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RELEASE PROFILE OF A MODEL PROTEIN DRUG DEPENDING ON THE STABILITY OF MICROSPHERES BASED ON POLYELECTROLYTE COMPLEX

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Abstract: Stability and disintegration of natural polyelectrolyte complex microspheres for protein drugs delivery have been extensively investigated because of their great influence on the drug release patterns. In this study, we tested stability of microspheres with alginate (Alg) core layered by either chitosan (Chi) or glycol chitosan (GChi) by examining release profiles of fluorophore-labeled bovine serum albumin (BSA) and lysozyme (Lys) from the microspheres. While GChi shell was disintegrated quickly, Chi-shell microspheres showed good stability in PBS. Disintegration of the coated layer induced the core material instable. The results indicated that while the charges of the shell material provided additional diffusion barrier against the protein release, the key factor to hold the proteins inside the microspheres was the integrity of the outer coating layer.

Introduction

Development of microspheres to control the release profile of an encapsulated drug has been extensively investigated by employing different materials. Among them, the natural polysaccharides-based polyelectrolyte complex has attracted advantages over synthetic ones especially in protein drugs releases. Alginate and chitosan have interesting properties such as biocompatibility, muco-adhesiveness, biodegradability as well as easy encapsulation of living cells [1] and drugs. Alginate microspheres have been fabricated by attaching its carboxylic acid side chains to divalent cations such as calcium (Ca^{2+}) or barium (Ba^{2+}), to induce ionic interactions which leads to formation of a gel. Chitosan has been recently recognized as an excellent natural polymer for microsphere fabrication. Chitosan possesses abundant cationic amino groups that are ready to be ionized under physiological pH (7.4) due to their low pKa value [2], ranging from pH 5 to 6 depending on the degrees of both deacetylation and polymerization. This polycation property leads to employment of chitosan as a coating material of a bead, providing the bead with an additional mechanical strength as well as control of drug diffusion out depending on its concentration and molecular weight [3].

Stability of the chitosan and alginate microspheres has attracted high interest in the issues of drug release. We here investigated profiles of disintegration and drug release from alginate-chitosan microspheres as functions of time and their concentrations as well as employment of chitosan derivatives such as glycol chitosan, by assuming that the stability of the carriers may be a primary factor in decision of release kinetics or profiles of the encapsulated drugs.

Materials and Methods

Materials. Chitosan (Chi, Mv 1,000 kDa, degree of deacetylation 80 %) and glycol chitosan (GChi, Mv 250 kDa, degree of deacetylation 82.5 %) were supplied from Wako Pure Chemical Industries

(Osaka, Japan). Sodium alginate (Alg), ethylene glycol-bis(β-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA), fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC), sodium tripolyphosphate (TPP), dibutyltin dilaurate, and most of chemicals were obtained from Sigma-Aldrich Co. (MO, USA). Millicell (pore size = 2 μm) was purchased from Millipore Corp. (MA, USA).

Polysaccharide Purification. 3 % (w/v) Chi was obtained by dissolving in 2 % (v/v) acetic acid solution. Insoluble particles containing residual proteins were removed by the membrane filter papers and the filtrate was dialyzed against excess water over 3 days. GChi (5 g) was purified as described above without using acetic acid. Alginate purification was performed as reported elsewhere with some modifications [4]. Briefly, alginate solution (10 g, 1 %, w/v) containing 1 mM EGTA was successively filtered by 5.0, 0.8, and 0.2 μm filter papers. The retrieved filtrate was precipitated against 70% ethanol, and then rinsed with ethyl ether. White solid was obtained in vacuum.

Fluorophore labeling. FITC solution in anhydrous DMSO (10 mg/mL) was slowly added in the 1% chitosan solution in 1 % (v/v) acetic acid. GChi (1 %, w/v) was also labeled with FITC as above. Alginate (1 g in 100 mL 0.5 N NaOH solution) was labeled, by mixing 10 mg RITC and 1 μL dibutyltin dilaurate in 1 mL anhydrous DMSO for 24 hours at RT and then dialyzing (MWCO 12–14 KDa) against excess water at 4 °C. Final products were obtained by freeze-drying and further dried by P_2O_5 under vacuum.

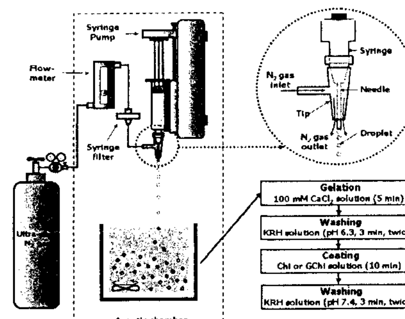

Figure 1. Instrument setup to prepare Alg/Chi or Alg/GChi microspheres (left panel) that had a house-made air jet (right upper). After jetting, preparation procedure is described at right lower panel.

Preparation of alginate-cored microspheres. Alginate-cored microspheres were prepared as shown in Fig. 1. In brief, polysaccharide solution was pushed down by syringe pump at a flow rate of 0.5 mL/h. High speed flow of nitrogen gas controlled by a flowmeter jetted out the solution so as to generate droplets. A jet tip (Fig. 1; right upper) was simply fabricated by connecting two micropipette tips, which were fitted to the 23 G syringe needle.

To produce microspheres, stock solutions were prepared by filtrating (0.45 μm) 3 % (w/v) FITC-labeled chitosan (ChiF) solution containing 2 % (v/v) acetic acid, 3 % (w/v) FITC-labeled glycol chitosan (GChiF) in PBS (pH 7.4) and 3% (w/v) RITC-labeled alginate (AlgR) solution in Ca^{2+}– and Mg^{2+}–free PBS (pH 7.4). The ChiF and GChiF stock solutions were further diluted with filtrated 2% (v/v) acetic acid solution or PBS (pH 7.4). Dropping AlgR solution (3 mL) into Krebs Ringer HEPES (KRH) solution (50 mL) containing 100 mM $CaCl_2$ led to rapid gelation of droplets. After 5 min, AlgR microspheres were harvested and washed twice by sterile KRH solution (pH 6.3). After coating microspheres with ChiF or GChiF for 10 min, rapid washing with KRH solution (pH 7.4) was performed. The prepared microspheres were transferred into 50 mL conical tube containing 10 mL sterile PBS (pH 7.4).

***In vitro* stability test.** Microspheres were split into 10 conical tubes containing 1 mL PBS. The amount of ChiF, GChiF, or AlgR was determined after washing and lyophilizing 3 samples. 7 samples were loaded into Millicell in a 24 well plate with 1 mL PBS and then incubated in the plate at 37 °C under humidified atmosphere with PBS replacement every 6 hours. The fluorescence intensities of ChiF, GChiF and AlgR were determined over a standard curve using a microplate reader with FITC (excitation 488 nm and emission 520 nm) and RITC (544 nm and 580 nm, respectively) filter sets by quantifying the amount of polysaccharide.

***In vitro* release of chromophore-labeled proteins.** FITC-labeled bovine serum albumin (F-BSA) and RITC-labeled lysozyme (R-Lys) were encapsulated to identify the effect of carrier stability on protein release. 3 % (w/v) alginate solution was obtained after dissolving each protein in PBS (1 mg/mL) and its filtering. Alg was clearly dissolved in each protein solution at 4 °C. Microspheres

were generated by the same method as described above (0.5 % Chi and GChi). The collected microspheres in 10 mL PBS were volumetrically distributed into Millicells (7 samples of 1 mL) and 1.5 mL microcentrifuge tubes (3 samples of 1 mL). Protein loading contents was determined after placing the Millicells into a 24-well culture plate containing 1 mL PBS. Microspheres in the Millicells were monitored by fluorescence microscopy using FITC and RITC channels for F-BSA and R-Lys, respectively, during the experimental period.

Results and Discussion

Stability of protein drug carriers from our polyelectrolyte complex was examined by hypothesizing that the release profiles of encapsulated protein drugs would be changed as a function of the carrier stability. All Chi and GChi microspheres have an alginate (Alg) core (3 %, w/v), a form of hydrogel cross-linked by calcium ions. Fig. 2 displays the stabilities of AlgR core and ChiF or GChi (GChiF) shell, demonstrating that the stabilities of the core and shell depended on the coated polycation material. The AlgR core of microspheres with the GChiF shell was more disintegrated (Fig. 2a, over 20 % at day 6) than that with ChiF core was (Fig. 2b, less than 6 % at day 6). While the concentration of GChiF had slightly influenced on the AlgR core stability (Fig. 2a.), ChiF had not done on the stability of AlgR core with ChiF shells (Fig. 2b).

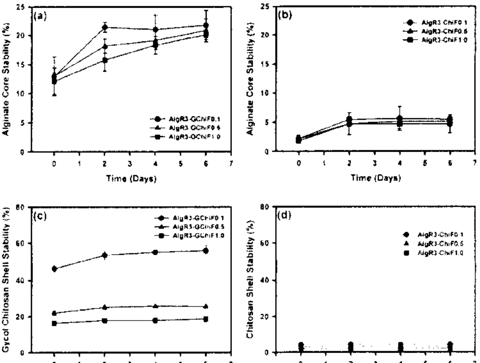

Figure 2. Disintegration of alginate core (AlgR, a and b for GChiF and ChiF, respectively) and, glycol chitosan (GChiF, c) or chitosan (ChiF, d) shell. Numerical values following abbreviation of polysaccharides mean their concentration (%, w/v).

The mechanical property of the Alg-cored microspheres has been reported in reliance to the coated polycation property such as molecular weight, concentration and coating time [3]. Therefore, the GChiF shell may not protect the microsphere, because the GChi is highly soluble in water unlike Chi which dissolves in only low pH water. Fig. 2c and 2d proved this hypothesis that the GChiF was more soluble in the buffer solution than ChiF. While the ChiF shell presented almost no disintegration regardless of its concentrations, the GChiF still showed concentration dependency, possibly due to the chain entanglement of long GChiF.

In order to investigate the influence of carrier stability on the release profile of protein drugs, both FITC-labeled BSA (F-BSA) and RITC-labeled lysozyme (R-Lys) were encapsulated into the microspheres; 0.5 % Chi and GChi solution were coated on 3 % Alg beads. Fig. 3a and 3b showed F-BSA and R-Lys release, respectively. In general, the GChi-shelled microsphere liberated more F-BSA or R-Lys than the Chi-shelled one did, but did not reach to hundred percent upto 14 days. During the first few hours, rapid release was observed, possibly due to initial detachment of protein from the outer wall of microspheres. Based on these results, we concluded that the carrier stability, especially shell disintegration, had greatly influence on the drug release.

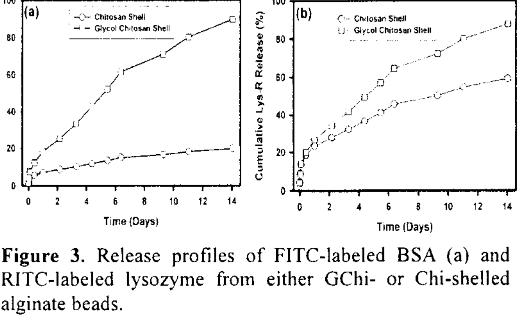

Figure 3. Release profiles of FITC-labeled BSA (a) and RITC-labeled lysozyme from either GChi- or Chi-shelled alginate beads.

One thing more to consider is that, besides the Alg is a negatively charged material, proteins have in nature their own isoelectric points (pIs), i.e. the pIs of BSA and Lys are approximately 5 and 11, respectively. At pH 7.4, since both the F-BSA and the R-Lys have positive charges, there was

additional electrostatic interaction between proteins and polysaccharide materials. In Fig. 3a and 3b, it is obvious that release rate of the R-Lys from the Chi-shelled microspheres is much faster than that of the F-BSA, although release profiles of each protein from the GChi-shelled microspheres were not much different. We could deduce that positively charged R-Lys did not freely pass through the positively charged Chi shell layer because of their electrostatic repulsion. As mentioned previously, property of the coating material is so important to determine the overall microsphere property. This is also employed to the solute diffusion [3]. Although F-BSA has negative charges, electrostatic interaction between F-BSA and Alg did not seem to greatly influence the F-BSA release kinetics. On the other hand, at the plots from GChi-shelled microspheres, unstable shell layer did not support any diffusion barrier. Based on the results, we concluded that the release rate of protein drugs was primarily controlled by the integrity of the coated material rather than the inner Alg core.

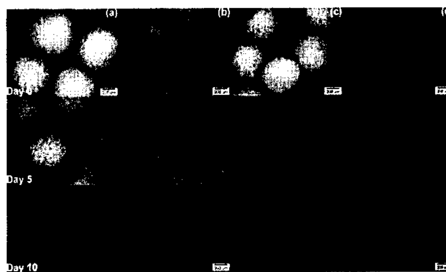

Figure 4. Fluorescence microscopic images of Chi- (a and b) or GChi-coated microspheres (c and d) encapsulating FITC-labeled BSA (a and c) and RITC-labeled lysozyme (b and d). Images at each raw were obtained at day 0, 5, and 10. The scale bar is 200 μm.

Fluorescence microscopic images (Fig. 4) gave additional supports to this conclusion. In the microspheres coated by the Chi shell, proteins trapped at the Chi coating layers were detected, which meant that the Chi layer still remained well up to day 10 (Fig. 4a and 4b). However, as shown in Fig. 4c and 4d, GChi shell disappeared from day 5 and led to even morphological rupture of microspheres. In the Chi-shelled microspheres, the R-Lys was more rapidly excreted than the F-BSA, which well agreed with our previous results in Fig. 3.

Conclusions

The influence of the carrier stability on the protein drug release was investigated. The results demonstrated that polyelectrolyte complex was not very stable so as to be disintegrated over time and the degree of the disintegration was largely dependent on the coated shell material and its concentration. Water-soluble GChi did not give the microspheres enough mechanical strength over a week, while Chi showed relatively good integrity. Protein release rate from the microspheres was under control of pI values of them, but integrity of the coated shell seemed to overwhelm the electrostatic interaction between proteins and core material (Alg). However, the charge of coating material provided additional diffusion barrier to control the protein release profile. As a conclusion, in order to develop and design reasonable protein drug carriers based on the polyelectrolyte complex, our results strongly suggested that the carrier stability should be considered as a primary factor in advance to any other controllable factors.

Acknowledgements

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All-*trans* Retin
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Keywords: All-trans reti
Drug-polymer complex, F

Abstract. To improve th
core-shell type of folate-
cationic PEI segments in
NMR, ELS, XRD, and T
function of drug content i
also evaluated by MTT
incorporated in folate-F
compared with *at*RA itsel

Introduction

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