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The pH regulated phycobiliproteins loading and releasing of polyelectrolytes multilayer microcapsules

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ABSTRACT

The polyelectrolytes multilayer microcapsules considered as a good matrix can meet the requirements of protein encapsulation and release. It is important to understand the factors affecting the encapsulation and release of proteins in capsules. In this study, the eight layers hollow capsules $(PSS/PAH)_4$ and nine layers hollow capsules $(PSS/PAH)_4PSS$ are fabricated. The protein, R-Phycoerythrins (R-PEs) is employed as a probe instead of fluorescein isothiocyanate labeled proteins to investigate protein loading capacities on capsules as a function of pH, since R-PEs demonstrate an excellent stability over a broad pH range. The loading capacities of R-PEs on capsules $(PSS/PAH)_4$ or $(PSS/PAH)_4PSS$ are demonstrated to be sensitive to pH. The R-PE encapsulated in capsules exhibit the largest load capacity around isoelectric point of the protein independent of outer most layer of polyelectrolytes. However, if the pH of buffer is far away from the isoelectric point of the protein, they are absorbed on the surface of capsules. Based on a Freundlich model, capsules take up proteins on their surface by monolayer adsorption. The release process of R-PEs from microcapsules to solution is also shown to be sensitive to pH. Proteins show a faster release process around isoelectric point. Therefore, the pH sensitive polyelectrolyte microcapsules may offer a promising delivery system for loading and releasing proteins in biological systems depending on environment.

1. Introduction

Proteins and peptides are currently the fastest growing class of prophylactic and therapeutic molecules. Nevertheless, inherent instabilities of protein under certain conditions are obstacles to achieve a successful application in medical fields [1]. Biological macromolecules such as proteins are sensitive to external conditions such as temperature and pH of the medium. In order to stabilize the macromolecule and keep them functional, it is necessary to protect them by a polymeric matrix to avoid denaturalization and degradation in the process of manufacturing and storage [2]. If one understands the interactions between polymeric matrix and biomolecules, it would aid in techniques such as bioencapsulations, biosensors, bioreactors, enzyme immobilization and drug release applications [3].

The polyelectrolyte multilayer microcapsules can meet the requirement of protein delivery [4]. Microcapsules have been fabricated via adsorption of oppositely charged polyelectrolytes onto the surface of colloidal particles with subsequent decomposition and removal of the core. The main driving forces for organizing layer-by-layer self-assembly are electrostatic interactions. The encapsulation of molecules in polymeric capsules is accomplished based on a fact that capsules have semipermeable properties. Fabricated capsules are not only permeable for small molecules, but also allow large biological macromolecules to diffuse into the interior of the capsule under certain circumstances. The permeability of polyelectrolyte multilayer capsule can usually be regulated by some exterior factors such as pH, temperature, ionic strength [5]. In addition, polyelectrolyte multilayers have strong affinities for proteins independent of the charge for either of them possess [6,7]. However, in many cases, biomolecules are adsorbed on the surface of capsule rather than diffused into the interior of the capsule due to surface charges, ionic strength and entropic force. Therefore, identification the factors that essentially affect protein encapsulation and release of polyelectrolytes multilayer microcapsules is an important task. Although polyelectrolyte multilayer capsules can be used as a potentially ideal carrier for protein bioencapsulation and drug delivery, only scattered studies have been devoted to this subject. Antipov et al. built a pH dependent capsule consisting of 4 PSS/PAH bilayers to have either an open or a closed state. FITC-labeled Albumin is shown to permeate into the capsules at low pH, but are excluded at a pH higher than 8 [8]. Similarly, Anandhakumar et al. fabricated the $(PAH/PMA)_2$ hollow capsules, which also shows reversible pH dependent open and closed state for FITC-Dextran and FITC-BSA. The hollow capsules are in open state at pH < 4 and in close state at pH > 5. FITC-BSA can be spontaneously encapsulated

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into capsule at pH < 4 [9]. Georgieva et al. had studied permeability of $(PSS/PAH)_2PSS$ prepared by coating red blood cells with the polyelectrolytes applying the layer-by-layer technique with subsequent dissolution of the core. Although permeability of capsules is reported to be dependent on salt concentration, no pH dependence for permeation is observed in their studies [10]. As described in examples earlier, FITC are widely used fluorescent labeling reagents for proteins. It is conjugated to proteins via primary amines thereby modifying chemical structure of the native proteins. This modified system in particular can influence their hydrophilic–lypophilic balance and isoelectric point [11]. In addition, FITC is unstable and easily cause self-quenching to reduce brightness. Fluorescence intensity of FITC is strongly vulnerable to changes in pH value [12]. Therefore accurate reading of bioencapsulation of the FITC labeled protein may differ essentially from that of natural protein if the bioencapsulation behaviors are controlled by surface charge of protein and external pH values. In this study, the eight layers hollow capsules $(PSS/PAH)_4$ (positively charged) and nine layers hollow capsules $(PSS/PAH)_4PSS$ (negatively charged) are fabricated. The native fluorescent protein, R-Phycoerythrin (R-PE) is selected to examine protein loading capacity on above-mentioned capsules regulated by pH.

The R-PE found in red algae is a phycobiliprotein [13]. It plays a crucial role in efficiently harvesting sunlight and then transferring this energy via a series of energy transfers in photosynthesis process [14]. The R-PE has optimal quantum yields (up to 98%), extinction coefficients and larger stokes shifts. The fluorescence of R-PE is not quenched by external agents. More importantly relevant to current studies, R-PE also has excellent stability over a broad pH range [15], since the fluorophores is protected by covalent binding to the protein backbone. R-PE therefore is more suitable choice for studying protein's loading capacity of capsules as a function of pH. The results presented here should advance our understanding of the loading properties of other biomolecules in the real situations. Moreover, polymeric matrix provides a mild route to shield the active molecule from the potentially harsh conditions for many practical applications.

2. Experimental

Potassium dihydrogen phosphate (Analytic grade), dipotassium hydrogen phosphate (Analytic grade), melamine (>99%), hydrochloric acid, sodium hydroxide, sodium salt, hydrogen peroxide (30%) and ammonium hydroxide were bought from Beijing chemical reagent company. Sodium poly(styrene sulfonate) (PSS, $M_w \sim 70{,}000$), and poly(allylamine hydrochloride) (PAH, $M_w \sim 50{,}000$) were obtained from Aldrich. The R-Phycoerythrin crystal from *Polysiphonia urceolata* was purchased from Hongrui biotechnology Co. Ltd. All chemicals were used as received without further purification. The hollow polyelectrolyte microcapsules with size around 3–4 μm, were prepared by layer-by-layer (LbL) adsorption technique, based on template of weakly crosslinked melamine formaldehyde (MF) latex microspheres according to a reported method [16–18]. Both eight layers hollow capsules $(PSS/PAH)_4$ and nine layers hollow capsules $(PSS/PAH)_4PSS$ were prepared in this work. The water used in all experiments was obtained in a three stage Millipore Milli-Q Plus 185 purification system and had a resistivity higher than 18.2 MΩ cm. The transmission electron microscopes are employed to determine the morphology of coated MF particles and capsules (JEM-1400 and JEM-100CX, Japan JEOL Company). The morphology of polyelectrolytes multilayer microcapsules has been given as supplementary information. Sample fluorescence imaging was measured on a confocal laser scanning microscope (Model: Leica TCS SP2, Germany Leica Company). The excitation wavelength was 488 nm. Microscope cover glasses were first cleaned by sonicating sequentially in 10% NaOH, acetone, and deionized water each for 20 min and then subjected to treatment according to a reported protocol in which cover glasses were immersed into a solution that contains H_2O_2, NH_4OH, and H_2O at 1:1:5 volume ratio for 20 min at 80 °C [19]. Afterward, the cover glasses were rinsed extensively with deionized water and dried with nitrogen gas. Absorption spectra at wavelength range of 400 nm to 800 nm were measured on a UV2001 UV-Vis spectrophotometer (Hitachi, Japan). Fluorescence emission spectra were recorded on a F4500 spectrofluorimeter (Hitachi, Japan). The pH adjustment was performed in solution containing 20 mM potassium phosphate buffer (pH 7.0) with HCl and NaOH modulating the pH of the solution. The ζ-potential of capsules was determined in buffer solution by a ζ-potential analyzer at 25 °C (Zetasizer Nano ZS90, Malvern, UK). The microcapsule number was determined using a counting chamber (hemocytometer, Qiu Jing, China).

The 100 μl (about $1.9 \pm 0.04 \times 10^7$ capsules, in desired pH phosphate buffer) hollow capsules suspension was mixed with 100 μl (0.044 mg/ml in desired pH phosphate buffer) R-PEs solution in an Eppendorf tube. The mixture was incubated for 30 min at 4 °C in the dark to allow adequate diffusion and accumulation of R-PE in the microcapsules. The heterogeneous solution was centrifuged. The supernatant was transferred to a clean centrifuge tube and diluted with 1800 ml of desired pH phosphate buffer. For reference samples (100 μl 0.044 mg/ml R-PEs and 1900 μl required pH phosphate buffer) were mixed and incubated for 30 min at 4 °C to obtain the initial concentration of R-PE used for the each experiment and to keep conditions constant. Both the sample and the reference solutions were subjected for the fluorescence spectral measurement. All fluorescent spectra were recorded under the same conditions.

For the release studies the R-PEs loaded capsules (100 μl) were mixed with 900 μl phosphate buffer (room temperature), aliquots were removed in time dependent manner over 24 h. The diffusion process of protein from hollow polyelectrolyte microcapsules to solution were detected by fluorescence emission wavelength of 578 nm at a performed pH.

The loading capacity is calculated by a using the following formula:

$$\text{Loading capacity} = \frac{[c]N_A}{M_w[N]}$$

where $[c]$ is concentration of loaded protein in mg/ml, M_w is the molecular weight of the loaded protein in g/gmol, $[N]$ is the number of capsules per liter, and N_A is the Avogadro's number.

3. Results and discussion

3.1. Effect of pH on the relative fluorescence intensity of FITC and R-PE

FITC is derivative of fluorescein with high fluorescent quantum yield and high photostability. The effect of pH on the relative fluorescence intensity of FITC is shown in Fig. 1. The relative fluorescence intensity of FITC increases with pH in the range of 3.0–10.0, then decreases in the range of pH 10.0–13.0. The R-PE on the other hand shows relatively stable fluorescence intensity over the pH range of 3.5–10. Outside of this range, a faster decrease in fluorescence intensity is observed. Moreover, a circular dichroism spectrum verifies that major R-PE structures are always remain in α-helix form (more than 95%) in the range of pH 3.5–10. No β-sheet structure is detected. When pH is lower than 3.5 or higher than 10, amounts of α-helix dramatically decrease, while amounts of β-sheet and random coil structures increase. Therefore, effect of pH on the secondary structure of R-PE can be neglected in the range of 3.5–10 [14]. Thus R-PE is more appropriate for the study of loading capacity of capsules as a function of pH.