Fabrication of bovine serum albumin microcapsules by desolvation and
destroyable cross-linking

Yi Zhu, a,b Weijun Tong, a,b Changyou Gao a and Helmuth Möhwald b

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Bovine serum albumin (BSA) mono-component microcapsules were fabricated by a method of
desolvation and destroyable cross-linking. Desolvation of BSA from its aqueous solution was achieved
by dropwise addition of ethanol. The dissolvable content of BSA in the supernatant decreased along
with the increase of ethanol volume. Adsorption of the desolvated BSA onto MnCO3 microparticle
surfaces and further cross-linking with disulfide-containing dithiobis(succinimidylpropionate)
smoothed the rough surfaces of the MnCO3 microparticles. Hollow and intact microcapsules were
obtained after core removal at low pH. Wall thickness of the microcapsules could be controlled by the
amount of ethanol. Measured by confocal laser scanning microscopy, about 70% of these microcapsules
were shown to be impermeable to fluorescein isothiocyanate-labeled dextran with a molecular weight of
2000 kDa. Destruction of the BSA microcapsules was achieved under the treatment with a reductive
agent (NaBH4) and ultrasonication. With the ease of fabrication and the features of biocompatibility,
degradability and environmentally controlled destroyability, these microcapsules could be attractive in
applications in the fields of pharmacology, medicine and cosmetics etc.

Introduction

Hollow capsules are of great interest because of their fundamental
importance and potential applications in medicine, drug
delivery, artificial cells or viruses, catalysis and cosmetics.1 A
wide range of methods have been used to fabricate capsules
made of polymer, metal and ceramic, such as the phase-separ-
technique.2,3 Also, emulsion polymerization and interfacial poly-
merization4 have been employed to prepare polymeric hollow
spheres. Recently, the layer-by-layer (LBL) technique has gained
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*Key Laboratory of Macromolecular Synthesis and Functionalization,
Institute of Polymer Science and Engineering, Zhejiang University, Hangzhou, 310027, China. E-mail: cgao@mail.hz.
zi.cn; Fax: +86-571-87951108

ab Max-Planck-Institute of Colloids and Interfaces, Potsdam, 14424, Germany

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The microcapsules in the current work are comprised mainly of cheap, biocompatible proteins and are relatively easy to fabricate. Also, the disulfide bonds in the cross-linkers are readily destroyable in the reductive environment in cells and ultrasonication treatment has been applied in clinical medicine. Thus we believe that they should be potentially useful as drug-delivery vehicles.

**Experimental**

**Materials**

Bovine serum albumin (BSA, $M_w$ 66 kDa), fluorescein isothiocyanate-labeled dextran (FITC-dextran, $M_w$ 2000 kDa), sodium fluorescein (FL), dithiobis(succinimidylpropionate) (DSP), manganese sulfate hydrate, ammonium hydrogen carbonate and sodium borohydride were all obtained from Sigma-Aldrich. BCA Protein Assay Kit was obtained from Novagen. Dimethyl sulfoxide (DMSO) and ethanol were purchased from Merck. All chemicals were used as received. Spherical MnCO$_3$ microparticles with an average diameter of 4–5 μm were synthesized according to a literature procedure by mixing MnSO$_4$ and NH$_4$HCO$_3$ solutions.

**Desolvation of BSA from aqueous solution**

Desolvation of BSA from its aqueous solution was implemented by addition of ethanol. Different volumes of ethanol ranging from 1 to 8 ml were added dropwise to 2 ml BSA aqueous solution (2 mg ml$^{-1}$). The mixtures were kept overnight and the desolvated BSA aggregates were separated by ultracentrifugation at 110 000 g for 40 min at room temperature. The supernatants were collected and diluted 1 : 10 with water. The BSA contents in the supernatants were determined using an Enhanced BCA Protein Assay according to the kit instructions and by referring to the BSA standard calibration curve.

**Fabrication of BSA microcapsules based on desolvation and cross-linking**

Approximately 20 mg of MnCO$_3$ microparticles were dispersed in 2 ml 2 mg ml$^{-1}$ BSA aqueous solution. After the different volumes of ethanol were added dropwise into the suspensions under vigorous shaking, adsorption of the desolvated BSA onto the MnCO$_3$ particles was allowed to proceed for 1 h. The particles were then centrifuged and washed with ethanol 3 times to remove the unadsorbed BSA. The BSA-adsorbed particles were re-dispersed in 4 ml DMSO containing 3 mg DSP for 3 h, followed by 3 cycles of centrifugation and washing in ethanol. Short pulses of ultrasonication were employed occasionally in ethanol or DMSO to keep the particles from possible aggregation. Hollow microcapsules were obtained with a membrane filtration apparatus by decomposing the MnCO$_3$ cores with 0.1 M HCl. The capsules were washed until a constant pH value was reached.

**Destruction of BSA microcapsules**

Typically, 200 μL capsule suspension and 0.2 M phosphate buffer (pH = 7.4) containing 6 mg NaBH$_4$ were mixed. 10 min later, the suspension was subjected to ultrasonication for 30 s. A control experiment was carried out without the use of NaBH$_4$ for 1 min. To illustrate the possible non-covalent forces stabilizing the shells, the BSA-adsorbed MnCO$_3$ particles were incubated in DMSO for 90 min. After being re-suspended in water, the BSA-adsorbed particles were subjected to an online core-removal procedure immediately with 0.1 M HCl and observed simultaneously by CLSM.

**Confocal laser scanning microscopy (CLSM)**

Confocal images were taken with a Leica confocal scanning system mounted to a Leica Aristoplan and equipped with a 100 × oil immersion objective with a numerical aperture (NA) of 1.4. FITC-BSA was used to obtain the capsules for visualization. To investigate permeability of the BSA microcapsules, an equal volume of capsule suspension and FITC-dextran (2 mg ml$^{-1}$) solution were mixed. Observation was carried out 20 min later.

**Scanning electron microscopy (SEM)**

Samples were prepared by applying a drop of the microparticle or capsule suspension onto clean glass slides. After being dried overnight, the samples were sputtered with gold and measured using a Gemini Leo 1550 instrument at an operation voltage of 3 keV.
Scanning force microscopy (SFM)

A drop of the capsule suspension was applied onto freshly cleaved mica and dried overnight at room temperature. The images were obtained using a Digital Instruments nanoscope IIIa Multimode SFM (Digital Instruments Inc., Santa Barbara, CA) in air at room temperature using tapping mode.

UV-vis spectroscopy

Absorption at 562 nm of the samples treated with BCA Protein Assay Reagent was recorded in cuvettes using a Cary 50 UV-vis spectrophotometer.

Ultrasonication

Ultrasonic treatment was performed using a Bandelin Sonorex Digital DK 255 P ultrasonic processor operating at 35 kHz at 20 °C with a power output of 640 W.

Results and discussion

Desolvation process of BSA from its aqueous solution was achieved by dropwise addition of ethanol. After the precipitated BSA was separated by ultracentrifugation, the supernatants were collected, diluted and analyzed using a BCA protein assay. As shown in Fig. 1, the content of dissolved BSA decreased along with the increase of volume ratio of ethanol to water. For example, about 3.7 mg BSA was dissolved when this ratio was 0.5, while the amount decreased to 0.4 mg when this ratio was increased to 4. The BCA protein assay reagent is compatible with a maximum concentration of 10% ethanol, so the interference of ethanol in the diluted samples (1 : 10) could be ruled out. Dynamic light scattering (DLS) measurements, which were conducted immediately after addition of various volumes of ethanol, showed that the desolvated BSA eventually formed particles with a mean size of 80 to 229 nm, depending on factors such as the addition rate of ethanol, the amount of ethanol used etc.

Proteins possess the ability to bind with various kinds of surfaces. Therefore, the desolvated BSA can adsorb onto the MnCO₃ template particles in the presence of the MnCO₃ microparticles. Here we chose the MnCO₃ microparticles instead of others such as CaCO₃ microparticles, because they are more easily re-dispersible after centrifugation. No apparent particle aggregation was observed during the process with the aid of short pulses of ultrasonication. Fig. 2a shows a typical rough texture of the bare MnCO₃ particle surface. After adsorption of the desolvated BSA, the MnCO₃ particle surfaces turn out to be smoother (Fig. 2b), indicating the successful adsorption of BSA. Moreover, energy-dispersive X-ray (EDX) spectroscopy verified the appearance of N and S elements in the BSA-adsorbed particles, which do not exist in the bare MnCO₃ particles. To stabilize the adsorbed BSA molecules, a further cross-linking with DSP – which can react with the amino groups in the BSA molecules – was conducted, resulting in a denser film structure (Fig. 2c). Reaction between the DSP and BSA yields an amide linkage between BSA molecules, which can not be spectroscopically detected since the BSA molecules have abundant amide groups as well as disulfide bonds. Although the direct evidence of cross-linking is absent so far for this system, a very similar reaction between the amino groups and the DSP molecules in DMSO has been confirmed by Gosselin and co-workers using polyethyleneimine. After removal of the MnCO₃ particles with 0.1 M HCl, hollow and intact microcapsules were obtained (Fig. 2d and the later figures). Similar to the capsules fabricated by the LBL technique, these hollow microcapsules collapse because of their thin walls and low mechanical strength and show typical folds and creases, indicating that the MnCO₃ cores were completely removed. However, a rough surface comprised of grains and some defects could usually be observed, which is different from the LBL capsules.

In order to fabricate microcapsules with the best quality, the minimum volume ratio of ethanol to water needed was then determined. Keeping the amount of MnCO₃ particles and DSP constant, parallel experiments were conducted with varied

![Fig. 1](image1.png)

**Fig. 1** Mass of BSA dissolved in the supernatant as a function of volume ratio of ethanol added to water. The original mass of dissolved BSA in 2 ml water was 4 mg. Each value was averaged from 4 parallel measurements.

![Fig. 2](image2.png)

**Fig. 2** SEM images of (a) bare MnCO₃ microparticles, (b) BSA-adsorbed MnCO₃ microparticles, (c) BSA-adsorbed MnCO₃ microparticles further cross-linked with DSP and (d) a hollow BSA microcapsule. The scale bar is 1 μm.
volume ratios, followed by DSP cross-linking. The results showed that more than 90% of the core–shell microparticles turned into hollow shells after online core removal with 0.1 M HCl with a volume ratio of 1. However, when the ratio decreased to 0.5, less than 5% of the particles yielded intact shells after core removal. Comparing the results with the desolvation data (Fig. 1), in which the desolvated BSA mass was about 0.3 mg with a volume ratio of 0.5, while this value increased to ~1.2 mg at a volume ratio of 1, it is reasonable to assume that adsorption of a minimum amount of BSA onto particle surfaces is required for fabrication of intact hollow shells and, for the amount of MnCO₃ microparticles used here (~20 mg), the amount of desolvated BSA at a volume ratio of 1 should be sufficient to form intact shells around the particles. After cross-linking, the shells are then strong enough to resist the osmotic pressure induced during the core-removal process.

Thickness of the capsule walls is important for many of their properties. It has been demonstrated that the amount of desolvated BSA can be controlled by varying the amount of ethanol used for desolvation, which should in turn influence the wall thickness of the resulted microcapsules. Therefore, the wall thickness of the capsules obtained with volume ratios of 1, 1.5, 3 and 4 was measured by SFM. Fig. 3a shows a typical SFM image of BSA microcapsules. Besides the creases and folds, a rather rough surface texture could be observed. The magnified image (Fig. 3b) reveals that the capsule surface is comprised of grains with size ranging from several tens to several hundreds of nanometres. Owing to this feature, the thickness of these capsules was determined by taking an average of all the peak and valley values of a double wall (Fig. 3c). Three parallel measurements were carried out on different “relatively smooth” areas. The mean thicknesses of the microcapsules fabricated with volume ratios of 1, 1.5, 3 and 4 turned out to be 20.1, 24.2, 37.8 and 34.2 nm, respectively. The substantially smaller height (of the order of tens of nanometres measured by SFM) than their size (of the order of hundreds of nanometres measured by DLS) reveals that the particles deform their shape upon adsorption and form spreading morphology. This is reasonable since DLS measures the hydrodynamic radius of BSA particles containing plenty of adsorbed water, while the SFM measures the dry film formed by the desolvated particles with most of the adsorbed water evaporated. Judging from the increment of more than 10 nm (from 20.1 nm to 37.8 nm), it is reasonable to believe that as more BSA molecules are desolvated, more would adsorb onto the particle surfaces, resulting in thicker shells. The adsorption at a lower ethanol concentration (a volume ratio of 1.5, for example) seems to be homogenous, as evidenced by the data of 24.3, 23.8 and 24.4 nm measured at 3 places. However, when a local equilibrium state is reached, further desolvation and adsorption (a volume ratio of 4, for example) could result in an inhomogeneous adsorption, as evidenced by the data of 36.4, 32.3 and 33.9 nm measured at 3 places. The inhomogeneous adsorption as well as the increasing velocity with increasing amount of ethanol may be the reason why the thickness decreases a little when the volume ratio increases to 4. In this case, the mean thickness could no longer be accurately measured. Of course the influence of the template surface roughness on the inhomogeneity of the shell thickness can not be ignored. Since shell thickness is crucial for capsule permeability as well as stability, a volume ratio of 5 was used to fabricate the BSA microcapsules for the studies below.

Permeability is crucial for microcapsules when considering their potential in drug delivery. The permeability of BSA microcapsules was therefore investigated using probes of different

![Fig. 3 SFM images of (a) a BSA microcapsule fabricated with volume ratio of ethanol to water at 1.5, and (b) its magnified surface morphology. (c) A typical section analysis profile for determination of the mean thickness of BSA microcapsules.](image)
molecular weights with CLSM. It was found that all the capsules were instantly permeable to fluorescein, a small molecular probe (Fig. 4b), while about 70% of the capsules were impermeable to FITC-dextran with a $M_w$ of 2000 kDa at least within 20 min, judging from 135 capsules (Fig. 4c). This is probably because the desolvation and adsorption process is very complicated. Also, the surface roughness of templates could possibly affect the shell structure. Thus, there is a high chance of creating relatively large defects in the capsule walls, which could also be observed in the SEM image (Fig. 2d), resulting in a relatively high percentage of “leaky” capsules.

Since the BSA capsules obtained here were cross-linked by DSP containing S–S bonds, one would expect that breaking these S–S bonds under reducing conditions or via a thiol-disulfide exchange reaction may cause destruction of the microcapsules. However, most capsules remained intact even after treatment with excessive NaBH$_4$ for 10 min. Yet the capsules after NaBH$_4$ treatment could be destroyed if they were further subjected to ultrasonication for 30 s, as indicated by the apparent reduction in capsule number as well as the appearance of tiny capsule pieces at the edge of the suspension drop (Fig. 5a). A control experiment without NaBH$_4$ pre-treatment under prolonged ultrasonication treatment for 1 min shows no apparent change in capsule number and most of the capsules remain intact, although a few may be distorted (Fig. 5b). A possible explanation for this observation could be that there are other forces besides the S–S covalent bonding, such as hydrophobic force and hydrogen bonding, which also play a role in stabilizing the capsule structure. After the treatment by excess NaBH$_4$, most of the S–S bonds are destroyed, but these non-covalent forces could still keep the capsules intact. Existence of the additional stabilizing forces is also supported by the fact that a simple incubation of the BSA adsorbed particles in DMSO for 90 min can produce intact capsules, as shown in Fig. 5c taken during an online core-removal process. However, these intact shells could not be obtained by either centrifugation or membrane infiltration. We assume that a partial dehydration of the shells might take place during the incubation in DMSO since DMSO has a lower dielectric constant (47.2) than water (80.0), making the shells more compact and facilitating more non-covalent interaction. Yet this interaction is not strong enough to maintain the hollow structures in water due to the good water solubility of BSA. A similar phenomenon has also been reported by Dejugnat and co-workers.$^{24}$ It has been demonstrated that ultrasonication is effective in damaging polyelectrolyte microcapsules based on electrostatic forces.$^{25}$ Therefore, with further treatment of ultrasonication, the NaBH$_4$ treated microcapsules are destroyed eventually.

**Conclusions**

A facile way has been developed to prepare BSA mono-component microcapsules by desolvation and destroyable cross-linking. Dropwise addition of ethanol can cause BSA to precipitate from its aqueous solution, which adsorbs simultaneously onto MnCO$_3$ microparticles. After cross-linking with disulfide-containing DSP and core removal with HCl, hollow and intact microcapsules are obtained, whose wall thickness can be controlled by the volume of added ethanol. Destruction results reveal that these microcapsules are stabilized not only by covalent bonds but also by other non-covalent forces. In summary,
the microcapsules reported in this work have the following advantages: 1) BSA is cheap, biocompatible, biodegradable and easy to obtain; 2) the fabrication process is relatively simple; 3) the S–S bonds in the cross-linkers are destroyable by organisms and cells; 4) the remaining amino groups provide possibilities for further modification and functionalization. Of course the method has some drawbacks such as low utilizing efficiency of the proteins, too. Nevertheless, the precipitated proteins can be collected and re-used. These microcapsules are attractive for potential applications in the fields of pharmacology, medicine and cosmetics etc., in particular as drug-delivery vehicles.

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