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Optimization and characterization of chitosan-coated alginate microcapsules containing albumin

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In order to obtain small microcapsules with high protein encapsulation efficiency and extended release characteristics various processing factors were studied. Bovine serum albumin-loaded alginate microcapsules were prepared by an emulsion method and further incubated in chitosan. Many process factors were tested including the concentration and molecular weight of alginate, the concentration and pH of chitosan, and surfactants, etc. Microcapsules were achieved with diameters less than 2 μm, high encapsulation efficiency (> 80%) and high loading rate (> 10% w/w). The results also showed that the initial BSA amount of 20% ~ 30% loaded alginate microcapsules coated with 0.2% ~ 0.5% chitosan solutions at pH 4 by the two-stage procedure present the best sustained releasing characteristics.

1. Introduction

Natural hydrophilic polymers as drug carriers have received much attention for several years, especially from the viewpoint of cost, environmental pollution and safety. Among them, polysaccharides such as alginates and chitosans are suitable candidates for sustained or controlled drug release due to their excellent biocompatibility and biodegradability. The alginate gel has been shown to protect proteins from hostile environments by cross-linking with divalent or trivalent metallic ions (Kikuchi et al. 1999), but its porous structure leads to an undesired fast release and unstable state (Polk et al. 1994). A membrane coat, such as the one formed by the electrostatic interaction of the alginate carboxyl groups with a polycationic amine coat of chitosan may be applied to increase stability and to reduce permeability (Ribeiro et al. 1999). Chitosan is the N-deacetylated product of chitin, a naturally occurring polymer which has been used extensively to prepare microcapsules for oral and intra-nasal delivery. Smooth and uniform chitosan coatings were confirmed both by optical microscopic photographs and the transmission electron microscopic photographs (Tamura et al. 2002). Moreover, the addition of chitosan increases the mechanical strength of the alginate microparticles (Ribeiro et al. 1999; Serp et al. 2000).

However, the sizes of alginate microcapsules are often relatively large, usually ranging from 150 μm to 800 μm (Filipović-Grčić et al. 1995; Ribeiro et al. 1999; Mi et al. 2002). An emulsification technique has been described, by which the mean diameter of alginate microcapsules was decreased from 150 μm (Wan et al. 1992) to 8 μm (Lemoine et al. 1998) and further to about 5 μm (Cho et al. 1998). Since only a few preparation methods and release characteristics of small alginate microspheres have been described in the literature, the purpose of this study was

Table 1: Effect of different formulation factors and preparation parameters on the morphology and size of alginate microcapsules

Factor		Size	Morphology
Alg concentration	5% (180 mps)	< 11 μm, homogeneous	nonaggregated
	2.5% (29 mps)	< 6 μm, homogeneous	nonaggregated
	1% (6 mps)	< 3 μm, homogeneous	nonaggregated
	0.5% (−)	< 2 μm, homogeneous	aggregated
Alg (1%)	high viscosity (37 mps)	> 3 μm, inhomogeneous	nonaggregated
	low viscosity (6 mps)	< 3 μm, homogeneous	nonaggregated
Lipophilic surfactant	5.0% span 80	< 11 μm, homogeneous	nonaggregated
	2.5% span 80	1–15 μm, homogeneous	aggregated
	5.0% span 85	8–85 μm, inhomogeneous	aggregated
Hydrophilic surfactant	30% tween 80	< 11 μm, homogeneous	nonaggregated
	20% tween 80	< 11 μm, inhomogeneous	aggregated
Tween 80/Span 80 (w/w)	3 : 10 ~ 1 : 2	< 2 μm, homogeneous	nonaggregated
	< 3 : 10	< 2 μm, homogeneous	aggregated

Table 2: Mean diameters of chitosan-coated, uncoated alginate microcapsules and the dried chitosan-coated alginate microcapsules

Microcapsules type	Mean diameter
Uncoated	1.85 ± 0.37 μm
Chitosan-coated	1.74 ± 0.45 μm
After being lyophilized	1.15 ± 0.21 μm

to optimize the process and formulation variables for preparing high protein load microcapsules with small size, high encapsulation efficiency and sustained release.

2. Investigations and results

2.1. Effects of formulation factors and process parameters on morphology of microcapsule

The effects of the various preparation factors on the morphology of the microcapsules are shown in Table 1.

The average diameter of the microcapsules was reduced when the alginate concentration decreased. Low viscosity alginate resulted in much smaller microcapsules than high viscosity alginate.

Small, smooth and homogeneous microcapsules were obtained only with a stirring rate between 10000 rpm and 18000 rpm and an addition rate of $CaCl_2$ of less than $4\ ml \cdot min^{-1}$. Therefore, 12000 rpm stirring rate, $3\ ml \cdot min^{-1}$ addition rate of $CaCl_2$ and 1% aqueous solution of low viscosity alginate were used in the further preparations.

Table 2 shows that the mean diameters of alginate microcapsules and those coated with chitosan were all about 1.8 μm, and decreased to 1.15 μm after being lyophilized.

Chitosan-coated alginate microcapsule had a spherical and smooth morphology. SEM micrographs of the dried alginate-chitosan microcapsules prepared with 1.0% (w/v) chitosan and 1.0% alginate are shown in Fig. 1.

2.2. BSA entrapment efficiency and loading rate in alginate-chitosan microcapsules

The encapsulation efficiencies in the uncoated microcapsules washed with either isopropyl alcohol alone or together with water were determined. The encapsulation efficiency in the water-washed microcapsules was only $18.8\% \pm 0.1\%$, sharply lower than $99.95\% \pm 0.06\%$ in the microcapsules washed only with isopropyl alcohol. The yield of microcapsules was reduced to almost half after being washed with water (Fig. 2). So only isopropyl alcohol was used in the washing step in the preparation of chitosan-coated microcapsules.

To assess the effect of chitosan concentrations on the encapsulation of proteins, 0.1%, 0.2%, 0.4% and 0.5% chitosan solutions were used to coat the alginate microcapsules. The resulted encapsulation efficiency is shown in Fig. 3. The effect of the added BSA amount on encapsulation and loading efficiency was also studied (Fig. 4). The BSA loading amount was proportional to the ratio of the

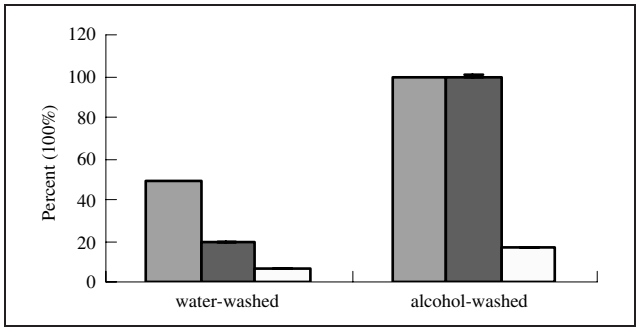

Fig. 2: The encapsulation yield ■, encapsulation efficiency ■, and loading rate □ of alginate microcapsules washed by water and isopropyl alcohol

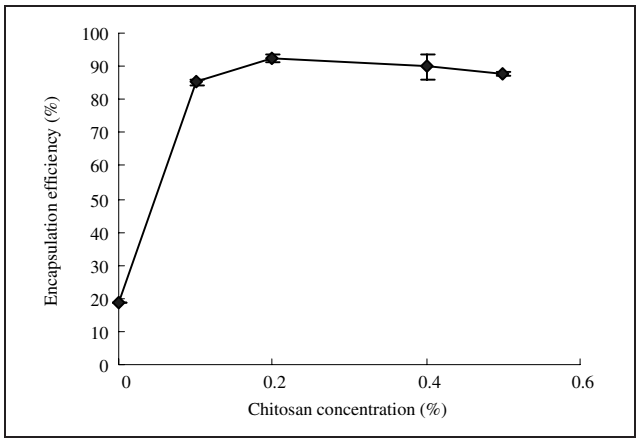

Fig. 3: Encapsulation efficiency of alginate microcapsules coated with different concentration of chitosan (0%, 0.1%, 0.2%, 0.4% and 0.5%)

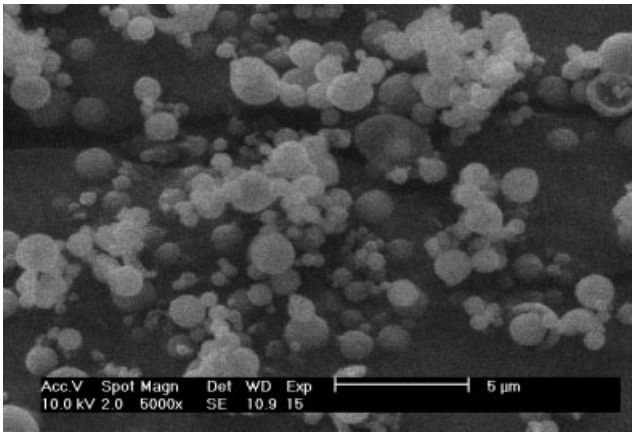

Fig. 1: Electron microscopic photographs of the dried alginate-chitosan microcapsules prepared with 1% low viscosity alginate and 1% chitosan

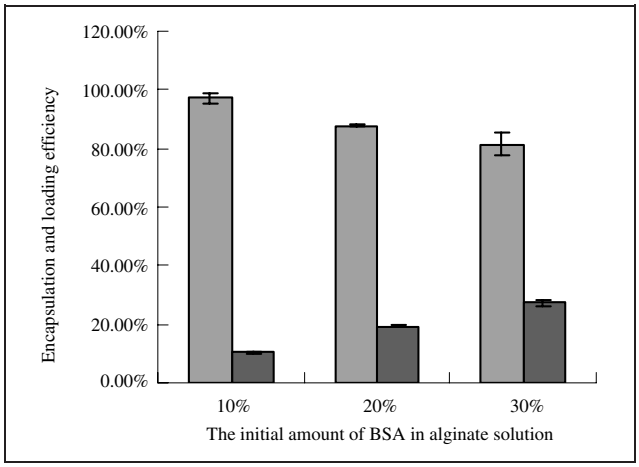

Fig. 4: Effect of the ratio of BSA: alginate on the encapsulation efficiency ■ and loading rate ■ of microcapsules coated with 0.5% chitosan

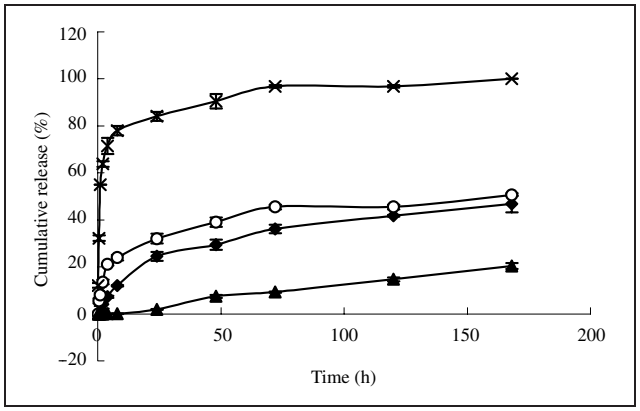

Fig. 5: Release profile of BSA in distilled water –○–, PBS (0.05M) –×–, saline –■–, and Tris-HCl (0.05M) –▲– from alginate-chitosan microcapsules made from 2% alginate and 1% chitosan at pH 4

added BSA to alginate up to 1 : 10. When the ratio further increased from 1 : 10 to 2 : 10 and 3 : 10, the encapsulation efficiency was slightly reduced from 97.2% ± 1.6% to 87.8% ± 0.4% and further to 81.4% ± 3.9%.

2.3. Release of BSA from alginate-chitosan microcapsules

The BSA release profiles from the microcapsules were studied in four different media as described in the Experimental. The cumulative release of BSA from alginate-chitosan microcapsules in PBS was 96.8% ± 0.6%, only 45.5% ± 0.9% in saline, 36.2% ± 1.8% in water and 9.3% ± 0.3% in Tris-HCl, respectively. The data are presented in Fig. 5. Therefore, the further release studies were carried out in saline containing 0.01% NaN_3.

The burst release from alcohol-washed alginate microcapsules was more obvious than that from the water-washed in the first 12 h, but their rapid release profiles were similar (Fig. 6).

The release rates from the microcapsules coated with various chitosan concentrations were also evaluated (Fig. 7). The cumulative release rate of BSA from 0.1% chitosan-coated alginate microcapsules was slightly delayed, 70.5% at 2 h and 93.2% at 48 h, compared with the release rate from the uncoated alginate microcapsules, 90.6% at 2 h and 99.5% at 48 h, respectively. When the chitosan concentration was elevated to 0.5% from 0.1%, the release rate of protein was greatly decreased, whereas no further release retardation effect was seen after the chitosan concentration increased to 1.0%. In addition, the influence of the chitosan solution's acidity on the release profile

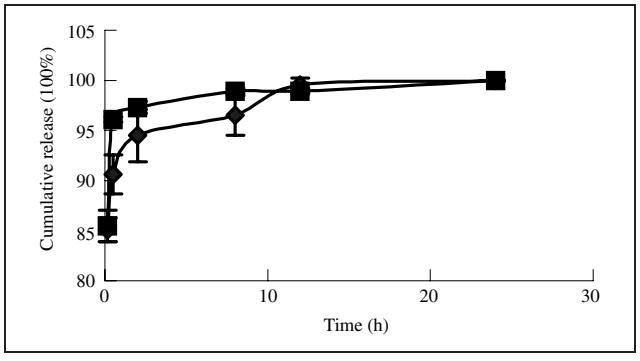

Fig. 6: Release profiles of BSA from alginate microcapsules washed with water –◆– and washed with isopropyl alcohol –■–

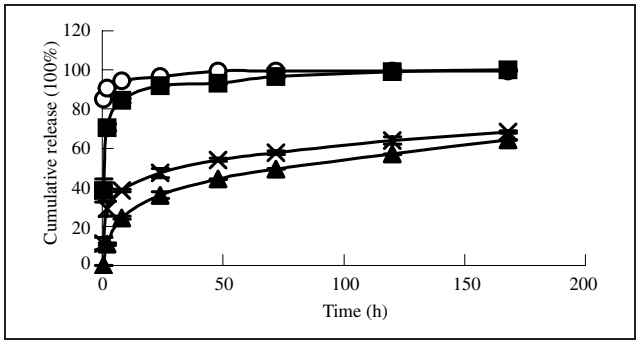

Fig. 7: Release profile of microcapsules prepared with BSA:alginate (2 : 10), 1% alginate and different concentrations of chitosan in the saline solution
–○– 0% chitosan, –■– 0.1% chitosan, –▲– 0.5% chitosan, –×– 1.0% chitosan

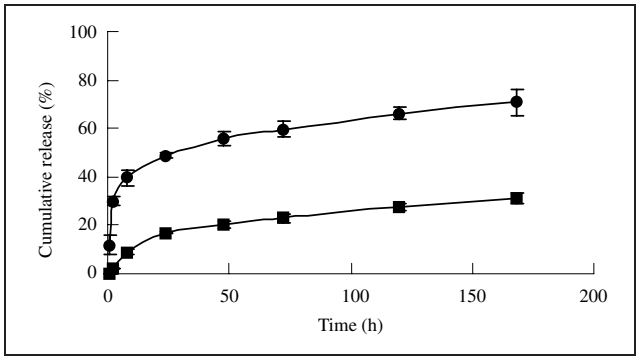

Fig. 8: BSA releasing from alginate-chitosan microcapsules prepared with different pH values of chitosan solution. 1% (w/w) Alginate and 1% (w/w) chitosan were selected
–■– pH = 4, –●– pH = 6

(Fig. 8) was evaluated. The protein release rate of the microcapsules coated with pH 6 chitosan solution was higher than that seen from the pH 4 chitosan solution (48.9% ± 0.9% vs. 17% ± 0.4% at 24 h, $p < 0.001$, 70.7% ± 5.7% vs. 31.2% ± 1.9% at 168 h, $p < 0.05$), while the encapsulation efficiency showed no difference from each other.

3. Discussion

In this study, uniform alginate microcapsules of less than 2 μm in diameter were prepared by a w/o emulsification method with BSA as a model protein. As expected, lower alginate concentration resulted in smaller microcapsule size and higher degree of aggregation. The mean diameter of microcapsules was gradually dropped as the alginate concentration was decreased from 5% to 0.5%. Increasing the alginate molecular weight may extend the total amount of cross-linking between the guluronic acid units and the calcium (Lemoine et al. 1998). Many factors seemed to be important in forming small and uniform microcapsules. We obtained much smaller microcapsules than Lemoine et al. (1998) when using Span 80 and Tween 80 as the surfactants, about 12000 rpm stirring rate and less than 3 ml · min^{-1} $CaCl_2$ addition rate even though the alginate concentrations were the same. In addition, slightly increasing the amount of Tween® 80 seemed to decrease the aggregation of the microcapsules.

Because of the low drug content in water-washed alginate microcapsules, isopropyl alcohol was used to wash the

microparticles and the entrapment efficiency was elevated to 5 times, approaching nearly 100% because of the insolubility of BSA in isopropyl alcohol. Maybe for this reason, the burst release from the alcohol-washed microcapsules was more visible than that from the water-washed in the first 12 h. However their total rapid release profiles were similar. It is necessary to retard the release profile by coating the alginate microcapsules with chitosan.

The electrostatic interactions of carboxyl groups of alginate with the amine groups of chitosan form a membrane to double protect the model protein (BSA) from the biological surroundings. DeGroot and Neufeld (2001) reported that rehydrated uncoated and chitosan-coated alginate beads retained 71% and 89% of the activity, following 10 min exposure to the enzyme.

The decreased size after lyophilization can be easily understood. Lyophilization dehydrates the microcapsules causing them to shrink, thus decreasing the microcapsules and the pore size.

To evaluate the release characteristics of the chitosan-coated alginate microcapsules, the determination method of encapsulation efficiency should be established first. To our surprise, in our experiment PBS was a more effective extraction medium than sodium citrate, which was usually used to liquefy the calcium alginate microspheres and retrieve the drug encapsulated in alginate or chitosan-alginate microparticles. This phenomenon may be explained by the cooperative effect of both sodium and phosphate ions in PBS. Calcium ions in the microcapsules are replaced by sodium ions in PBS and then combined with phosphate ions, another ion in PBS, to form the insoluble $Ca_3(PO_4)_2$. In recent years, more and more scientists have selected PBS as a medium to determine drug loading (Liu et al. 1997; Mi et al. 2002; Lucinda-Silva and Evangelista 2003; Lee et al. 2003).

Then the release media were selected. Obviously, BSA may release rapidly in PBS for the same reason for it is suitable for the entrapment efficiency determination. Tris-HCl is an organic compound, which is not a proper medium as it makes protein release too slow. The saline solution contains sodium. So the release rate of BSA in it was a little higher than that in water. Since the saline solution is also similar to physiological conditions, it was chosen as release medium for further studies.

In saline, the highly porous structure of alginate microcapsules made the release of the encapsulated BSA fast. In order to delay the protein release, the microcapsules were coated chitosan solutions of various concentsations. The enhancement of tensile strength was achieved by adjusting the pH of the coating solution (Tamura et al. 2002). The release profiles of alginate-chitosan microcapsules appeared highly dependent on the concentration of chitosan used for coating. The microcapsules prepared with 0.1% chitosan did have significantly elevated encapsulation efficiency, but still showed greater burst release of BSA than those prepared by 0.5% or 1% chitosan solutions. Since the complex layer of alginate-chitosan microcapsules was formed by the electrostatic interaction between the amine group of chitosan and the carboxyl groups of alginate, this was mainly because the concentration of chitosan was not high enough to interact thoroughly with alginate so that only a thin membrane was formed. There was no big difference between those coated with 0.5% and 1% chitosan solutions, because the concentration of 0.5% was high enough to make strong membrane.

Though the release of protein from alginate microcapsules could be inhibited by chitosan coating, the release rate did not constantly present the inverse ratio to the chitosan concentration. In contrast to a previous report (Polk et al. 1994) that the controlled ability of chitosan weakened when the concentration increased to 0.2% from 0.1%, our results indicated that the shift was at the higher concentration from 0.5% to 1.0%. Perhaps the used volume of chitosan solution or the total amount of chitosan of the same concentration was different.

Alginate microcapsules demonstrated a low protein release rate after chitosan coating, and the release rate was less for microcapsules prepared with the lower pH chitosan solution (pH = 4) than those prepared with the higher pH chitosan solution (pH = 6). The pKa of chitosan and alginate are 6.3 and 3.5, respectively. At higher pH, chitosan has less free amine groups due to the increased deprotonation. As the result, the extent of the interaction between alginate and chitosan is reduced, and the alginate-chitosan complex layer becomes less dense.

4. Experimental

4.1. Materials

Chitosan (≥80% deacetylation, M.W. 80000) was obtained from Yuhuan Oceanic Biochemistry Co. Ltd. (China). Low viscosity sodium alginate (6 mps and 24 mps for 1% and 2% aqueous solutions at 25 °C, respectively) and high viscosity sodium alginate (37 mps and 880 mps for 1% and 2% aqueous solutions at 25 °C, respectively) were purchased from China Medicinal Group Shanghai Chemical Agent Co. (China). Bovine serum albumin (BSA) was bought from Sino-American Biotechnology Co. Bicinchoninic acid (BCA) protein assay kits were supplied by Beyotime Co. (China). Sorbitan trioleate (Span 80), polyoxyethylene sorbitan trioleate (Tween 80), iso-octane, isopropyl alcohol, calcium chloride, and all other reagents were of analytical grade supplied by Huadong Medical Company.

4.2. Preparation of alginate microcapsules

The preparation method of alginate microcapsules was adopted from an emulsification method described by Lemoine et al. (1998). In order to prepare much smaller microcapsules, the types of surfactant and some procedures were modified. Sodium alginate aqueous solutions in the concentration range of $10-50\ g \cdot L^{-1}$ were prepared by dissolving the required amount of sodium alginate in 100 ml of deionized water. BSA was dissolved in the alginate solution at a BSA/alginate ratio of 1 : 10, 2 : 10 and 3 : 10 respectively. Span® 80, a lipophilic surfactant was dispersed in iso-octane at the concentration of 5% (w/v). And then 40 ml of the oil phase (iso-octane) was poured into 20 ml of the alginate aqueous solution. The mixture was emulsified for 3 min using a mechanical stirrer (FJ-200 homogenizer, Shanghai Biaoben Model Factory, China) at 10000 rpm ~ 18000 rpm. 2.0 ~ 3.3 ml of Tween® 80 aqueous solution (30%, w/w) was added as the second emulsifier to provide the necessary HLB value and the mixture was further stirred at the same speed for 3 min. Then 8 ml of calcium chloride solution (8%, w/v) was added dropwise. The distance from the dropper tip to the liquid surface was about 4 cm and the addition rate of $CaCl_2$ was less than $4\ ml \cdot min^{-1}$. The cross-linking process lasted for 3 min. Then 40 ml of isopropyl alcohol was added to harden the solidified microcapsules and to separate the microcapsules from the organic phase. The preparation was stirred for another 3 min. Then the alginate microcapsules were collected by centrifugation and washed twice with isopropyl alcohol. Half of the microcapsules were air-dried, and the rest washed twice by distilled water and then lyophilized.

4.3. Preparation of chitosan-coated alginate microcapsules

The chitosan powder was dissolved in 1% (v/v) aqueous acetic acid solution to prepare $1\ g \cdot L^{-1}$ and $10\ g \cdot L^{-1}$ solutions. The pH of chitosan solutions was adjusted to 4.0 or 6.0 by dropwise addition of 4% NaOH aqueous solution. The alginate microcapsules prepared according to the above mentioned procedures were dispersed into the different chitosan solutions with various pH values. Then the mixture was shaken gently for 30 min to form the alginate-chitosan complex membrane. The microcapsules were centrifuged and collected, then washed once in the same chitosan solution, and twice in distilled water, and finally lyophilized.

4.4. Morphology observation

Morphology and size of the microcapsules were studied with an optical microscope, and a scanning electron microscope (SEM, XL30, PHILIPS). The size and distribution of the microcapsules were determined with a particle size analyzer (Zetasizer 3000 HAS, Malvern Instrument, England).

4.5. Determination of BSA content in the microcapsules

The encapsulation efficiency of BSA was determined by an extraction method. The liquefaction and extraction efficiency were compared among citrate sodium solution, PBS and their mixtures. Briefly, the microcapsules were dispersed in 3 ml of the extract solution and incubated in a shaking water bath at 37 °C, 100 rpm for 9 h. Then the sample was centrifuged at 2000 rpm to remove the non-soluble residuals and the supernatant was collected. This extraction step was repeated 3 times. The protein concentration in the combined extraction was determined using a BCA protein assay kit (Beyotime Biotechnology, China) at a wavelength of 570 nm. From these results, the percentage (w/w) of BSA entrapped per dry weight of the microcapsules (BSA loading) and the encapsulation efficiency (actual BSA content /theoretical BSA content) were calculated.

4.6. In vitro release study of BSA from alginate-chitosan microcapsules

Since the *in vitro* release of BSA from the alginate-chitosan microcapsules was closely related to the types and concentrations of ions contained in the media, the release study was carried out in four different solutions including saline, PBS (pH 7.4, 0.05 mol $\cdot$ L^{-1}), Tris-HCl buffer solution (pH 7.4, 0.05 mol $\cdot$ L^{-1}) and distilled water, each containing 0.01% sodium azide as the preservative.

For the *in vitro* release, accurately weighted amounts (about 30 mg) of alginate microcapsules were placed in Eppendorf tubes containing 3 ml of the release medium. The tubes were then incubated at 37 °C under shaking. At pre-determined time intervals, the tubes were centrifuged. 0.5 ml of the supernatant was removed and 0.5 ml fresh solution was added. The BSA concentration was determined by the BCA assay.

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