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Communication

Release of Insulin from Calcium Carbonate Microspheres with and without Layer-by-Layer Thin Coatings

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Abstract: The release of insulin from insulin-containing $CaCO_3$ microspheres was investigated. The microspheres were prepared by mixing aqueous solutions of $CaCl_2$ and Na_2CO_3 in the presence of insulin. The surface of the insulin-containing $CaCO_3$ microspheres was coated with a layer-by-layer thin film consisting of poly(allylamine hydrochloride) and poly(styrene sulfonate) to regulate the release kinetics of insulin. The release rate of insulin from the coated $CaCO_3$ microspheres was significantly suppressed compared with that of uncoated $CaCO_3$ microspheres, and depended on the thickness of the films. Rhombohedral calcite crystals of $CaCO_3$ formed from the microspheres during the release of insulin, suggesting that the $CaCO_3$ microspheres dissolved and recrystallized during the release of insulin.

Keywords: microsphere; polymer coating; calcium carbonate; insulin delivery; layer-by-layer film; controlled release

1. Introduction

Organic and inorganic nano- and micro-particles have been extensively studied for the development of catalysts, biosensors, reagents for imaging, and drug delivery systems [1–8]. $CaCO_3$ microspheres are widely used owing to their facile preparation, biocompatibility, and low cost [9–11]. $CaCO_3$ microspheres are promising materials for encapsulating biological molecules such as proteins, because the microspheres can be prepared in aqueous media under mild conditions. Protein-loaded $CaCO_3$

microspheres have been prepared by mixing a Na_2CO_3 solution and protein-containing $CaCl_2$ solution at room temperature, exploiting the limited solubility of $CaCO_3$ in water [12–16]. In addition, protein-loaded $CaCO_3$ microspheres can be used for preparing polymer microcapsules by coating the surface of $CaCO_3$ microspheres with polyelectrolyte layer-by-layer (LbL) films, and then dissolving the core in solution [17–20]. The amount of proteins loaded in $CaCO_3$ microspheres depends on the preparation conditions, including the concentration of proteins and salts in the solutions, the relative volume of the solutions, and the reaction time. These parameters must be optimized to obtain $CaCO_3$ microspheres containing the desired amount of proteins. Therefore, we have optimized the operational variables for the preparation of $CaCO_3$ microspheres using insulin as a model protein.

The release of drugs and proteins from $CaCO_3$ microspheres and polymer microcapsules has been studied for developing controlled delivery systems. For example, the release of doxorubicin (DOX) from $CaCO_3$ microspheres with and without polymer coatings has been investigated for temperature- and pH-sensitive release systems [21]. The release profile of DOX depended on the temperature and pH of the solution owing to the stimuli-sensitive nature of the polymer coatings, showing that $CaCO_3$ microspheres are useful as vehicles for controlled drug delivery. In the present study, we have prepared insulin-loaded $CaCO_3$ microspheres and coated the surface with LbL thin films consisting of poly(allylamine hydrochloride) (PAH) and poly(sodium styrenesulfonate) (PSS) to regulate the kinetics of insulin release. We report the effects of solution pH and LbL film coatings on the release profile of insulin.

2. Experimental

2.1. Materials

PAH (MW, ~70,000) and PSS (MW, ~70,000) were purchased from Nitto Bouseki Co. Ltd. (Tokyo, Japan) and Sowa Science Co. Ltd. (Tokyo, Japan), respectively. Insulin (human, recombinant) was obtained from Wako Pure Chemical Co. Ltd. (Osaka, Japan). All other reagents used were of the highest grade available. Fluorescein-labelled insulin (F-insulin) was prepared by the coupling reaction of fluorescein isothiocyanate and insulin according to a previously reported procedure [22].

2.2. Preparation of Uncoated and LbL Film-Coated $CaCO_3$ Microspheres

$CaCO_3$ microspheres containing insulin were prepared by mixing 0.2 M Na_2CO_3 aqueous solution (10 mL) and 0.2 M $CaCl_2$ aqueous solution (10 mL) containing insulin (0.5–5 mg). The mixture was stirred for 30 min at ambient temperature. The precipitated $CaCO_3$ microspheres were filtered off and dried. The amount of insulin loaded in the $CaCO_3$ microspheres was determined by high-performance liquid chromatography of a dialyzed solution of microspheres (80 mg) in 1 M HCl (Shimadzu, LC-20AB (Kyoto, Japan) with COSMOSIL 5Diol-II packed column (Nacalai USA, Inc., San Diego, CA, USA), 1 mM carbonate buffer at pH 8.0 and 1 mM acetate buffer at pH 4.0 as eluents). The surface of $CaCO_3$ microspheres was coated with the LbL films by immersing $CaCO_3$ microspheres alternately in 0.5 $mg \cdot mL^{-1}$ PAH solution (10 mM HEPES buffer at pH 7.4) and in 0.5 $mg \cdot mL^{-1}$ PSS solution (10 mM HEPES buffer at pH 7.4) for 15 min each. After each deposition, $CaCO_3$

microspheres were rinsed for 5 min in the working buffer. Sedimentation or aggregation of the microspheres did not occur during the film deposition and ζ-potential measurement.

2.3. ζ-Potential and Scanning Electron Microscopy

To monitor the film deposition, ζ-potentials of LbL film-coated $CaCO_3$ microspheres were recorded with a ζ-potential analyzer (Zeecom/ZC-2000, Microtec, Funabashi, Japan). Scanning electron microscope (SEM; S-3200N, Hitachi Co., Tokyo, Japan) images of $CaCO_3$ microspheres and crystals were obtained for platinum-sputtered samples at 15 kV.

2.4. Release of Insulin from Microspheres

In vitro release of insulin was studied using F-insulin and the amount of released insulin was determined by UV-visible spectroscopy. F-insulin-loaded $CaCO_3$ microspheres (100 mg) were dispersed in 10 mM HEPES buffer (5 mL) at pH 7.4 under gentle stirring. The dispersion was centrifuged every 60 min and the absorption intensity at 494 nm of the supernatant was recorded to determine the amount of F-insulin released.

3. Results and Discussion

Insulin-loaded $CaCO_3$ microspheres were prepared by using $CaCl_2$ solutions containing varying amounts of insulin to evaluate the effect of insulin concentration on the loading of insulin in the microspheres. Table 1 shows the weights of $CaCO_3$ microspheres produced by the reaction and their insulin contents. The reaction produced 184–188 mg of $CaCO_3$ microspheres, which corresponded to a 92%–94% yield. Thus, $CaCO_3$ microspheres were obtained nearly quantitatively with this protocol. The insulin loading in the microspheres increased with the insulin concentration in the $CaCl_2$ solution. The insulin loading was approximately 18 mg/g in the $CaCO_3$ microspheres for 5 mg of insulin in 10 mL $CaCl_2$ solution, showing that 64% of the insulin was immobilized in the $CaCO_3$ microspheres. The insulin loading in the $CaCO_3$ microspheres was lower when $CaCl_2$ solutions containing smaller amount of insulin were used. In addition, we have evaluated the effect of additives on the preparation of insulin-containing $CaCO_3$ microspheres. When Na_2CO_3 solutions (10 mL) containing 1–40 mg additives such as dextran sulfate (DS), PSS, or PAH were employed, $CaCO_3$ microspheres were successfully prepared. However, the loading of insulin in the microspheres could not be improved by the addition of these polymers. Therefore, in the following experiments, $CaCO_3$ microspheres were prepared using 5 mg insulin in 10 mL $CaCl_2$ solution without additives.

Figure 1 shows the release profiles of insulin from $CaCO_3$ microspheres without LbL film coating in solutions of pH 6.0, 7.4, and 9.0. The release of insulin was suppressed in the first 300 min, irrespective of the pH of the solution. After the inductive period, the release rate of insulin depended on the solution pH. The release was faster at pH 6.0 than at pH 7.4 and 9.0. This may arise from the difference in solubility of $CaCO_3$ microspheres at pH 6.0–9.0. In insulin-containing $CaCO_3$ microspheres, the $CaCO_3$ core dissolves in solutions of pH 6.5 or lower, whereas $CaCO_3$ is practically insoluble at higher pH [23]. The insulin is probably released from the $CaCO_3$ microspheres at the same time as the $CaCO_3$ core partially dissolves. Figure 2 shows SEM images of insulin-containing $CaCO_3$

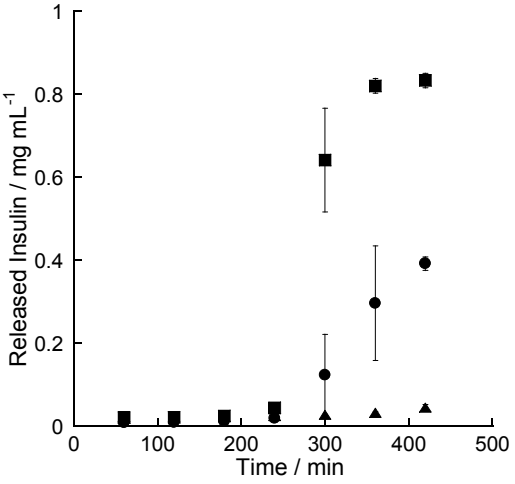
microspheres before and after the microspheres were immersed in the buffer solution at pH 7.4. The as-prepared $CaCO_3$ microspheres were spherical with a rough surface, which is typical for vaterite morphology [24]. However, after soaking the $CaCO_3$ microspheres in the buffer solution, the microspheres changed to rhombohedral crystals characteristic of calcite [25]. It is clear that the phase transition in the crystal form of $CaCO_3$ occurred during the insulin release in the buffer solution as a result of the simultaneous partial dissolution of $CaCO_3$ microspheres and precipitation of calcite crystals. A similar phase transition in $CaCO_3$ microspheres has recently been reported [24]. The crystalline phase of $CaCO_3$ readily changes from metastable vaterite to stable calcite in solution [26,27]. These results suggest that the dissolution of the $CaCO_3$ core is involved in determining the release rate of insulin from the microspheres.

Table 1. Preparation of insulin-containing $CaCO_3$ microspheres [(1)].

Insulin in $CaCl_2$ Solution (mg/10 mL)	$CaCO_3$ Precipitated [(2)] (mg)	Insulin Loading in $CaCO_3$ [(2)] (mg/g)
0.5	185	1.4 ± 0.3
1.0	184	2.6 ± 0.4
2.0	188	4.4 ± 0.2
5.0	186	17.5 ± 0.8

(1) $CaCO_3$ microspheres were prepared by mixing 0.2 M Na_2CO_3 (10 mL) and 0.2 M $CaCl_2$ (10 mL) containing insulin (0.5–5.0 mg); (2) Average values of three preparations are listed.

Figure 1. Amount of insulin released from uncoated $CaCO_3$ microspheres in buffer solutions at pH 6.0 (■), 7.4 (●), and 9.0 (▲). Average values of three measurements are plotted.



The surface of insulin-loaded $CaCO_3$ microspheres was coated with LbL films consisting of PAH and PSS to evaluate the effect of LbL film coatings on the insulin release. Figure 3 shows the ζ-potentials of LbL film-coated $CaCO_3$ microspheres as a function of the number of bilayers. The unmodified microspheres showed a negative potential, and the potential was reversed upon deposition of first PAH layer because of the positive charge of PAH. The sign of the ζ-potential alternated depending on the sign of electric charges of polymeric materials deposited on the outermost surface of the microspheres, suggesting the successful formation of the LbL film coatings on the surface of the

microspheres [28]. It is reasonable to assume that PAH and PSS are deposited on the surface through electrostatic bonds. Figure 4 shows SEM images of $(PAH/PSS)_5$ film-coated $CaCO_3$ microspheres, in which microspheres are well-dispersed without significant aggregation. The partial aggregation of the microspheres observed in the SEM images might probably be caused during drying process for preparing SEM samples.

Figure 2. SEM images of insulin-containing $CaCO_3$ microspheres (**a**) before and (**b**) after the microspheres were immersed in buffer solution for insulin release.

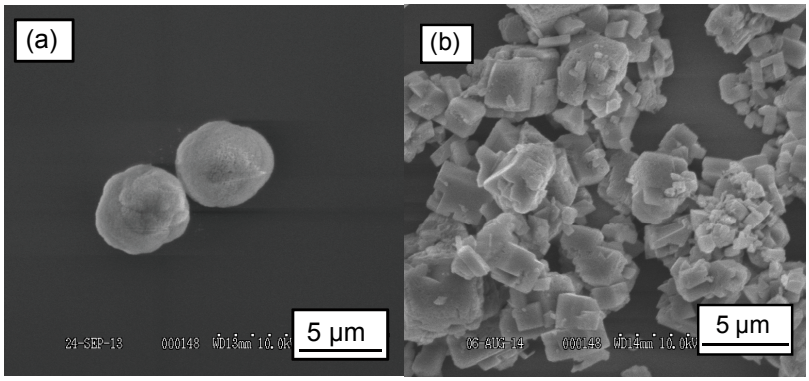


Figure 3. ζ-Potentials of $(PAH/PSS)_n$ film-coated $CaCO_3$ microspheres at pH 7.4. The average values of ζ-potentials for *ca*. 50 particles are plotted with standard deviations. The outermost surface of the microspheres was covered with PSS for the integer bilayer numbers.

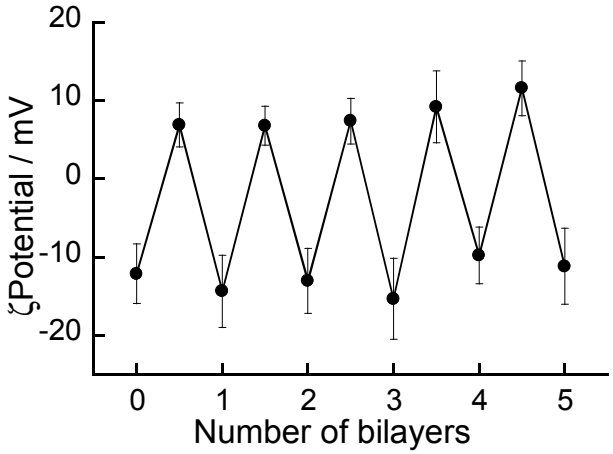


Figure 5 shows the effects of LbL film coatings on the release of insulin from $CaCO_3$ microspheres. The LbL film coatings significantly suppressed the release of insulin. The amount of insulin released from the $(PAH/PSS)_1$ film-coated $CaCO_3$ microspheres after 7 h was approximately 40% of that released from uncoated microspheres, showing the substantial effect of the film coating. The effects of thicker $(PAH/PSS)_3$ and $(PAH/PSS)_5$ films were more significant; the amount of released insulin after 7 h was less than 5% of that released from uncoated microspheres. These results suggest that the transport of insulin across the LbL films determined the overall release rate from the microspheres. The significant variations in the amounts of released insulin from uncoated $CaCO_3$ microspheres at 300 and 360 min may result from the fact that a burst release of insulin occurred at this stage after

induction period. The effects of the LbL film coating and its thickness on the stability and permeability of ions and drugs have been reported [29–34]. However, the suppressive effect of the film coatings on the release is more clearly demonstrated here for insulin, probably because of the large size of the protein drug. The phase transition of $CaCO_3$ microspheres to calcite crystals during the insulin release was also observed for the LbL film-coated $CaCO_3$ microspheres (data not shown). Thus, the release rate of insulin from $CaCO_3$ microspheres can be regulated by coating the surface of microspheres with LbL films.

Figure 4. SEM images of $(PAH/PSS)_5$ film-coated $CaCO_3$ microspheres.

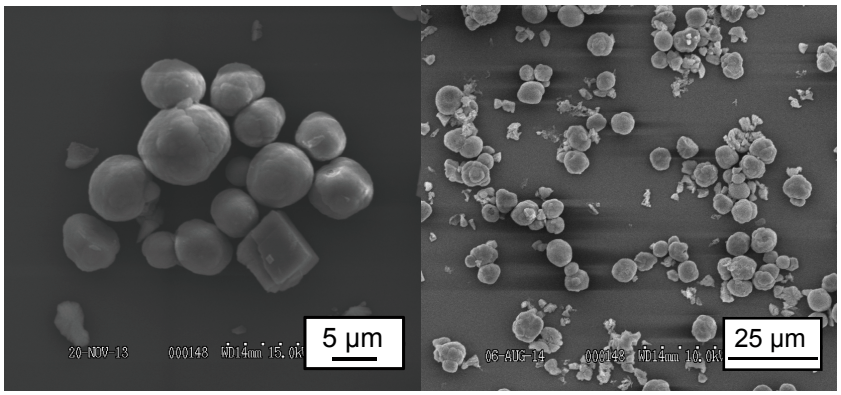
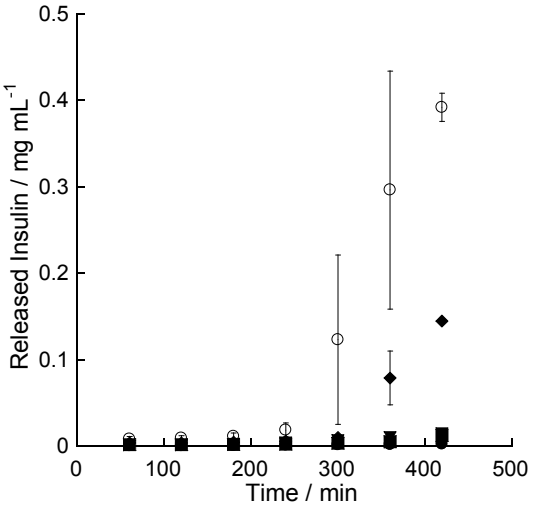


Figure 5. Amount of insulin released from $(PAH/PSS)_n$ film-coated $CaCO_3$ microspheres in buffer solutions at pH 7.4. The number of bilayers (n): 0 (○), 1 (◆), 3 (■), and 5 (●). Average values of three measurements are plotted.



4. Conclusions

We have prepared insulin-containing $CaCO_3$ microspheres with and without polymer film coatings. The release of insulin from the microspheres depended on the pH of the medium and the thickness of the polymer film coating on the surface. The release rate of insulin from uncoated $CaCO_3$ microspheres was faster at pH 6.0 than in neutral and basic solutions, probably because of the higher solubility of $CaCO_3$ in weakly acidic solutions. SEM images showed that a phase transition in $CaCO_3$ microspheres from vaterite to calcite crystals occurred during the release of insulin in the solution.

The polymer thin films on the surface of the $CaCO_3$ microspheres substantially suppressed the release of insulin, depending on the thickness of the films. The results suggest LbL film coatings are effective for regulating the release rate of macromolecular drugs such as insulin from $CaCO_3$ microspheres.

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Author Contributions

All authors were involved equally in the experimental works and the manuscript preparation.

Conflicts of Interest

The authors declare no conflict of interest.

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