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Immobilization and bioactivity of glucose oxidase in hydrogel microspheres formulated by an emulsification–internal gelation–adsorption–polyelectrolyte coating method

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Abstract

The purpose of this study was to develop a novel microsphere formulation of glucose oxidase (GOX) with high drug loading, encapsulation efficiency and bioactivity. GOX was encapsulated in alginate/chitosan microspheres (ACMS) using an emulsification–internal gelation, followed by GOX adsorption and polyelectrolyte coating method. The factors influencing GOX loading, encapsulation efficiency and activity of the loaded GOX were investigated. The resultant ACMS in wet state were spherical with a mean diameter of about 138 μm. GOX loading was found to be pH dependent. High GOX loading and encapsulation efficiency were achieved when the pH of the adsorption medium was lower than the isoelectric point (p*I*) of GOX. GOX loading and encapsulation efficiency increased with increasing GOX concentration in the loading solution, but decreased with increasing chitosan concentration in the coating solution. The activity of loaded GOX increased and then decreased with increasing chitosan concentration. The activity of GOX in ACMS was maintained and showed sustained production of H_2O_2 as compared to free GOX. Around 90% of the original activity of immobilized GOX remained after lyophilization and storage at −20 °C for a month. These results suggest that the ACMS and the fabrication method are suitable for microencapsulation of proteins like GOX.

Keywords: Alginate; Chitosan; Microspheres; Glucose oxidase; Enzymatic activity

1. Introduction

In recent years, proteins and enzymes become promising therapeutics as a result of advances in biotechnology and genetic engineering. More than 300 protein-based drugs have been approved for marketing, are in human clinical trials or are under review by regulatory agencies. However, their acidic and/or enzymatic degradation in the gastrointestinal tract after oral administration or their short circulation time and biological half-life after intravenous injection place extreme limits on their application. Various kinds of protein formulation to deal with these limitations have been reported, including hydrogels, nano/microspheres and lipid-based systems such as liposomes, solid lipid nanoparticles and water-in-oil (W/O) emulsions (Gombotz and Pettit, 1995; Alcon et al., 2005; Schubert and Muller-Goymann, 2005). Of various preparation methods, encapsulation using polymers is the most widely used protein formulation technology aimed at maintaining the activity and prolonging the circulation time of protein-based drugs. Proteins immobilized in polymeric microparticles have the advantages of improved biological and thermal stability, pH suitability, extended therapeutic effect and possibility for targeted delivery.

In the polymeric protein delivery systems, the entrapment efficiency and retention of protein activity are largely dependent on the polymer and reagents used, the type of immobilization technique, and other process variables (Gombotz and Pettit,

Abbreviations: ACMS, alginate/chitosan microspheres; ACMS-GOX, encapsulated glucose oxidase in microspheres; ACMS-16, 16 mU/ml encapsulated glucose oxidase in microspheres; CaAlg, calcium alginate; DDI, distilled and deionized; FR-GOX, free glucose oxidase; FR-16, 16 mU/ml free glucose oxidase; G, α-L-guluronic acid; GOX, glucose oxidase; KPS, potassium persulfate; M, β-D-mannuronic acid; p*I*, isoelectric point; PLGA, poly(D,L-lactic-co-glycolic acid); POD, peroxidase; SEM, scanning electron microscope; TGF-β_1, transforming growth factor-β_1; W/O, water-in-oil

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1995; Sinha and Trehan, 2003). Normally high shear forces, organic solvents and/or elevated temperatures are employed for immobilization of proteins. For the preparation of the most popular protein delivery system, i.e., poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres, an organic solvent, such as methylene chloride, is used to dissolve PLGA and form a water-in-oil-in-water (W/O/W) double emulsion. The presence of a water/organic solvent interface was believed to be responsible for the low encapsulation efficiency, inactivation and aggregation of proteins (van de Weert et al., 2000; Pérez-Rodriguez et al., 2003). In contrast, hydrogel systems, with a relatively high water content and soft consistency that is similar to natural tissue, are more compatible with proteins, which make such system more desirable for protein delivery (Peppas et al., 2000).

Alginate, as a hydrogel, has been well studied and is one of the most popular materials for cell encapsulation owing to its high biocompatibility (Lim and Sun, 1980; Hisano et al., 1998). In addition, the unique properties of alginate make it an ideal material for protein delivery. These properties include a mild hydrogel formation condition at room temperature without use of an organic solvent, the relatively inert aqueous environment inside the gel, a controllable gel porosity by coating with polycationic polymers, and degradability under physiological conditions. Alginate has been studied for sustained release or immobilization of bioactive agents such as enzymes (Hearn and Neufeld, 2000), vaccine (Bowersock et al., 1999), insulin (Hari et al., 1996) and cytokines (Mumper et al., 1994; Gu et al., 2004). The studies indicate that the encapsulation efficiency, protein stability and enzymatic activity are dependent on the properties of protein loaded, e.g. isoelectric point (p*I*), molecular weight and other experimental variables.

Alginate is a polysaccharide obtained from brown algae and is composed of 1,4-linked-β-D-mannuronic acid (M) and α-L-guluronic acid (G). The pK_a values of M and G are 3.38 and 3.20, respectively (Martinsen et al., 1992). Encapsulation using alginate is most often performed by extruding alginate solution together with encapsulant from a syringe into a gelling bath containing calcium chloride. Calcium alginate (CaAlg) gel beads are formed immediately upon contact of alginate solution and calcium ions by the formation of an ionic complex between G residues of alginate and calcium ions. The gelation starts from outer surface and proceeds towards the center. Thus this method is called an external gelation method. This method may encounter problems when the production of large amounts of CaAlg gel beads is attempted. Additionally, the diameter of CaAlg gel beads is normally in the range of 400 μm–1 mm or greater, which is too large for many clinical applications via injection. Compared with the external gelation method, the internal gelation method through emulsification provides a method that is easily scaled up and can result in a particle size as small as 50 μm (Poncelet et al., 1992). In this method, insoluble calcium carbonate is added to an alginate solution. This mixture is added to a vegetable oil containing surfactant and stirred at high speed resulting in the formation of a W/O emulsion. The gelation reaction is initiated by lowering the pH of W/O emulsion to release the calcium ions. In both the external and internal gelation methods a problem of low encapsulation efficiency may occur. This problem is caused by the release of the encapsulant to the gelling medium during particle manufacture (Wheatley et al., 1991; Vandenberg and De La Noüe, 2001). A greater protein loss was reported for the internal gelation method compared with the external method due to the greater porosity of CaAlg gel beads prepared by the former method. Mixing protein drugs with alginate and then forming an emulsion under high shear force could cause inactivation of protein at the W/O interface.

In order to stabilize and reduce the porosity of CaAlg gel beads, the beads were coated with a cationic polymer such as chitosan by polyelectrolyte complexation (Polk et al., 1994). Chitosan is a derivative of chitin, a natural polysaccharide and is second only to cellulose in its occurrence in nature. Chitosan exhibits several favorable properties including biodegradability, lack of toxicity and mucoadhesiveness. Chitosan has been extensively studied as a promising protein carrier (Liu et al., 1997; Lee et al., 2004; Wang et al., 2004). However, most of work up to date focuses on the loading and release of proteins. Our previous work demonstrated that when immobilized in a hydrogel phase of a polymeric composite membrane, glucose oxidase (GOX) maintained up to 80% activity as compared to free GOX (Zhang and Wu, 2002). In the present work, GOX was immobilized in alginate/chitosan microspheres (ACMS) using the emulsification–internal gelation method, followed by GOX adsorption to a hydrophilic solid surface of CaAlg gel beads, which avoided the exposure of GOX to W/O interface and shear force of forming emulsion, and chitosan coating. GOX is a microbial enzyme that oxidizes glucose to gluconic acid and hydrogen peroxide. It is a relatively stable enzyme with a molecular weight of 160 kDa and a p*I* of 4.2 (Swoboda and Massey, 1965), which means that it is anionic at physiological pH. Factors influencing GOX loading, encapsulation efficiency and the activity of the loaded GOX were investigated in the present work.

2. Materials and methods

2.1. Materials

Alginate (sodium salt, medium molecular weight, viscosity of 2% solution at 25 °C is 3500 cps), glucose oxidase (Type X-S, from *Aspergillus niger*), peroxidase (Type II, from horseradish), potassium persulfate (KPS), Span 80 and Tween 80 were all purchased from Sigma (St. Louis, MO, USA). Chitosan with a molecular weight of 150 kDa was purchased from Fluka (Buchs, SG, Switzerland) and modified by the KPS degradation method (Hsu et al., 2002). The molecular weight of chitosan was determined by viscometry (Roberts and Domszy, 1982) and the degree of deacetylation was characterized by a titration method (Roberts, 1992). Calcium carbonate, acetic acid and light mineral oil were purchased from Fisher (Fairlawn, NJ, USA). Distilled and deionized (DDI) water was prepared by a Millipore (Billerica, MA, USA) system.

2.2. Preparation of ACMS

A modified emulsification–internal gelatin method was employed to prepare CaAlg gel beads (Poncelet et al., 1992).

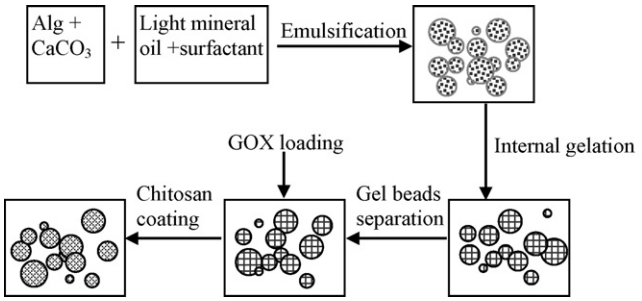

Fig. 1. Schematic illustration of a process for preparation of CaAlg gel beads by emulsification–internal gelation method, GOX loading and chitosan coating.

As shown in Fig. 1, the W/O emulsion was performed with a sodium alginate aqueous solution and calcium carbonate as the internal phase and light mineral oil as the external phase. The calcium ions were released from calcium carbonate by the addition of acetic acid thus initiating the gelation reaction of the calcium ions with the sodium alginate. Briefly, 0.02 g of calcium carbonate was added to 6.0 ml of 1.5% (w/v) sodium alginate solution and placed in ultrasonic bath for 4 min. The suspension was dispersed into 30 ml of light mineral oil containing 1.5% (v/v) Span 80 and 0.2% (v/v) acetic acid at a stirring speed of 760 rpm at room temperature for 5 min. Next, 120 ml of DDI water was added to the above emulsion. Stirring continued for 40 min at a speed of 300 rpm. The CaAlg gel beads were rinsed with 200 ml of 1% (v/v) Tween 80 in aqueous solution and then by 100 ml of DDI water, three times, to remove any traces of oil from the CaAlg gel beads surface. Blank ACMS were prepared by immersing 0.5 ml of the CaAlg gel beads into 0.5 ml of 1% (w/v) chitosan acetic acid solution (1%, v/v) for 10 min at room temperature. During the coating process, the CaAlg gel beads were gently shaken to make the reaction uniform.

2.3. Characterization of ACMS

The morphology of the CaAlg gel beads in the GOX buffer, during the process of chitosan coating, was observed under an optical microscope (LEITZ DM IL, Leica, Wetzlar, Germany) equipped with a digital camera (Coolpix 995, Nikon, Tokyo, Japan). CaAlg gel beads and ACMS were freeze-dried by a lyophilizer (Benchtop 3.3, VirTis, Gardiner, NY) and examined by scanning electron microscope (SEM, S2500, Hitachi, Tokyo, Japan). Samples were mounted onto metal holders using double-sided adhesive tape, coated with gold under vacuum prior to the examination. The particle size and size distribution of the CaAlg gel beads and the ACMS were determined by a particle size analyzer (Mastersizer S, Malvern, Worcestershire, UK).

2.4. Determination of GOX loading and encapsulation efficiency

GOX loaded ACMS was obtained by the incubation of CaAlg gel beads into a GOX buffer solution followed by chitosan coating. In a typical GOX microencapsulation experiment, 0.5 ml of the CaAlg gel beads (dry weight of 3.95 mg) was added into 0.5 ml of 0.2 mg/ml GOX in a 0.2 M buffer solution of pH 3–5 ($KHC_6H_4(COO)_2$–HCl) or pH 6–7 (KH_2PO_4–NaOH). The adsorption experiments were carried out for 30 min at 4 °C, followed by incubation of the CaAlg gel beads in 1% (w/v) chitosan solution for 10 min. The preparation process is illustrated in Fig. 1. The concentration of GOX before and after loading and coating was characterized with the BioRad (Hercules, CA, USA) protein microassay. At the end of reaction, the microspheres were centrifuged and rinsed with 15 ml of DDI water five times to remove unencapsulated GOX. The percentage of GOX loading and encapsulation efficiency were calculated based on the dry weight of the particles using the following equations:

$$\text{Percentage of GOX loading} = \frac{\text{weight of GOX loaded}}{\text{weight of total particles}} \times 100\%$$

$$\text{Encapsulation efficiency} = \frac{\text{weight of GOX loaded}}{\text{total weight of GOX added to the preparation}} \times 100\%$$

2.5. Measurement of the activity of GOX

The activity of free GOX (FR-GOX) and encapsulated GOX in microspheres (ACMS-GOX) was measured by two methods. The first method, the Trinder method (Kaplan, 1987), was used as a quick endpoint activity assay to determine the relative activity among the formulations. In this method, glucose is oxidized by GOX to glucuronic acid and hydrogen peroxide. The hydrogen peroxide then oxidized a reduced dye, 4-aminophenazone (colorless) catalyzed by peroxidase (POD). The color change of the dye was measured photometrically after 15 min at 527 nm at room temperature. The assay consisted of 2.4 ml of 50 mM 4-aminophenazone, 0.1 ml of 60 U/ml POD, and 0.5 ml of 18% (w/v) glucose aqueous solution, which was prepared 1 h before use. The ACMS-GOX activity was measured immediately after rinsing the microspheres.

The second method measured the activity of GOX as characterized by measuring the concentration of H_2O_2 at various times generated by FR-GOX or ACMS-GOX in cell culture medium by using PeroXOquant™ quantitative peroxide assay kit (Pierce, Rockford, IL, USA). Briefly, ACMS-GOX, loaded at different concentrations of GOX (0.4, 2, 10 U/ml) as compared with equivalent dose of FR-GOX (0.016, 0.08, 0.4 U/ml), was incubated in cell culture medium in a humidified incubator with 95% air/5% CO_2 at 37 °C. At different times, 800 μl of culture medium was taken and centrifuged for 1 min through a Centricon YM-50 (Millipore, Billerica, MA, USA) that has a cut off molecular weight of 50 kDa. Then 20 μl of supernatant was added to a 200 μl of assay kit and incubated for 15 min at room temperature and assayed using a microplate reader (Molecular Device, Sunnyvale, CA, USA) at 595 nm.

For determination of the storage stability of ACMS-GOX, the samples were lyophilized and stored at −20 °C. Their activity was measured after different storage times using the first method described above.

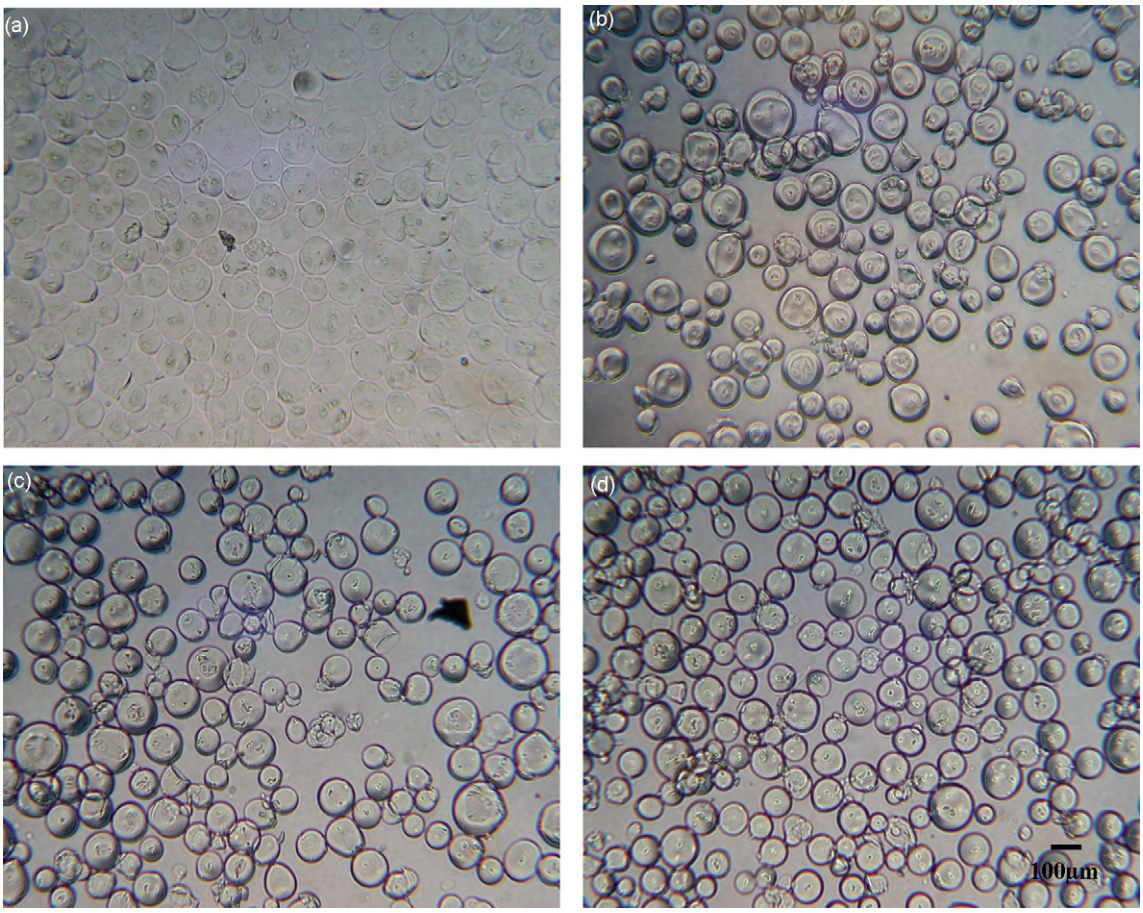

Fig. 2. Optical images of CaAlg gel beads produced by emulsification–internal gelation method during GOX loading and chitosan coating over time. (a) CaAlg gel beads immersed in 0.5 mg/ml GOX buffer solution of pH 4.0; the GOX-loaded CaAlg gel beads after coating with 1% (w/v) chitosan solution of pH 4 at (b) 2 min, (c) 4 min and (d) 10 min.

2.6. Statistical analysis

Each experiment was repeated at least three times. The results are expressed as means ± S.D. A Student's *t*-test was applied to compare the effect of pH value of adsorption medium, initial GOX solution concentration, and chitosan coating on the activity of loaded GOX between two groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Morphology and particle size of CaAlg gel beads and ACMS

As shown in Fig. 2a, CaAlg gel beads are highly spherical and nearly transparent in GOX solution when observed under an optical microscope. As the chitosan coating began, the front of CaAlg gel beads and chitosan complexation was visible. It moved inwards gradually as shown in Fig. 2b–d, taken at 2 min (b), 4 min (c) and 10 min (d). By 10 min, the reaction front almost reached the center of the gel core and the microspheres became uniform (Fig. 2d). Fig. 3 shows the SEM photographs of lyophilized CaAlg gel beads (left) and ACMS (right). It is seen that the CaAlg gel beads have a collapsed and crumpled surface whereas ACMS have more rigid and compact structure. The particle size and size distribution of a representative preparation of ACMS dispersed in water are presented in Fig. 4 and Table 1. The microspheres in the wet state have a volume-based mean diameter of 138 μm.

3.2. pH effect on the kinetics of GOX adsorption and GOX encapsulation efficiency

GOX is stable at various pH values ranging from pH 4 to 7 (Bright and Appleby, 1969) allowing the study of pH effect

Table 1
Volume-based particle size distribution percentile of ACMS in water

Percentile (%)	Particle size of ACMS (μm)
10.0	48.2
20.0	75.3
50.0	132.0
80.0	196.5
90.0	234.9
Mean	137.5

ACMS were prepared according to method part.

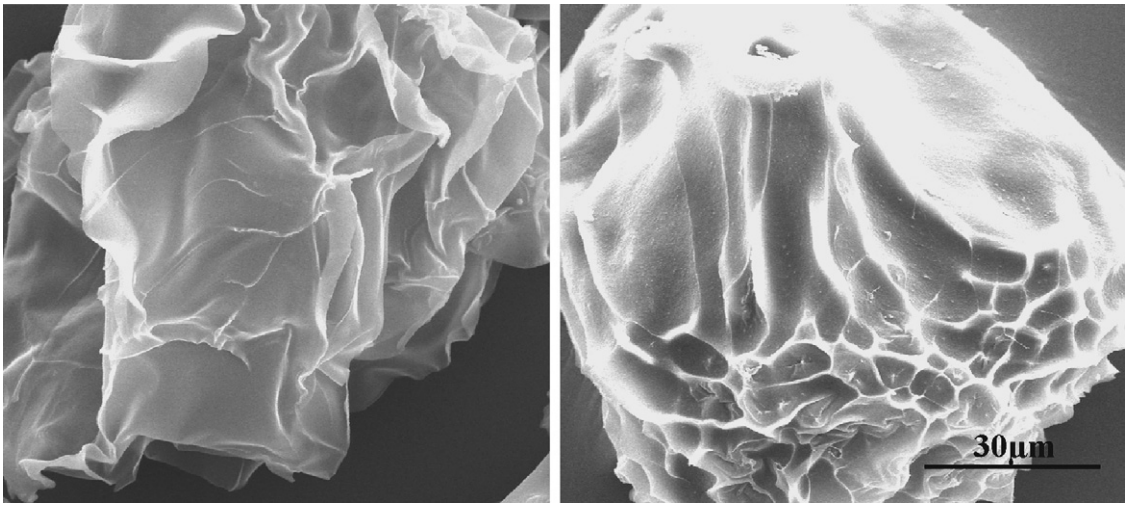

Fig. 3. SEM photographs of lyophilized CaAlg gel beads with collapsed and crumpled surface (left) and ACMS (right) with more rigid and compact structure after coating with 1% (w/v) chitosan solution of pH 4 for 10 min. The pictures were taken at 20 kV with magnification of 1K.

on the kinetics of GOX adsorption into CaAlg gel beads. The amount of GOX loaded was calculated in terms of the change of the GOX concentration in the supernatent before and after incubation of CaAlg gel beads in GOX solutions of various pH values. In order to see this change, a greater amount of CaAlg gel beads (dry weight of 12.7 mg corresponding to 1.8 ml) was used than that (dry weight of 3.95 mg corresponding to 0.5 ml) in later experiments. As shown in Fig. 5, GOX adsorption reaches saturation in less than 10 min. The rate and extent of adsorption substantially increases with decreasing pH of the medium from pH 6 to pH 3. The hightest rate and extent of adsorption is achieived at pH 3.

As illustrated in Fig. 6, the GOX loading and encapsulation efficiency are, respectively, around 10 and 80% after chitosan coating when the GOX loading was performed in media with pH values lower than the p*I* of GOX (i.e., pH 3 and 4). However, when the pH values increase above the p*I*, GOX loading and encapsulation efficiency decrease significantly with increasing pH. The enzymatic activity of loaded GOX in ACMS prepared at various pHs was determined in pH 6 buffer by the Trinder method. The GOX activity is shown in Fig. 7 as compared to that of ACMS-GOX prepared at pH 3. It is seen that the relative activity increases as the loading pH increases from 3 to 4 and then decreases with increasing pH. The highest relative activity is achieved in pH 4 adsorption medium.

3.3. Effect of GOX concentration

The influence of GOX concentration in the loading solution on GOX loading, encapsulation efficiency, and GOX activity was investigated and the results are summarized in Table 2. Both GOX loading and relative activity of encapsulated GOX increase with increasing initial GOX concentration from 0.05, 0.2, 1 to 5 mg/ml, whereas encapsulation efficiency experiences insignificant change.

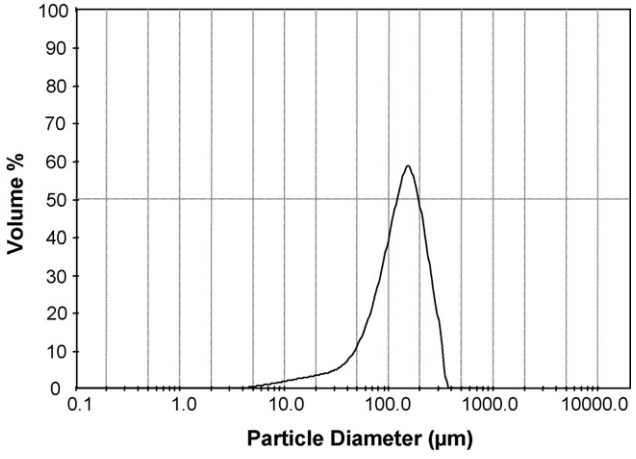

Fig. 4. Particle size distribution of ACMS in water reported as volume-based particle diameters.

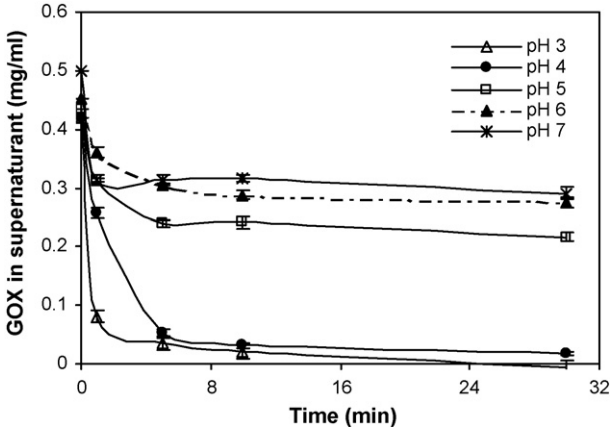

Fig. 5. Kinetics of GOX adsorption into CaAlg gel beads as a function of pH of the adsorption medium. The experiments were conducted by incubating CaAlg gel beads (dry weight of 12.7 mg) in 2 ml of 0.5 mg/ml GOX in 0.2 M buffer solution of pH ranging from 3 to 7. The data points and the error bars represent means ± S.D. ($n = 3$); where not seen, the S.D. values lie within the symbols.

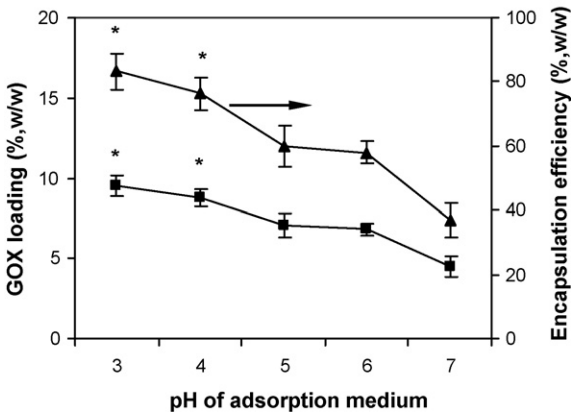

Fig. 6. Effect of pH of the adsorption medium on the GOX loading and encapsulation efficiency. The experiments were conducted by incubating CaAlg gel beads (dry weight of 3.95 mg) in 0.5 ml of 1.0 mg/ml GOX in 0.2 M buffer solution of pH ranging from 3 to 7, and then coated with 1% (w/v) chitosan solution. Data points and the error bars represent means ± S.D. ($n = 3$). Asterisk (*) indicates significantly different from the value at pH 5 of the adsorption medium ($p < 0.05$).

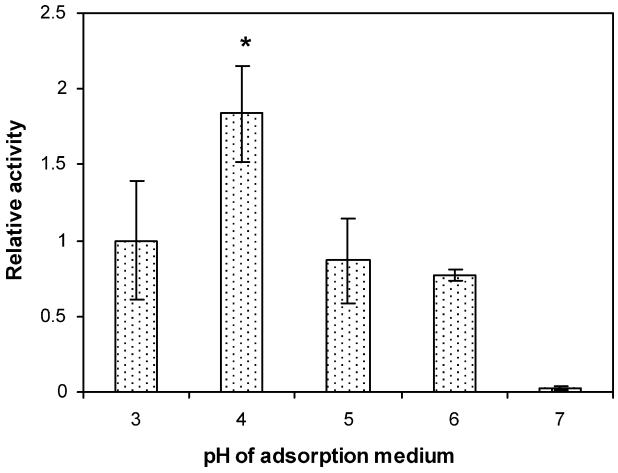

Fig. 7. Effect of pH of the adsorption medium on the relative activity of loaded GOX as relative to the activity at pH 3. The experiments were conducted by incubating CaAlg gel beads (dry weight of 3.95 mg) in 0.5 ml of 1.0 mg/ml GOX in 0.2 M buffer solution of pH ranging from 3 to 7, and then coated with 1% (w/v) chitosan solution. The data points and the error bars represent means ± S.D. ($n = 3$). Asterisk (*) indicates significantly different from the value at pH 5 of the adsorption medium ($p < 0.05$).

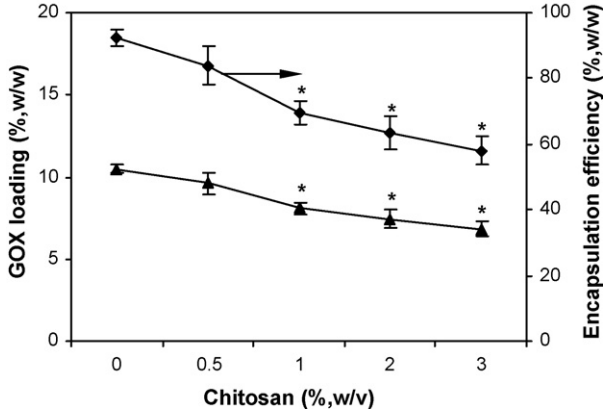

Fig. 8. Effect of chitosan concentration on GOX loading and encapsulation efficiency. The experiments were conducted by incubating CaAlg gel beads (dry weight of 3.95 mg) in 0.5 ml of 0.5 mg/ml GOX in 0.2 M buffer solution of pH 4. The data point and the error bars represent mean ± S.D. ($n = 3$); where not seen, the S.D. values lie within the symbols. Asterisk (*) indicates significantly different from the value at 0.0% of chitosan concentration ($p < 0.05$).

3.4. Effect of chitosan concentration on GOX encapsulation

The effects of various concentrations of chitosan coating on GOX loading and encapsulation efficiency were evaluated. Fig. 8 shows that GOX loading and encapsulation efficiency decreased gradually with increasing chitosan concentration. The GOX loading decreased from 10 to 7% and encapsulation efficiency decreased from about 90 to 60%, respectively, as chitosan concentration increased from 0 to 3%. The decrease of GOX loading and encapsulation efficiency become statistically significant, relative to 0% chitosan concentration, as chitosan concentration reaches 1% or higher.

In contrast to GOX loading and encapsulation efficiency, the relative activity of encapsulated GOX exhibits a strong dependence on the chitosan concentration. As shown in Fig. 9, the GOX activity of the ACMS prepared using 0.5% (w/v) chitosan is six-fold higher than that with 0% chitosan. The activity then gradually decreases as the chitosan concentration is further elevated.

3.5. Kinetics of hydrogen peroxide generation by FR-GOX and ACMS-GOX

The kinetics of H_2O_2 generation by FR-GOX and ACMS-GOX containing equivalent amounts of GOX was determined

Table 2
Effect of initial concentration of GOX in solution on GOX loading, encapsulation efficiency and activity of loaded GOX

GOX initial concentration (mg/ml)	Mass ratio of GOX to CaAlg gel beads in the adsorption medium	GOX loading (%, w/w)[a]	Encapsulation efficiency (%, w/w)	Relative activity[b]
0.050	0.0063	0.48 ± 0.10	76.5 ± 15.4	1.00 ± 0.07
0.20	0.025	1.88 ± 0.14	75.5 ± 5.6	1.35 ± 0.28
1.0	0.13	9.17 ± 0.37	79.8 ± 3.5	52.8 ± 12.4
5.0	0.63	34.5 ± 0.17	83.1 ± 0.6	174 ± 18.3

The adsorption was performed by incubating of CaAlg gel beads (dry weight of 3.95 mg) in 0.5 ml of 0.2 M pH 4 buffer solution containing 0.05, 0.2, 1, 5 mg/ml GOX and then coated with 1% chitosan solution.

[a] Values are presented as mean ± S.D., $n = 3$.

[b] The relative activity was calculated by taking the GOX activity at 0.05 mg/ml initial concentration as unity.

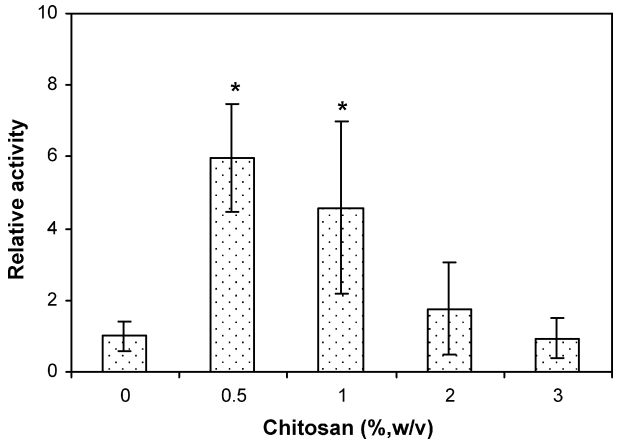

Fig. 9. Effect of chitosan concentration on the activity of loaded GOX relative to the activity at 0% of chitosan. The experiments were conducted by incubating CaAlg gel beads (dry weight of 3.95 mg) in 0.5 ml of 1.0 mg/ml GOX in 0.2 M buffer solution of pH 4 and then coated with chitosan solution of various concentrations. The data points and the error bars represent mean ± S.D. ($n = 3$). Asterisk (*) indicates significantly different from the value at 0.0% of chitosan concentration ($p < 0.05$).

using PeroXOquant™ quantitative peroxide assay kit and is compared in Fig. 10. In the figure the numerals represent the concentration of GOX (mU/ml) either as a free form or encapsulated in ACMS. For example, FR-16 represents 16 mU/ml free GOX and ACMS-16 stands for 16 mU/ml ACMS-GOX equivalent to free GOX. The ACMS-GOX were prepared by incubating CaAlg gel beads in 0.5 ml of 0.4 U/ml (ACMS-16), 2 U/ml (ACMS-80) and 10 U/ml (ACMS-400) GOX, respectively, with an encapsulation efficiency of 80%. H_2O_2 generation by ACMS-GOX

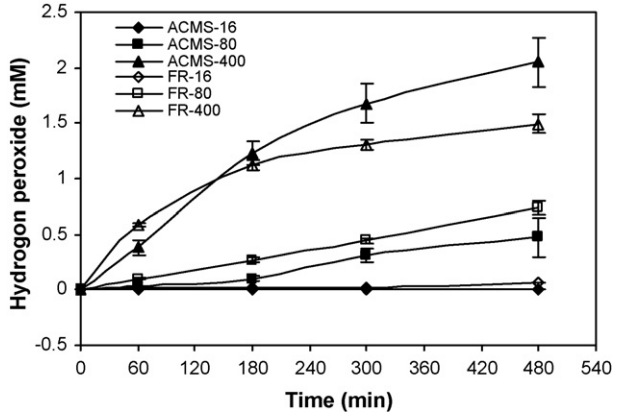

Fig. 10. Kinetics of H_2O_2 generation by FR-GOX and ACMS-GOX with different doses. The ACMS-GOX were prepared by incubating CaAlg gel beads (dry weight of 3.95 mg) in 0.5 ml of various GOX concentrations in 0.2 M buffer solution of pH 4 and then coated with 1% (w/v) chitosan solution. The numerals in the legends indicate the GOX concentrations in mU/ml as a free form or encapsulated in the ACMS in 10 ml of the medium for the H_2O_2 generation study. For example, Free-16 represents 16 mU/ml free GOX and ACMS-16 stands for the GOX concentrations for the ACMS-GOX equivalent to free GOX. The ACMS-GOX were prepared by incubating CaAlg gel beads in 0.5 ml of 0.4 U/ml (ACMS-16), 2 U/ml (ACMS-80) and 10 U/ml (ACMS-400) GOX, respectively, with an encapsulation efficiency of 80%. The data points and the error bars represent mean ± S.D. ($n = 3$); where not seen, the S.D. values lie within the symbols.

and FR-GOX at low GOX concentrations (FR-16 and ACMS-16) was not significant. At a higher GOX concentration, the H_2O_2 production by ACMS-80 lags FR-80 initially but gradually approaches a similar rate. At an even higher GOX concentration, ACMS-400 outperforms FR-400 in the H_2O_2 production after 2.5 h.

To examine the stability of loaded GOX, ACMS-GOX were lyophilized, stored for up to one month at −20 °C, and rehydrated. The GOX activity in the ACMS-GOX was found to be 90% of its original activity (data not shown).

4. Discussion

4.1. Internal gelation in relation to the formation of CaAlg gel beads structure and GOX encapsulation

The chitosan coating process appears to reach the gel core within 10 min as shown in Fig. 2. This rate and extent of chitosan molecule diffusion into the gel network, produced by the internal gelation method, is much greater than that of the CaAlg gel beads formed by the external gelation method (Gåserød et al., 1998). In the latter method the chitosan, with a molecular weight of 61 kDa, bound to the CaAlg gel beads surface with only a very limited diffusion into the gel network after 4 h. This indicates that the porosity of the CaAlg gel beads formed by internal gelation is greater than the porosity of CaAlg gel beads by external gelation. As shown in Fig. 3, the CaAlg gel beads appeared to collapse after lyophilization due to the highly porous gel network. As a result, high protein loss would be anticipated during a preparation process in which GOX was encapsulated by mixing it with the alginate solution by itself (Wheatley et al., 1991). In contrast, CaAlg gel beads coated with chitosan had a more rigid, compact and less porous structure. Moreover, the chitosan–alginate complexation occurs throughout the gel, rather than at the surface in the external gelation method (Gåserød et al., 1998). This is probably due to the homogeneous structure of the CaAlg gel beads produced by the internal gelation method (Poncelet et al., 1995). In the external gelation process, a high gradient of alginate and calcium ion concentration near the surface of CaAlg gel beads results in a dense shell that acts as a major barrier to protein diffusion. In the internal gelation process, calcium carbonate is distributed uniformly in the alginate solution resulting in in-site gelling induced by faster proton diffusion (Quong et al., 1998). Therefore, the CaAlg gel beads formed by internal gelation have a homogenous and less compact structure than the CaAlg gel beads formed by external gelation. It was expected that protein diffusion into this higher porosity CaAlg gel beads would be greater than that of the CaAlg gel beads produced by external gelation. It has been reported that the diffusion of proteins is dependent on molecular weight of the molecule. The γ-globulin with a molecular weight of 154 kDa, similar to the GOX (160 kDa), had no diffusion into the CaAlg gel beads formed by the external gelation method (Tanaka et al., 1984). The high encapsulation efficiency of 80–90% shown in the present study was achieved because of the relatively more porous structure allowing higher molecular weight proteins to diffuse into the CaAlg gel beads. However, the higher porosity

of CaAlg gel beads also allows chitosan to diffuse deep into the gel core and possibly cause desorption of GOX which is not desired.

4.2. Effect of adsorption medium on GOX encapsulation

As shown in Fig. 5, the pH of the adsorption medium has a substantial effect on GOX adsorption. A possible explanation for this is that as the adsorption medium pH became less than 4.2 (the p*I* of GOX), greater than 50% the GOX was positively charged and more GOX adsorbed to negatively charged CaAlg gel beads due to electrostatic interactions. Whereas when the pH was greater than 4.2, less of the GOX was negatively charged and the adsorption became more diffusion controlled instead of being controlled by electrostatic interactions. It was also seen that the relative activity of ACMS-GOX prepared at pH 3 was lower than that at pH 4 (Fig. 7) althougth GOX loading and encapsulation efficiency were approximately 10% higher at pH 3 (Fig. 6). This was probably due to reduced stability of GOX at pH 3.

4.3. Adsorption capacity of CaAlg gel beads and desorption of GOX induced by chitosan coating

The increment of GOX loading decreased significantly from 4.9- to 3.8-fold from the range of 0.2–1 to 1–5 mg/ml, respectively, indicating that it was approaching its adsorption capacity limitation in the latter concentration range. The extremely low GOX activity in the low GOX loading concentration range (0.05–0.2 mg/ml) was probably due to the low sensitivity of Trinder method at low concentrations of GOX (Table 2). In this range, PeroXOquant^{TM} assay kit was more applicable as later characterized and shown in Fig. 10. In high concentration range (1–5 mg/ml), higher relative activity could be obtained effectively by increasing the initial concentration of GOX. However, as GOX concentration increases, at a certain value, GOX probably starts aggregate causing deactivation of GOX.

Chitosan is composed of glucosamine residues and the positively charged amino groups of glucosamine bind with the negatively charged carboxyl groups of alginate. This reaction likely competes with GOX adsorption to the CaAlg gel beads although chitosan was introduced right after GOX loading. Therefore, the higher the concentration of the chitosan the more competition with the GOX adsorption occurred and the more desorption of the bound GOX from the CaAlg gel beads. As a result, lower GOX loading and encapsulation efficiencies result (Fig. 8). The protein concentration in the supernatant was measured before and after chitosan coating. When chitosan was added, the protein concentration increased. The more chitosan added the more the protein concentration increased. This is likely due to the desorption of bound GOX from CaAlg gel beads. As chitosan concentration in the coating solution increased six-fold from 0.5 to 3%, the GOX loading and encapsulation efficiency decreased by 30% as seen in Fig. 8. At low concentrations of chitosan, fewer amino groups were available for complexation with alginate. The lower cross-linking density between amino groups and carboxyl groups resulted in a more porous polymer network, which allowed a greater amount of GOX to diffuse out from the polymer network. This interpretation is supported by the increased GOX concentration in the supernatant after chitosan coating. The activity of loaded GOX was the lowest without chitosan coating (i.e., 0% chitosan in the solution) perhaps due to washing off of bound GOX from the microspheres. It was also found that at high concentrations of chitosan, microspheres shrank significantly, resulting in smaller surface area for the contact with the glucose substrate and possibly blocking the binding site of loaded GOX with glucose leading to a decrease in the apparent activity of loaded GOX.

4.4. Activity of ACMS-GOX versus FR-GOX

Similar H_2O_2 generation behavior was found for the ACMS-GOX formulation compared with FR-GOX. In fact, at a higher level of GOX loading (ACMS-400) and at later times (after 2.5 h) the concentration of H_2O_2 generated by ACMS-GOX exceeded that of FR-GOX (Fig. 10). This result indicates that the GOX activity was well maintained in ACMS using the present formulation. The formulation may even prevent deactivation of GOX by the external environment, for example, gluconic acid and H_2O_2, the products of the reaction. The delayed H_2O_2 production by the ACMS-GOX was likely caused by diffusion in of its substrate, glucose, and diffusion out of its product, H_2O_2.

Different enzymes and cytokines immobilized in CaAlg gel beads have been reported with various relative activities by different authors. For instance, a 31 and 60% of the urease activity was maintained (Shah et al., 1995 and Hearn and Neufeld, 2000), respectively. Lipase activity was retained to a level of 9% (Betigeri and Neau, 2002). Nearly all the activity of the transforming growth factor-β_1 (TGF-β_1) immobilized in CaAlg gel beads was lost without additives (Mumper et al., 1994). It has also been reported that when GOX was immobilized in poly(hydroxybutyrate-co-hydroxyvalerate) nanocapsules, only 4.5% of GOX activity was retained (Baran et al., 2002).

5. Conclusion

A novel microsphere formulation of ACMS for therapeutic protein was developed using an emulsification–internal gelation method to produce CaAlg gel beads, followed by GOX adsorption and coating by chitosan. The highly porous structure of CaAlg gel beads and the opposite charges on GOX contributed to the high encapsulation efficiency of GOX. A number of factors influencing GOX loading, encapsulation efficiency and loaded GOX activity were investigated. GOX loading was pH dependent with much higher loading and encapsulation efficiency at pH values of the adsorption medium lower than the p*I* of GOX. The competition between chitosan coating and GOX binding to alginate affected GOX loading, encapsulation efficiency, and apparent activity. The activity of ACMS-GOX was well maintained and ACMS-GOX showed prolonged H_2O_2 production as compared to FR-GOX. The majority of the activity ACMS-GOX was retained after lyophilization and storage at low temperature. The results of this study suggest that ACMS has the potential to serve as a useful and efficient delivery system for proteins like GOX.

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