was used. A 2.0% (w/v) sodium alginate solution had a viscosity of 717 cps at 25°C, which was determined with a LAB-LINE 4535-1 viscometer (LAB-LINE Instruments, Inc., Melrose Park, Illinois, United States). BSA [molecular weight = 68,000, isoelectric point (pI) = 5.0] was provided by the Yuanheng Shengma Biotechnology Research Institute (Beijing, China). Coomassie Brilliant Blue G250 was purchased from Fluka (Buchs, Switzerland). All other reagents were reagent-grade.

Preparation of calcium alginate microspheres by spraying

A mixture of 2.0% (w/v) sodium alginate and 80 mg/mL BSA was dissolved in purified water to reach a final theoretical alginate/BSA ratio of 2.5 : 1 (w/w) with a natural pH value of 9.2. Spraying was concurrently performed with a jet head with a standard 1.56-mm nozzle under the following conditions optimized in our previous study[15]: a liquid flow rate of 13 mL/min, an inner needle diameter of 0.42 mm, and a distance between the jet head and coagulation solution of 15 cm. Individual parameters, including the gas flow rate (0.50, 0.60, 0.70, and 0.85 m^3/h) and pH of the coagulation solution, were evaluated separately. The pH of the coagulation solution containing 1.1% calcium chloride was adjusted with an HAc–NaAc buffer (0.2 mol/L) ranking between 9.0 and 3.4. The microspheres were spontaneously hardened for half an hour and then were collected by filtration and washed with distilled water. The morphology of the properly dried microspheres was measured by scanning electron microscopy (JSM-5600LV, Tokyo, Japan). Three batches of the microspheres were prepared for further study.

Preparation of calcium alginate microspheres by the SEF method

The experimental setup of the SEF method was similar to the spraying configuration with the appending of two infinite plane-parallel electrodes. The diameter of the upper electrode was 31 cm with a center hole of 3 cm, and a high direct-current voltage ranging from +5 to 20 kV was applied to the upper electrode against a plate earth electrode. The distance between the two plane-parallel electrodes was fixed at 0.32 m throughout the study. The other parameters were the same as those of the spraying method. All the experiments were performed in triplicate.

Particle size and distribution analysis

Before freeze drying, the mean diameter and polydispersity of at least 500 alginate microspheres were determined by laser diffractometry with a Coulter LS 230 (Beckerman Coulter Co., Fullerton, California, United States). The microsphere size distribution was expressed by the polydispersity index (PDI).[16] It was defined as follows:

$$\mathrm{PDI} = \left(\sum_{i=1}^{N} D_i^4 \Big/ \sum_{i=1}^{N} D_i^3\right) \Big/ \sum_{i=1}^{N} D_i/N \qquad (1)$$

where N is the total number of microspheres counted and D_i is the diameter of microsphere i. PDI < 1.05 was considered monodispersity.

EE

EE of the microspheres was calculated as follows:

$$\mathrm{EE}\ (\%) = 100 \times \frac{\text{Total BSA amount} - \text{Free BSA amount}}{\text{Total BSA amount}} \qquad (2)$$

After centrifugation at 10,000g for 10 min, the free nonencapsulated BSA in the supernatant of the aqueous preparation medium (1 mL), after hardening for half an hour, was assayed by the Bradford method[17] at a wavelength of 595 nm. All the experiments were performed in triplicate.

Fourier transform infrared (FTIR) spectra

FTIR spectra of BSA, blank alginate microparticles, and alginate microparticles loaded with BSA under

different conditions were determined by the KBr pellet method. FTIR spectra were taken in the range of 4000–400 cm^{-1} with an Equinox 55 FTIR spectrophotometer (Bruker, Ettlingen, Germany) operated in the transmission mode.

In vitro release studies

The release of BSA was determined by the incubation of 15 mg of microspheres in 100 mL of different media with horizontal shaking (100 rpm) at 37°C. The different media were simulated gastric fluid (SGF; 0.1 mol/L HCl, pH 1.2), NaCl (0.9%, pH 7.0), SIF [0.2 mol/L phosphate-buffered saline (PBS), pH 7.4], and SGF for 2 h and then SIF for an additional 22 h. At appropriate time intervals, 1 mL of the medium was periodically removed and replaced by the same quantity of the medium. The concentration of BSA released at time t was assayed by the Bradford method at a wavelength of 595 nm. The corresponding drug release profiles were represented through the plotting of the cumulative percentage of the drug released [calculated from the total amount of BSA contained in each matrix (EE)] versus the time. All the experiments were performed in triplicate.

Structural integrity of encapsulated BSA

The structural integrity of BSA released from alginate microspheres prepared by SEF was detected by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE), and it was stained with silver for comparison with native BSA and reference markers. SDS–PAGE was carried out with a DYY-2C system (Beijing Liuyi Factory, Beijing, China) using Tris–glycine gels of 15% polyacrylamide (1.0 mm, 10 wells). The running condition was 45 mA for 160 min.

Statistical analysis

One-way analysis of variance was performed to compare multiple groups. Data acquisition was analyzed with Origin 7.0 software (OriginLab Co., Northampton, Massachusetts, United States). The differences were considered to be significant at a level of $P < 0.05$. All data are presented as average values, with the standard deviation of three experiments indicated (mean ± standard deviation).

RESULTS AND DISCUSSION

Effect of the crosslinking solution pH on EE of loaded BSA

In a previous work,[18] we optimized the physical conditions (e.g., concentrations of sodium alginate and $CaCl_2$ solutions, ratio of sodium alginate to BSA, and gas and liquid flow rates) for the purpose of obtaining small and fine globular-shaped alginate microspheres. It was observed that even under the optimized conditions, the main disadvantage of spraying was the low EE of BSA.

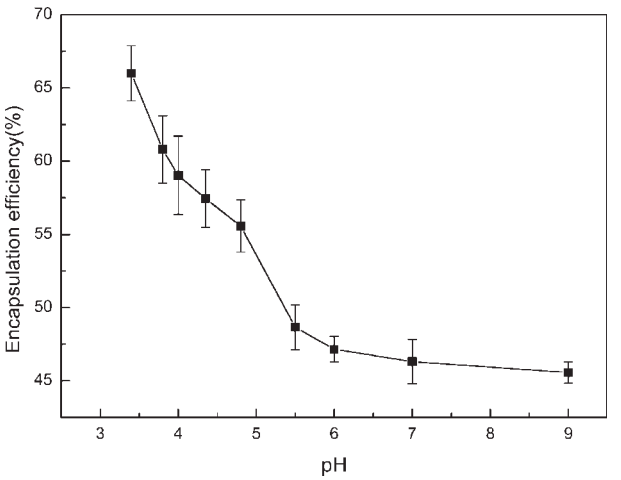

Figure 1 Effect of the pH of the $CaCl_2$ solution on EE of BSA.

In an attempt to reduce protein loss and modify release rates from highly porous alginate microspheres, we favored increasing the electrostatic attraction between BSA and alginate. As shown in Figure 1, EE of BSA was rather low (<50%) at pH 9.2, and the protein retention was almost invariant when the pH decreased to 5.5 ($P > 0.05$). This was reasonably attributed to an electrostatic repulsion between BSA and alginate due to negatively charged BSA when the pH of the $CaCl_2$ solution was higher than the pI (5.0) of BSA. However, when the pH was adjusted below the pI, there was an interaction between the positively charged BSA and the negatively charged alginate chains. Consequently, at pH 3.9, BSA was encapsulated with almost 30% higher efficiency than that in microspheres with electrostatic repulsion prepared by Wheatley et al.[8] and by Chretien and Chaumeil.[19] Although protein retention was further increased at pH values lower than 3.6, alginate was protonated at this pH value,[6] and the complex that formed at pH 3.5 was a white laminar film, not a spheroid. Moreover, the lower pH of the $CaCl_2$ solution might have resulted in the acid-catalyzed degradation of proteins. Consequently, the pH of the $CaCl_2$ solution was fixed at 3.9 in our further studies.

The electrostatic attraction of BSA to alginate was also confirmed by FTIR analysis. Figure 2 shows the FTIR spectra of BSA, sodium alginate, blank alginate microspheres, and microspheres containing BSA prepared at pHs 3.9 and 9. As shown in the spectrum of sodium alginate, the peaks observed at 1618 and 1406 cm^{-1} were assigned to the asymmetric and symmetric stretching peaks of carboxylate

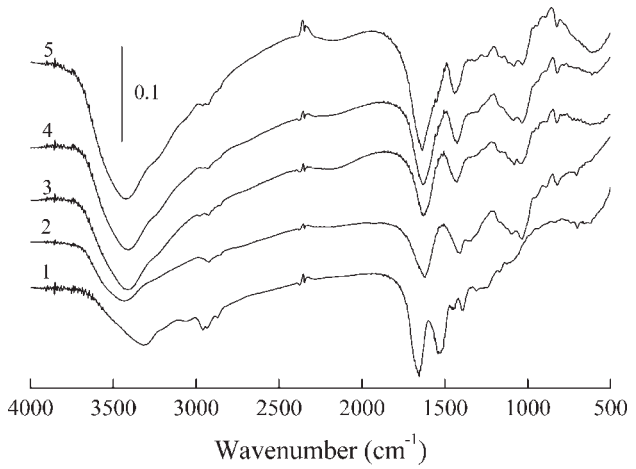

Figure 2 FTIR spectra of (1) pure BSA, (2) pure sodium alginate, (3) blank alginate microspheres, (4) microspheres containing BSA at pH 9, and (5) microspheres containing BSA at pH 3.9.

groups.[10,20] In the spectra of alginate microspheres, $v_{as}(COO^-)$ and $v_s(COO^-)$ were shifted slightly to higher frequencies of 1630 and 1425 cm^{-1}.[21] On the other hand, the bands at 1654 and 1533 cm^{-1} present in the IR spectrum of BSA were the characteristic absorption bands of amide I and amide II, respectively. After the loading into alginate microspheres, the peak intensities of both aforementioned peaks (1654 and 1533 cm^{-1}) decreased dramatically. A broad band appeared between 1750 and 1500 cm^{-1}, which might have been brought about by the interaction of the amino groups of BSA with the carboxyl groups of alginate. Moreover, in the structure of alginate microspheres, because of the crosslinking formation between COO^- groups of alginate and Ca^{2+}, the difference between the asymmetric and symmetric stretching peaks of carboxylate salt groups, $\Delta = [v_{as}(COO^-) - v_s(COO^-)]$, was 205 cm^{-1}, which was less than that of the ionic compound of sodium alginate ($\Delta = 212$ cm^{-1}).[22] After the pH was adjusted to 3.9, the difference further decreased to 197 cm^{-1}, and this indicated that there existed an electrostatic interaction between some carboxyl groups of alginate and BSA. A similar result was observed in the peak intensity ratio of $v_{as}(COO^-)$ to $v_s(COO^-)$. The ratio for blank alginate microspheres was 0.92, and the ratio for microspheres with BSA at pH 9 was 0.89, whereas in the spectrum of microspheres at pH 3.9, that ratio decreased to 0.85. All these results confirmed the presence of an electrostatic interaction between BSA and carboxylic groups of alginate.

Characterization of the alginate microspheres

The effects of the gas flow rate and electric voltage on the properties of alginate microspheres are listed in Table I. In general, both increasing the gas flow rate and applying a high electrostatic field had a marked effect on the decrease in the microsphere size ($P < 0.05$). The population of microspheres prepared by spraying or SEF was close to monodispersity (PDI < 1.90). The value of PDI was significantly reduced by the application of a high electrostatic field to a polymer solution through a spraying setup, and this was similar to the trend obtained by the method of dripping under a high electrical field.[13] The decrease in the diameter caused by an increase in the gas flow rate was due to the huge effects of the air shear force and airflow turbulence, which disrupted the spontaneous formation of drops.[9] The decrease in the diameter caused by a high electrostatic field was attributed to the addition of the electrostatic force to the balance of gravity and surface tension in the process of drop formation.[14]

When the gas flow rate increased from 0.70 to 0.85 m^3/h, although the diameter decreased 17.10%, EE of BSA decreased more seriously ($\sim$ 28.16%; Table I). However, under high electrostatic voltages of 10 and 15 kV, the characteristic feature of the polymer droplets, the mean diameter, decreased 5.58 and 11.65%, respectively. The efficiency of encapsulated BSA separately increased 4.09 and 5.48% ($P < 0.05$). This may be attributed to the morphological and electric charge variations of alginate microspheres obtained with a high electrostatic field. From Figure

TABLE I
Effects of the Preparation Conditions on the Microsphere Properties ($n = 3$)

Conditions				
Gas flow rate (m^3/h)	Voltage (kV)	Mean diameter (μm)	PDI	EE (%)
0.50	0	90.23 ± 1.26	1.86 ± 0.13	64.54 ± 1.39
0.60	0	76.07 ± 1.53	1.69 ± 0.17	61.31 ± 1.58
0.70	0	71.35 ± 0.66	1.60 ± 0.06	62.61 ± 0.69
0.85	0	59.15 ± 1.92	1.43 ± 0.07	45.70 ± 0.90
0.70	10	67.37 ± 0.94	1.38 ± 0.07	65.17 ± 0.67
0.70	15	63.04 ± 0.83	1.33 ± 0.04	66.04 ± 0.39

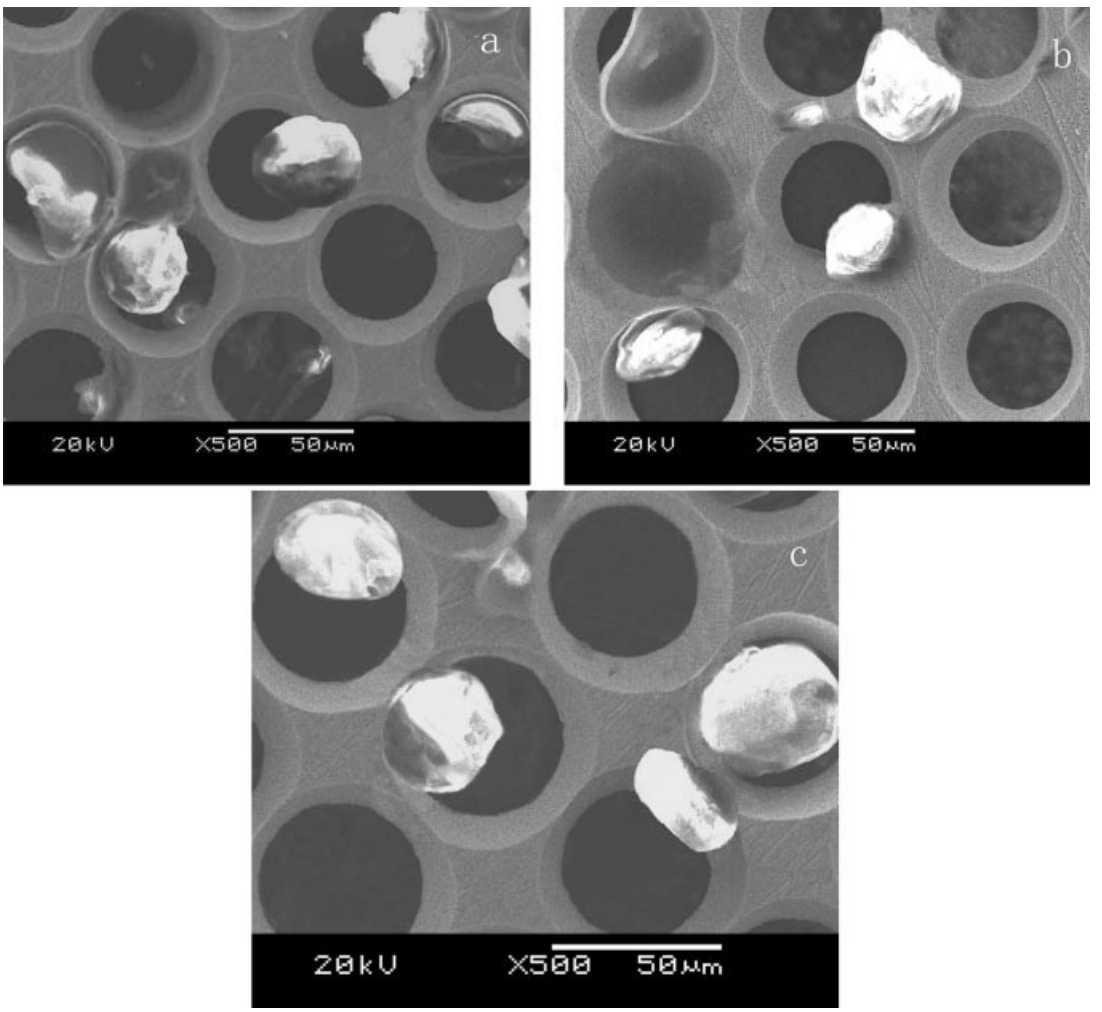

Figure 3 Scanning electron micrographs of alginate microspheres: (a) gas volume = 0.70 m^3/h and $U = 0$ kV, (b) gas volume = 0.85 m^3/h and $U = 0$ kV, and (c) gas volume = 0.70 m^3/h and $U = 15$ kV.

3(a,b), we can see that the fragments and microspheres with smaller diameters increased when the gas flow rate increased from 0.70 to 0.85 m^3/h. Thus, the amount of protein that was availably entrapped in the prepared microspheres obviously decreased. However, no other significant morphological variations could be found. The material used was probably unaltered, and the preparation method was the same. When alginate microspheres were prepared under a high electrostatic voltage, the sphericity of the alginate microspheres with a uniform diameter seemed slightly improved [Fig. 3(c)]. This was probably due to the increase in electric charges induced by the electrostatic field, which created a repulsive outside-directed force against the balance of gravity and surface tension in the process of drop formation. Thus, a mass of electric charges would not only improve the sphericity of microspheres[23] but also increase protein retention adsorbed on the surface of the microspheres during manufacture. These results implied that the application of an electrostatic field to the spraying setup was really efficient in improving the characteristics of alginate microspheres, especially EE of BSA.

Effect of the gas flow rate on BSA release *in vitro*

Comparing the release profiles of BSA from alginate microspheres prepared at gas flow rates of 0.50 [Fig. 4(a)], 0.70 [Fig. 4(b)], and 0.85 m^3/h [Fig. 4(c)], it seems that the released drug content increased with the gas flow rate increasing ($P < 0.05$). For example, 70% of BSA was released into SIF over a 10-h period when the microspheres were prepared at gas flow rates of 0.50 and 0.70 m^3/h; however, alginate microspheres prepared at 0.85 m^3/h exhibited a higher released drug content (ca. 85% in SIF). As shown in Table I and Figure 3, increasing the gas flow rate resulted in a decrease in the microsphere mean diameter. This led to more specific surface area being exposed to the release media and deeper disruption of the calcium alginate matrix. Moreover,

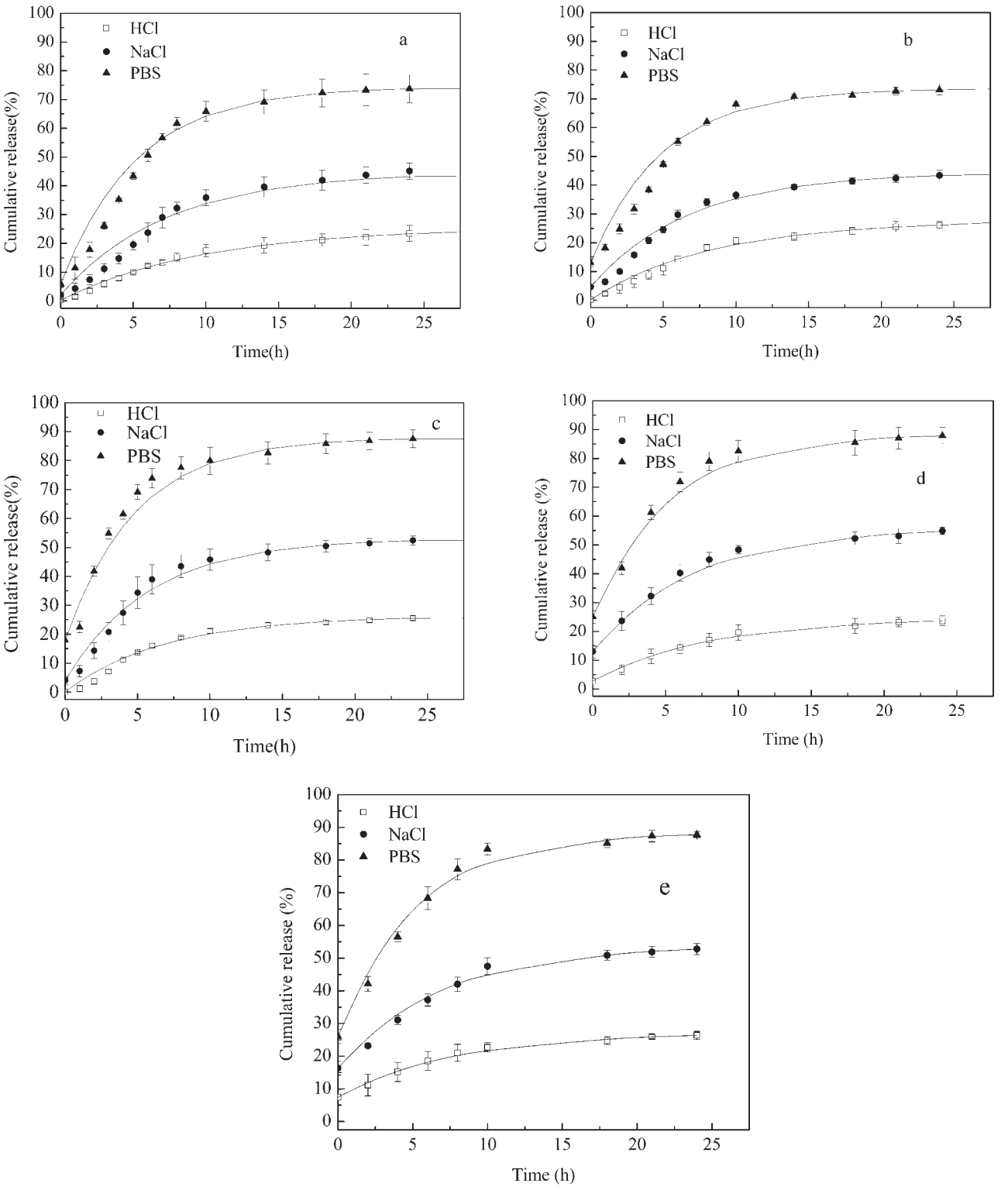

Figure 4 Release profiles of BSA in different media from alginate microspheres under different conditions: (a) gas volume = 0.50 m^3/h and $U = 0$ kV, (b) gas volume = 0.70 m^3/h and $U = 0$ kV, (c) gas volume = 0.85 m^3/h and $U = 0$ kV, (d) gas volume = 0.70 m^3/h and $U = 10$ kV, and (e) gas volume = 0.70 m^3/h and $U = 15$ kV. The points represent experimental data, and the solid curves show the results calculated with eq. (5).

when the gas flow rate increased, the capacity for encapsulating BSA in alginate microspheres decreased because of the increase in the number of fragments and smaller microspheres [Fig. 3(a,b)]. Consequently, as calculated with eq. (2), the initial burst percentage of BSA was more prominent. The

BSA release rate was faster because of the easier drug diffusion and the faster swelling of microspheres with a smaller diameter.

Effect of an electrostatic field on BSA release *in vitro*

Figure 4(d,e) demonstrates that the addition of a high electrostatic field had a significant effect on the initial burst and total release amount of BSA. They were both increased significantly ($P < 0.01$). The addition of a voltage of 10 [Fig. 4(d)] or 15 kV [Fig. 4(e)] increased the content of BSA released into SIF over a 10-h period from 75 (without a high electrostatic field) to 88%. This was also in agreement with previous studies showing a positive relationship between protein retention in microspheres and electric charges induced by the electrostatic field. Microspheres formed with the SEF method had more electric charges,[14,24] and thus higher BSA retention and a higher BSA gradient were found near the microsphere surface, which slightly improved the smoothness of alginate microspheres [Fig. 3(c)]. The BSA content decreased as the center was approached. In the release study, this high surface BSA content induced the significant initial burst and the increase in the total release amount of BSA.

Effect of the release medium on BSA release *in vitro*

As shown in Figure 4, it was evident that the alginate microspheres presented excellent pH-responsive release characteristics. In SGF (pH 1.2), BSA release was very low (<20% of entrapped BSA). No significant swelling of microspheres occurred at this pH value together with insignificant BSA release because of the pH sensitivity of alginate.[4,5,11] Therefore, more proteins would be protected by microspheres from gastric juice when the protein-loaded microspheres were administrated by an oral route.

Calcium ions have been reported to exchange with monovalent nongelling ions, mainly sodium and potassium ions, under physiological conditions.[10,25] This exchange would increase the swelling potential and at the same time reduce the stabilizing forces. Even if the microspheres did not burst, large swelling occurred and thus increased matrix porosity.[26,27] Therefore, the extent of drug release in physiological saline was 20–30% higher than that in SGF.

The release of BSA in SIF was more rapid than that in SGF or in a solution of NaCl ($P < 0.05$), and the release profile was characterized by an initial burst effect (10–20%) followed by a sustained release phase. This may be attributed to the highly porous matrix structure, drug solubility, a greater degree of matrix swelling, and thus erosion in this medium.[11,28] Anal and Stevens[29] found that the disruption of the calcium alginate matrix occurred faster in a phosphate buffer above pH 5.5 because of the chelating action of phosphate ions. At pH 7.4, the affinity of calcium to phosphate was higher than that to alginate, and consequently, BSA was released from alginate microspheres through the slow and continuous erosion of the matrices.

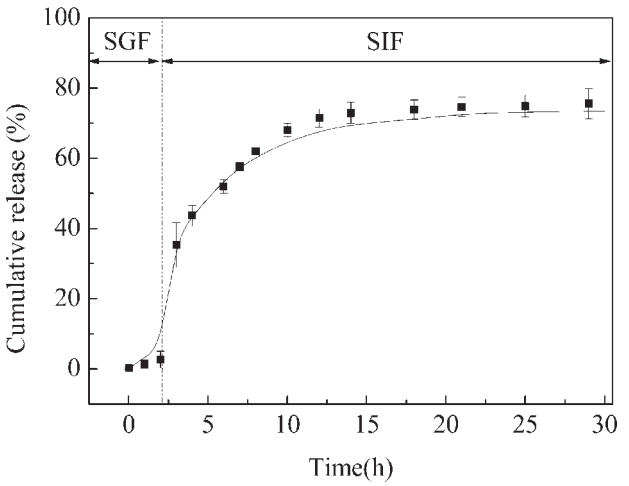

Figure 5 Influence of the usual pH changes on BSA release from alginate microspheres prepared by spraying at 0.70 m^3/h. The points represent experimental data, and the solid curves show the results calculated with eq. (5).

Effect of usual pH changes on BSA release *in vitro*

As shown in Figure 5, the sample in a simulated usual gastrointestinal transit showed a three-phase behavior of BSA release due to the pH-dependent release characteristics mentioned previously. Similar findings were also reported by Pillay and Fassihi.[30] Only about $\sim$ 5% of BSA was released into SGF within 2 h, and this could protect the protein encapsulated in alginate microspheres from destruction in the acidic medium and maximally maintain their activity in oral delivery. During the second burst phase, 30% of the loaded BSA was delivered in 2.5 h. Immediately, the sustained phase arrived, and all the remaining BSA was released because of the disintegration of the microspheres when they were transferred to SIF for 10 h. This three-phase release behavior was suitable for oral delivery of therapeutic proteins in a controlled and sustained manner.

Release kinetics of BSA from microspheres

Mathematical models of release kinetics

For drug release from alginate microspheres, besides diffusion out of the alginate matrix,[3,10] swelling and erosion of microspheres have been described as the major pathways for drug release.[30,31] In general, the

TABLE II
r^2 Values for Linear Relationships of the First Order and the Higuchi Kinetic Equation with Release Data Obtained by Spraying

	r^2					
	HCl		NaCl		PBS	
Gas flow rate (m^3/h)	First order	Higuchi	First order	Higuchi	First order	Higuchi
0.50	0.906	0.931	0.900	0.954	0.847	0.929
0.70	0.914	0.914	0.876	0.926	0.849	0.902
0.85	0.920	0.902	0.922	0.903	0.906	0.850

release kinetics of encapsulated drugs are determined by the fitting of the release data to distinct models, first-order release kinetics [eq. (3)] and the Higuchi matrix model [eq. (4)][32]:

$$Q_t = Q_\infty(1 - e^{-k_1 t}) \quad (3)$$

$$Q_t/Q_\infty = k_H t^{1/2} \quad (4)$$

where Q_t is the amount of drug released at time t, Q_∞ is the total amount of drug in the matrix, k_1 is the first-order kinetic constant, and k_H is the Higuchi rate constant.

However, many investigations have shown that the aforementioned models yield unsatisfactory adjustments if an obviously initial burst release phase exists.[19,32] Recently, the studies of Vueba et al.[33] and Rodriguez et al.[34] led to the conclusion that the first-order kinetic model yields remarkably better adjustments than Higuchi's model when the diffusional exponent value (n) of the Korsmeyer–Peppas model ranges from 0.45 to 0.70. In this range, both drug diffusion and matrix erosion modulate drug release from swellable matrices. Thus, to adjust the initial burst release phase and simulate the whole release phases in continuous gastrointestinal fluid, the first-order release kinetics model is modified, and a linear transformation (logarithms) of eq. (3) is performed by the replacement of the amount of drug released (Q) with the cumulative drug release percentage (y).

Then, the modified first-order release kinetics are represented as follows:

$$\ln(y_\infty{}' - y_0) = \ln(y_\infty{}' - y_t) + k(t - t_1) \quad (5)$$

where $y_\infty{}'$ denotes the theoretical terminal release percentage of the drug from the microspheres, y_0 denotes the initial drug release percentage, y_t denotes the release percentage in time t, t_1 is the initial time lag, and k is the release kinetics constant.

Fitting the data to the model

To better understand the drug release mechanism for the microsphere system studied, the n values in the Korsmeyer–Peppas model were obtained through the fitting of the data in Figure 4. The values varied from 0.451 to 0.625, indicating that the release mechanism of BSA from these matrices was modulated by both drug diffusion and matrix erosion.

Accordingly, the description of the release profiles by a model function was attempted with eqs. (3) and (4). However, the release profiles of BSA from alginate microspheres could not be simulated by the models properly because the correlation coefficient (r^2) values were not close to 1 ($r^2 < 0.95$; Table II). However, r^2 increased when the initial data were excluded. Similar results were found by Chretien and Chaumeil[19]: a serious deviated effect occurred because of an initial burst phase.

TABLE III
k and r^2 Values for the Linear Relationship of eq. (5) with Release Data Obtained by the Spraying and SEF Methods

Conditions		HCl		NaCl		PBS	
Gas flow rate (m^3/h)	Voltage (kV)	k	r^2	k	r^2	k	r^2
0.50	0	0.101	0.996	0.122	0.991	0.191	0.990
0.70	0	0.121	0.994	0.148	0.992	0.203	0.988
0.85	0	0.145	0.993	0.172	0.992	0.206	0.987
0.70	10	0.127	0.994	0.146	0.992	0.196	0.989
0.70	15	0.132	0.993	0.148	0.991	0.198	0.990

Then, the BSA release characteristics of alginate microspheres prepared at different gas flow rates were calculated with eq. (5) (Table III). As shown in Figure 4 and Table III, all the calculated release data derived from eq. (5) appeared to correlate well with experimental observations ($r^2 > 0.98$). There was a positive correlation between the value of k and the gas flow rate (with the highest value for alginate microspheres prepared at 0.85 m^3/h), despite the infection of incubated media. This result further confirmed that the drug release rate in the determined medium was in an inverse ratio to the diameter of the obtained microspheres.

For the alginate microspheres incubated at pH 1.2, the release profiles fitted eq. (5) well because of the simple diffusion of BSA from the almost unchanged matrix structure. However, for alginate microspheres incubated in NaCl and SIF, an erosion of alginate microspheres led to an increase in k values and a slight deflection of calculated results to experimental values.

From the analysis of the k values obtained from the samples prepared at different voltages with the same gas flow rate, it can be seen that the k values were apparently higher in SGF than those calculated at $U = 0$; this also suggests that the drug release rate was in an inverse ratio to the diameter of the microspheres. However, the increase in the k values was inconspicuous in NaCl or in SIF. This difference might be attributed to the electric charges induced by the electrostatic field. Although the diameter of the microspheres was decreased by the application of an electrostatic field, there was an increase in the surface crosslink density aroused by opposite electric charges. As a result, these microspheres slightly withstood the erosion of release media to the alginate matrix and controlled the release of the encapsulated drug to some extent. Additionally, it is reported in Table I that the PDI values decreased and were near monodispersity when the microspheres were prepared by SEF. Thus, the release kinetics behavior of microspheres obtained by SEF was more concurrent than that obtained from the simples prepared by the simple spraying method ($r^2_{SEF} > r^2_{spraying}$).

At last, the fitting curve of BSA released from alginate microspheres incubated with continuous pH changes was also determined by eq. (5), and this was performed with the parameter values (k, y_∞', and y_0') obtained previously. As shown in Figure 5, the modified first-order release kinetics equation fit the release data well, and r^2 approached 1 ($r^2 = 0.998$) in a simulated gastrointestinal fluid. Thus, the modified first-order release kinetics equation best described the release behaviors when both the diffusion of entrapped drugs through a porous structure and an extent of anomalous erosion caused by the considerable swelling and disruption of the alginate matrix were considered.

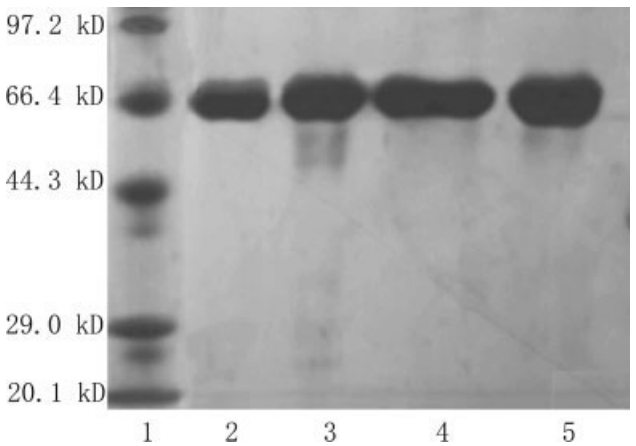

Figure 6 Electrophoretic patterns of BSA released from alginate microspheres prepared by SEF (gas volume = 0.70 m^3/h and $U = 15$ kV). Lanes represent (1) the molecular weight marker, (2) native BSA, (3) BSA released from alginate microspheres in HCl at 10 h, (4) BSA released from alginate microspheres in PBS at 24 h, and (5) BSA released from alginate microspheres in SGF for 2 h and then transferred to SIF for another 22 h.

Structural integrity of BSA released from the microspheres

It is well known that the structure and stability of proteins are easily denatured by harsh conditions, such as the air shear force during preparation and acidolysis in the release medium. These potential destructive effects are the main challenges for the application of the spraying method to protein encapsulation. In our previous study, it was found that the encapsulation process of spraying had no effect on the molecular structure and antigenicity of BSA by Western blotting and circular dichroism spectra, respectively.[15] Here, the effect of an electrostatic field on the structure integrity is still unknown and should be further studied.

SDS–PAGE of BSA prepared by SEF is presented in Figure 6. There were no additional peaks of high-molecular-weight BSA and low-molecular-weight BSA for all the samples investigated. This finding indicates that the protein was entrapped virtually within the alginate microspheres to prevent its direct contact with the harsh conditions, and this was helpful in preserving the structural integrity and activity of the protein.

CONCLUSIONS

In this study, alginate microspheres were prepared via the spraying of an alginate–BSA mixture into a $CaCl_2$ solution. Both the size of the obtained microspheres and EE of BSA decreased with increasing

gas flow rates. In a gelation medium with a low pH, high protein retention in manufacturing could be obtained by interaction with negatively charged alginate. Although the diameter and size distribution of the microspheres obtained by SEF significantly decreased, EE of BSA evidently increased. An *in vitro* release analysis demonstrated that these alginate microspheres had an excellent pH-responsive property, which could protect protein drugs by avoiding the acidity in gastric fluid and releasing all remaining drugs when they were transferred to intestinal juice. A modified first-order kinetic equation was postulated and well described the release kinetics of BSA in SGF or SIF with an evidently initial burst release. The drug release rate increased with increasing gas flow rates or under a high electrostatic field. The delivery system prepared by the spraying and SEF methods for intestinal digestive enzymes and cytokines shows some advantages in improving the protein loading efficiency, achieving a controlled\sustained release profile, and preserving the structural integrity and activity of the encapsulated protein.

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