Enzyme-Responsive Release of Encapsulated Proteins from Biodegradable Hollow Capsules

Yuki Itoh,[†,‡] Michiya Matsusaki,[†,‡] Toshiyuki Kida,[†,§] and Mitsuru Akashi*[,†,‡,§]

Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan, Center of Excellence (COE) Program for 21st Century, Osaka University, Japan, and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

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Biodegradable hollow capsules encapsulating proteins were prepared via layer-by-layer assembly of chitosan and dextran sulfate on protein-entrapping mesoporous silica particles and the subsequent removal of the silica. The enzymatic degradation of the capsules in the presence of chitosanase was explored by scanning electron microscopy (SEM). With increasing time, the chitosan component was degraded by chitosanase, and the capsules began to deform and were finally destroyed. Sustained release of the encapsulated proteins was attained by using the enzymatic degradation of the hollow capsules. The release behavior was successfully manipulated by altering the charge of capsule surface.

Introduction

Recently, the development of new drug carriers for drug delivery systems (DDS) have been actively pursued in order to construct new controlled and sustained release systems.[1−5] In particular, particles are applicable to DDS because of possessing many properties such as drug delivery or controlled drug release.[3−5] Hollow capsules prepared by the layer-by-layer (LbL) assembly[6] of oppositely charged polyelectrolytes have attracted a great deal of attention as novel drug carriers, since their diameters, membrane thickness, and permeability can be easily controlled.[7,8]

In general, drug release from hollow capsules can be controlled by changes in the membrane permeability based on changes in the physicochemical properties of the membrane constituent polymers, which respond to stimuli such as pH or ionic strength.[9−17] In most of these capsules, however, the release of encapsulated drugs was a rapid "burst" release due to a rapid and large change in the capsule permeability induced by physiological triggers. For example, Sukhorukov and co-workers reported that encapsulated peroxidase was completely released from dextran sulfate/protamine capsules within 1 h by a change in pH.[16] Caruso and co-workers reported that catalase was released from poly(L-lysine)/poly(L-glutamic acid) capsules within 30 min by pH and ion strength changes.[18] Thus, it has been difficult to achieve sustained release of encapsulated drugs from hollow capsules. This point is a serious drawback for the application of hollow capsules as drug carriers. To solve this problem, we propose a novel strategy for the sustained release of encapsulated drugs from hollow capsules. Our group previously demonstrated that ultrathin biodegradable polymer films coated onto a Au plate can be gradually degraded by a specific enzyme.[19,20] Therefore, using the enzymatic degradation of the capsule membrane, the membrane thickness and the permeability of the capsule can be expected to be gradually changed. Therefore, sustained release can be accomplished by optimizing the conditions of the enzymatic degradation of capsule membranes.

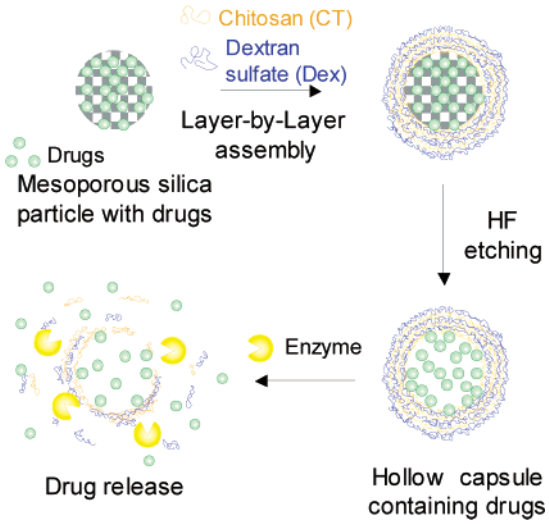

Figure 1. Schematic illustration outlining the preparation of the biodegradable hollow capsules and the encapsulated-drug release system due to enzymatic degradation.

In this study, we report a novel sustained release system in which the encapsulated proteins are released over a long period from hollow capsules by the enzymatic degradation of the capsule membranes (Figure 1). We previously reported the preparation of biodegradable hollow nanocapsules by a combination of the LbL assembly of chitosan (CT) and dextran sulfate (Dex) and the silica template method.[21] Mesoporous silicas (MS), which have the appropriate pore sizes for these proteins, were chosen as a template for incorporating the proteins into the capsules.[18,22,23] We demonstrated an effect of capsule surface charge on protein release profile. There have never been any reports that proteins were released from biodegradable hollow capsules due to enzymatic degradation, and we reported

* To whom correspondence should be addressed. Tel.: +81-6-6879-7356. Fax: +81-6-6879-7359. E-mail: akashi@chem.eng.osaka-u.ac.jp.

† Department of Applied Chemistry, Graduate School of Engineering, Osaka University.

‡ Center of Excellence (COE) Program for 21st Century, Osaka University.

§ Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST).

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the basic research results on the enzymatic degradation of hollow capsules and controlled protein release from the capsules.

Experimental Section

Materials. Chitosan (CT, $M_w = 6.5 \times 10^5$), dextran sulfate (Dex, $M_w = 5.0 \times 10^5$), sodium chloride (NaCl), and hydrofluoric acid (HF) were purchased from Wako Pure Chemicals Inc and were used without further purification. Chitosanase (835 units g^{-1}) was purchased from Wako and was dissolved in 0.1 M acetic acid buffer (pH 5.6) before use. Fluorescein isothiocyanate labeled albumin (FITC-albumin) was purchased from Sigma Aldrich.

Preparation of Biodegradable Hollow Capsules. Mesoporous silicas (MS) were exposed to protein solution (FITC-albumin, 1.0 mg mL^{-1}, pH 5.6) for 12 h at 4 °C. The MS were then immersed into an aqueous CT solution (1.0 mg mL^{-1}, 0.5 M NaCl) containing 25% formic acid for 15 min at ambient temperature. The excess polyelectrolytes were removed by washing with high-purity water three times. Next, the MS were immersed into an aqueous Dex solution (1.0 mg mL^{-1}, 0.5 M NaCl) for 15 min, and the same procedure was repeated six or seven times. The MS template cores were dissolved by the treatment with 2.0 M HF solution at ambient temperature for 1 h, followed by washing seven times with high-purity water.

Enzymatic Degradation and Protein Release. Protein-loading hollow capsules (1.0 mg) were added to 10 mL of chitosanase solution (3.3 μM, 100 mM acetic acid buffer pH 5.6, 4 °C or 37 °C) or acetic acid buffer solution without chitosanase. After a predetermined time interval, the suspension was fractionated and centrifuged. The amount of protein released from the hollow capsules was determined by measuring the fluorescent intensity of the supernatant.

Characterization. The microelectrophoresis of MS dispersed in water was measured on a Malvern Zetasizer 2000 instrument. Fourier transform infrared spectroscopy (FT-IR) spectra of these capsules were recorded using Perkin-Elmer Spectrum One. Inductively coupled plasma (ICP) analysis was recorded using JOVIN YVON, ULTIMA2. Scanning electron microscopy (SEM) observations were performed using a HITACHI S-800 electron microscope. Confocal microscopic images were taken with an Olympus confocal system equipped with a 100× oil immersion objective. The UV/vis absorbance spectra were recorded with a JASCO FD-6500 spectrophotometer. The fluorescent intensity was measured with a Pharmacia Biotech Ultraspec 2000 spectrofluorometer. Quartz crystal microbalance (USI System, Japan) measurements were carried out using an Iwatsu model SC7201device.

Result and Discussion

Fluorescein isothiocyanate-labeled albumin (FITC-albumin) was used as the model protein drug. The model protein-entrapping hollow capsules were prepared via the LbL assembly of CT and Dex onto the protein-entrapping MS as template core. The assembly of the $(CT/Dex)_3$ ultrathin polymer films on the MS was confirmed by microelectrophoresis (pH 5.6). The MS coated with CT/Dex ultrathin polymer films yielded an alternating ζ-potential of ca. +20 mV (CT-outermost surface) and ca. −30 mV (Dex-outermost surface). This result suggests that sequential CT/Dex ultrathin polymer films were formed on the MS (Figure 2a). We obtained hollow capsules after the removal of the MS from the CT/Dex polymer films-coated MS by HF etching. The complete removal of silica was confirmed by Fourier transform infrared spectroscopy (FT-IR) and inductively coupled plasma (ICP) analysis (data not shown). The shapes of the hollow capsules were then observed by scanning electron microscopy (SEM) (Figure 2b,c). The hollow capsules maintained their spherical shape in the dry state without puncturing. The long-term sonication of hollow capsules generated holes,

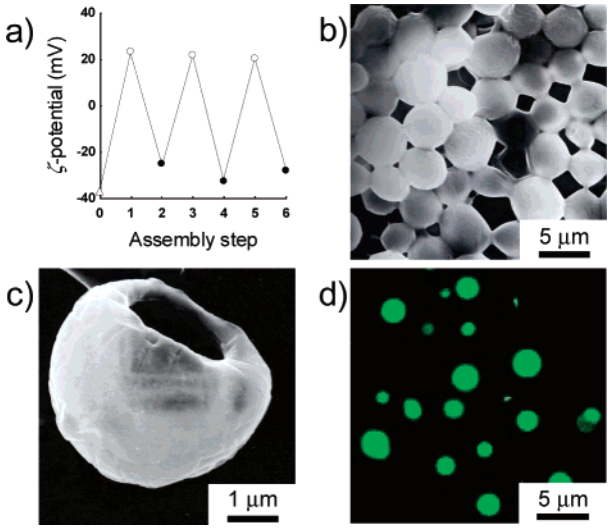

Figure 2. ζ potential of MS (open triangle) coated with CT (open circles)/Dex (filled circles) ultrathin polymer films (a), SEM images of $(CT/Dex)_3$ hollow capsules (b, c), and confocal microscopic images of $(CT/Dex)_3$ hollow capsules incorporating FITC-albumin (d). Panels b and c show the images of capsules before and after sonication, respectively.

clearly indicating that the obtained particles have a hollow structure (Figure 2c). The confocal microscopic image shows that FITC-albumin is incorporated into the hollow capsules (Figure 2d). Since the size of albumin is relatively large, its encapsulation into hollow capsules was successfully carried out without any leakage. The amount of encapsulated FITC-albumin was estimated to be 104 μg per 1.0 mg of hollow capsules based on their fluorescent spectra. On the other hand, it was difficult to encapsulate rhodamin 6G as a model of low molecular weight molecules, because most of rhodamin 6G molecules were leaked from hollow capsules during the preparation process.

We examined the enzymatic degradation and shape changes of $(CT/Dex)_3$ hollow capsules. Chitosanase was employed as a model enzyme in this process. The enzymatic degradation of CT was determined with the Schales reagent[24,25] by a relative analysis of the amount of reductive ends in CT in the supernatant following enzymatic degradation (see the Supporting Information). To study the effect of enzymatic degradation on the shape of $(CT/Dex)_3$ hollow capsules, we performed SEM observations of the hollow capsules before and after enzymatic degradation (Figure 3). Hollow capsules exposed to a chitosanase solution (3.3 μM, pH 5.6, 37 °C) for specific time periods were fractionated, and the shapes of the obtained capsules were observed. The shapes of the hollow capsules were spherical before the enzymatic reaction (Figure 3a). After reaction for 5 min, however, the spherical shapes changed to concave shapes including folds (Figure 3b,c). The CT/Dex ultrathin polymer films were desorbed from the capsule surface by enzymatic degradation of CT, possibly due to the formation of the water-soluble polyion complex between degraded CT and Dex.[19,20,27] It seems that the capsule membrane thickness and strength became weaker with the progress of enzymatic degradation, leading to a shape change to a concave capsule. The longer the reaction time, the more wrinkled the hollow capsule shapes became. After 24 h, almost all of the hollow capsules were destroyed (Figure 3d). On the other hand, the shapes of capsules immersed in the same buffer without chitosanase and those immersed in the enzyme solution at 4 °C were not changed (data not shown). These observations suggest that the degradation and deformation of hollow capsules incubated in a

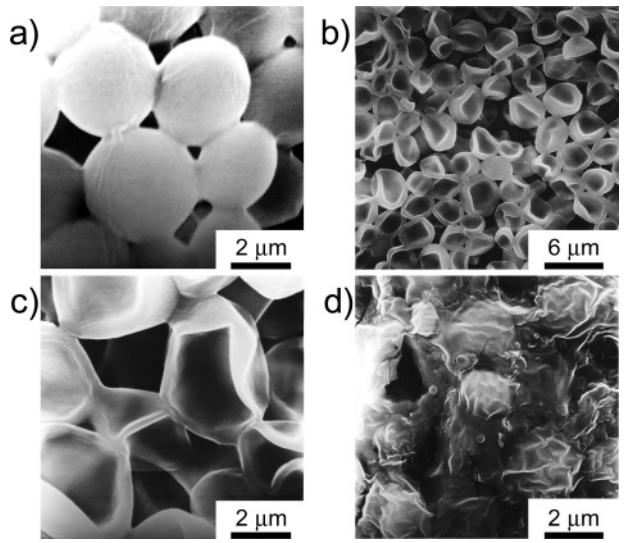

Figure 3. SEM images of the $(CT/Dex)_3$ hollow capsules before (a) and after (b−d) enzymatic degradation at 37 °C respectively for 5 min (b, c), and for 24 h (d). Panel c is a higher magnification of panel b.

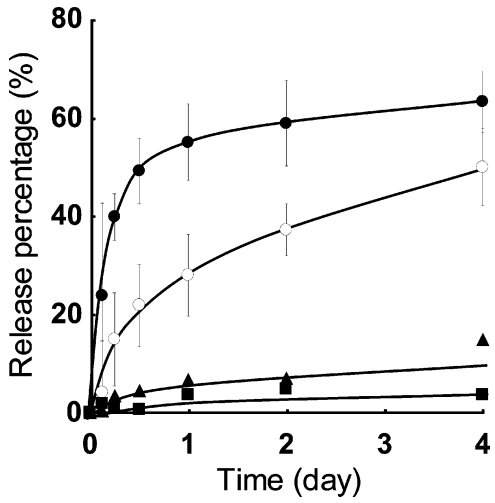

Figure 4. Release profile of FITC-albumin from $(CT/Dex)_3$ hollow capsules (Dex surface) (filled circles) and $(CT/Dex)_3$/CT hollow capsules (CT surface) (open circles) at 37 °C in the presence of chitosanase, $(CT/Dex)_3$ hollow capsules at 4 °C (filled squares) in the presence of chitosanase, and at 37 °C in the absence of chitosanase (filled triangles). The results in the presence of chitosanase at 37 °C are presented as means ±SD ($n = 3$). The other results represent the average mean from two separate experiments.

chitosanase solution occurred due to the enzymatic degradation of CT in the capsule membranes.

By exploiting the biodegradable property of the hollow capsules, it should be possible to control the release of encapsulated proteins from the hollow capsules by controlling the enzymatic degradation of the capsule membranes. The release behaviors of encapsulated proteins from the hollow capsules were examined in the presence and absence of chitosanase. The hollow capsules encapsulating FITC-albumin were immersed into a chitosanase solution (4 and 37 °C), and the amount of released FITC-albumin was determined by the fluorescent spectra of the supernatant (Figure 4). The hollow capsules were not expected to be degraded by chitosanase at 4 °C, a temperature at which chitosanase is inactive. Thus, the little amount of released proteins at 4 °C was observed. At this temperature, the stable polyion complex between the CT and the Dex prevented the protein from penetrating through the capsule membrane. In the case of the absence of chitosanase at 37 °C, a small amount of protein was released. This result is due to increasing the capsule permeability with raising the medium temperature.[26] On the other hand, when the enzyme was activated at 37 °C, remarkable protein release was observed, indicating that the degradation of the hollow capsules had occurred. The amount of protein released from the hollow capsules reached 63% after 4 days. The complete release of protein was prevented, possibly due to the entrapment of some of the protein molecules into the capsule membrane and/or degraded chitosan, likely through electrostatic interactions between the protein and the polyelectrolytes. The release behavior of encapsulated protein saturated after a day. The result was in agreement with the data of SEM observation that almost all of the capsules were destroyed due to enzymatic degradation after a day (Figure 3). These results showed that the release of the encapsulated protein triggered by enzymatic degradation was successful.

To achieve sustained release, one must control the degradability of the hollow capsules. We also prepared $(CT/Dex)_3$/CT hollow capsules, which have a cationic CT surface via LbL assembly (7 steps), and then studied the release behavior of the encapsulated protein from the capsules. In the case of using $(CT/Dex)_3$ hollow capsules (Dex surface), the amount of protein released from the hollow capsules reached 55% and 63% after a day and 4 days, respectively. On the other hand, in the case of a cationic CT surface, the release rate was decreased: the amount released reached only 28% after a day and 50% after 4 days. These unique results can be explained by considering the difference in the surface charge of hollow capsules.[19] The surface charge of hollow capsules has a large effect on the adsorption of chitosanase onto the capsule surface. In the buffer solution (pH 5.6) examined here, chitosanase (pI 9.3) is cationic. Therefore, larger amounts of chitosanase should adsorb onto the anionic Dex surface due to the electrostatic interactions, rather than onto the cationic CT surface, leading to the faster enzymatic degradation of the capsules membrane with a Dex surface. The difference in the amount of chitosanase adsorbed onto $(CT/Dex)_3$/CT hollow capsules (cationic CT surface) or $(CT/Dex)_3$ hollow capsules (anionic Dex surface) was calculated from the frequency shifts of quartz-crystal microbalance (QCM). The weights of the chitosanase on the capsules (1.0 g) with CT surface and Dex surface were 4.5×10^{-2} and 9.6×10^{-2} g, respectively. The amount of chitosanase adsorbed onto the CT surface was half of that onto the Dex surface. The smaller amount of adsorbed chitosanase leads to the slower enzymatic degradation of the hollow capsules. These results imply that the release behavior can be controlled by altering the surface charge of the hollow capsules. Using CT surface hollow capsules, a longer sustained release than that on the Dex surface was attained. In most of the previous hollow capsules, the encapsulated drugs were almost completely released in response to the pH or salt concentration within a few minutes to a few hours.[9−17] This type of rapid "burst" release should be overcome by using our system triggered by enzymatic degradation.

Conclusion

We have developed a novel enzyme-responsive controlled and sustained release system utilizing the enzymatic degradation of biodegradable hollow capsules. Protein-encapsulating hollow capsules were successfully prepared by a combination of LbL assembly of oppositely charged biodegradable polyelectrolytes

and protein-entrapping mesoporous silica templates. The release profile of the encapsulated protein due to enzymatic degradation of the hollow capsules can be controlled by altering the surface charge of the hollow capsules and can be sustained for a longer period. This system possesses the favorable features, such as stimuli-responsive controlled release and prolonged sustained release, for the construction of an effective DDS. The release system, in response to specific enzymes present locally in the diseased organ of a living body, would be useful for local and sustained drug release. We believe that altering the thickness of capsule membranes or the kind of biodegradable polymers constructing the capsules may be able to achieve the efficient protein release. Studies on the entrapped-protein release from biodegradable hollow capsules by using enzymes in mammals, such as lysozyme, and the capsule uptake by cells are now in progress.

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Supporting Information Available. Details of the enzymatic degradation of chitosan in the hollow capsules and analysis of the amount of reductive ends in chitosan with the Schales reagent. This materials is available free of charge via the Internet at http://pubs.acs.org.

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