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Ma Xiaoteng, Han Guangda, Zhao Hanying

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Degradable Protein-loaded Polymer Capsules Fabricated by Thiol-disulfide Cross-linking Reaction at Liquid-liquid Interface

Xiaoteng Ma[†], Guangda Han[†], and Hanying Zhao^{*}

Key Laboratory of Functional Polymer Materials, Ministry of Education, College of Chemistry, Nankai University, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin 300071, China

 Electronic Supplementary Information

Abstract In these years, the encapsulation of proteins for protection and delivery purpose has attracted great interest. In this research, W/O emulsion droplets were used as soft templates and bovine serum albumin (BSA) encapsulated hollow capsules were prepared by liquid-liquid interfacial thiol-disulfide exchange reaction. Block copolymer chains with pendant pyridyl disulfide groups are located at liquid-liquid interface, and upon addition of a macromolecular crosslinking agent with multiple pendant thiol groups into an emulsion, thiol-disulfide interfacial crosslinking reactions lead to the formation of BSA encapsulated hollow capsules. The cleavage of disulfides on the membranes results in the degradation of hollow structures and the release of encapsulated protein molecules. Transmission electron microscopy, scanning electron microscopy, atomic force microscopy, and confocal laser scanning microscopy were employed to characterize the hollow capsules. In comparison with native BSA, BSA molecules encapsulated in the hollow structures show higher catalytic efficiency due to higher local concentration of reactants in the structures. The membranes of the hollow capsules can efficiently protect the encapsulated BSA from hydrolysis by trypsin.

Keywords Polymer capsules; BSA; Interfacial thiol-disulfide reaction

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INTRODUCTION

Polymer hollow capsules are a type of interesting micro- or nanosized structures with polymer shells and interior voids. Polymer capsules have found a diverse range of applications, including nanoreactors for chemical reactions and nanocontainers for encapsulating chemical compounds or drug molecules.^[1–6] Many synthetic strategies have been employed in the synthesis of hollow structures, such as hard templating,^[7,8] soft templating,^[9,10] sacrificial templating,^[11,12] and template-free method.^[13] In soft templating method, oil-in-water (O/W) and water-in-oil (W/O) emulsions are used as templates, and polymer shells are fabricated around the liquid-liquid interface.^[14–16]

In these years, more and more researches are focused on the encapsulation of proteins for protection and delivery purpose, and many platforms, including polymeric micelles, vesicles, emulsion droplets, liposomes, hydrogels, and nanocapsules, have been built up.^[17–29] The encapsulation of proteins not only protects the proteins from outside interference,^[30] but also endows the particles with structural resemb-

lance to cells. For example, Peters and coworkers reported encapsulation of free enzymes and enzyme-filled nanoreactors in hollow structures. An original cofactor-dependent three-enzyme cascade reaction could be performed in the multicompartimentalized structures.^[31] In many cases, the encapsulated proteins, especially therapeutic proteins, are required to be released from the carriers so as to realize the functionalities of the proteins. Previously, Zhang and coworkers studied the release of lysozyme from double-network cryogels and they demonstrated the structure and bioactivity of the enzyme were kept unchanged.^[32] The release of the active proteins from hybrid organosilica nanocapsules was reported by Prasetyanto and coworkers.^[33] Knowles and coworkers studied sequential release of proteins from structured multi-shell microcapsules.^[34]

In this research, we studied the fabrication of degradable protein-loaded hollow capsules based on interfacial thiol-disulfide cross-linking reaction. Because of high encapsulation efficiency and simple manipulation under mild conditions, W/O emulsion droplets were used as soft templates. Bovine serum albumin (BSA) encapsulated hollow capsules were prepared by liquid-liquid interfacial thiol-disulfide exchange reactions at room temperature. Poly(oligo(ethylene glycol) monomethyl ether methacrylate-block-poly(di(ethylene glycol) methyl ether methacrylate-co-2-(2-pyridyl)disul-

* Corresponding author: E-mail hyzhao@nankai.edu.cn

[†] These authors contributed equally to this work.

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fide) ethyl methacrylate)) (POEGMA-*b*-(PDEGMA-*co*-PPDMSMA)) and macromolecular crosslinking agent were synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization. In W/O emulsions, POEGMA-*b*-(PDEGMA-*co*-PPDMSMA) block copolymer chains are located at liquid-liquid interface reducing the interfacial tension (Scheme 1). Upon addition of a macromolecular crosslinking agent with multiple pendant thiol groups into the emulsion, thiol-disulfide interfacial crosslinking reactions between crosslinking agent and the block copolymer chains at the interface result in the formation of hollow capsules. The hollow capsules protect protein molecules from trypsin hydrolysis. The disulfides can be cleaved in the presence of glutathione or DTT and the encapsulated protein molecules can be released. Because the encapsulation of protein molecules was conducted at room temperature, the structure and bioactivity of protein molecules were well maintained.

EXPERIMENTAL

Materials

4,4'-Azobis(4-cyanopentanoic acid) (ABCPA, Aldrich, 97%) was recrystallized from methanol and dried under vacuum at room temperature. Chain transfer agent (CTA) (4-cyanopentanoic acid) dithiobenzoate (CPADB) was synthesized in our laboratory. Oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA, $M = 500$ g/mol) and di(ethylene glycol) monomethyl ether methacrylate (DEGMA, $M = 188$ g/mol) were purchased from Sigma Aldrich and purified by passing through a column filled with basic alumina. 2-Mercaptoethanol (Tianjin Guang Fu Chemical Co., 98%) and bis(2-hydroxyethyl) disulfide (BHEDS, Aldrich, technical grade) were distilled before use. BSA (Dingguo, 96%), fluorescein isothiocyanate (FITC, Aldrich, 95%), 2,2'-dithiodipyridine (Aldrich, 98%), trypsin (Shanghai Macklin Biochemical Co., potency > 2500 units/mg), tributylphosphine (Bu_3P , 10% in hexane), *p*-nitrophenyl acetate (NPA, Alfa Aesar, 98%),

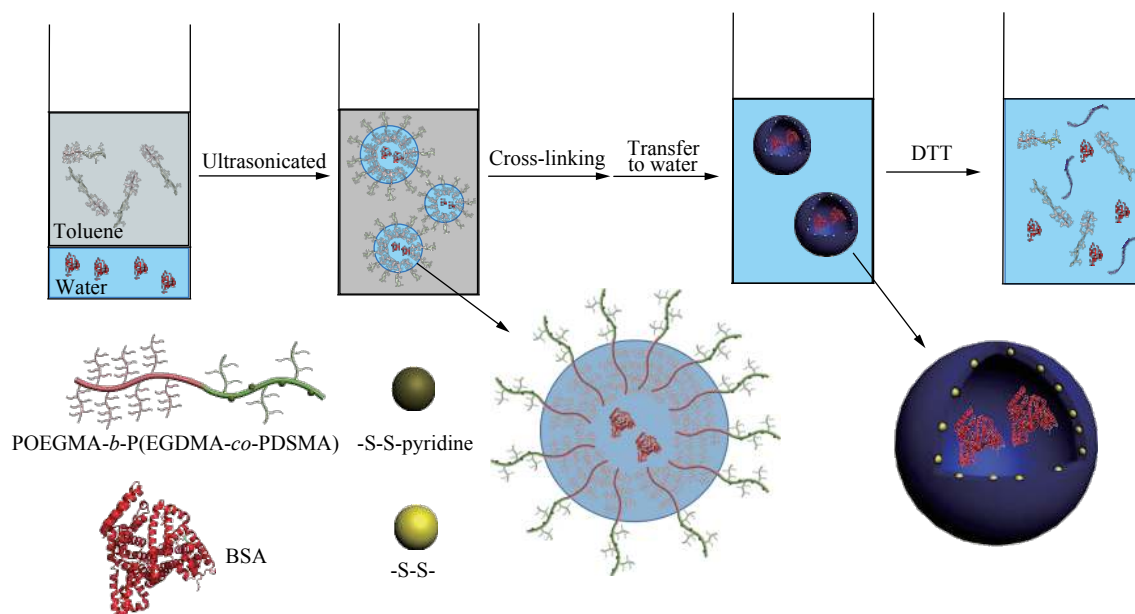
DL-dithiothreitol (DTT, Alfa Aesar, 99%) and methacryloyl chloride (TCI, 80%) were all used as received. The details for the synthesis of pyridyldisulfide ethylmethacrylate (PDSMA) monomer, POEGMA, and POEGMA-*b*-(DEGMA-*co*-PDSMA) block copolymer can be found in our previous paper.^[30] 2-(Dimethylamino) ethyl methacrylate (DMAEMA) (Aldrich, 99%) was purified by passing through a basic alumina column, drying with CaH_2 and distilling under reduced pressure.

Synthesis of 2-(Methacryloyl)oxyethyl-2'-hydroxyethyl Disulfide (MAOHD)

BHEDS (2 g, 12.8 mmol) and triethylamine (0.73 mL, 7.2 mmol) were dissolved in 20 mL of THF, and the solution was cooled to 0 °C. Methacryloyl chloride (0.64 mL, 7.2 mmol) dissolved in 5 mL of dry THF was added into the solution at 0 °C. After 24 h of stirring and subsequent filtration, the solution was washed successively with NaHCO_3 saturated solution, NaCl saturated solution and water. The organic layer was dried over MgSO_4 and concentrated on a rotary evaporator. The crude product was purified by column chromatography (petroleum ether: CH_2Cl_2 :methanol = 70:10:1, $V:V:V$), and the final product was obtained (1.5 g, yield: 65%). $^1\text{H-NMR}$ spectrum of MAOHD is shown in Fig. S1 (in electronic supplementary information, ESI).

Reversible Addition-fragmentation Chain Transfer (RAFT) Polymerization of PDMAEMA-*co*-PMAOHD

ABCPA (4.0 mg, 0.015 mmol), DMAEMA (3.1 g, 20 mmol), MAOHD (0.44 g, 2.0 mmol), and chain transfer agent CPADB (28 mg, 0.10 mmol) were dissolved in 6.0 mL of 1,4-dioxane in a 25 mL Schlenk flask. The solution was degassed by three freeze-pump-thaw cycles. The polymerization was conducted at 60 °C for 12 h and then stopped by quenching the Schlenk flask in liquid nitrogen. The polymer was precipitated in hexane, and dried at reduced pressure. The monomer conversions of DMAEMA and MAOHD were 23% and 35%, respectively.



Scheme 1 Schematic depiction for the fabrication of protein-loaded hollow capsules based on thiol-disulfide exchange reaction

Preparation of Macromolecular Crosslinking Agent Containing Pendant Thiol Groups

PDMAEMA-*co*-PMAOHD (100 mg, 0.012 mmol) was dissolved in 1.0 mL of methanol in a 10 mL Schlenk flask. After one freeze-pump-thaw cycle, Bu₃P (0.2 mL) was added into the flask under argon atmosphere and the solution was stirred at room temperature for 1 h. The polymer was precipitated in hexane/ether mixture and the purified polymer was dried under reduced pressure.

Preparation of BSA-loaded Hollow Capsules

POEGMA-*b*-P(DEGMA-*co*-PDSMA) (50 mg, 1.67 μ mol) was dissolved in 4.0 mL of toluene in a 10 mL centrifuge tube; BSA (5.0 mg) was dissolved in 0.5 mL of distilled water. The aqueous solution was added to the toluene solution under 5 min of ultrasonication. An emulsion containing toluene as the continuous phase and distilled water as the dispersed phase was formed. BSA molecules were dissolved in the water phase. Macromolecular crosslinking agent, reduced PDMAEMA-*co*-PMAOHD (18 mg), was dissolved in 0.5 mL of toluene and added into the emulsion under another 5 min of ultrasonication. The BSA-loaded capsules were collected after centrifugation and washed with distilled water.

Determination on the Encapsulation Efficiency of Protein Molecules in Hollow Capsules

FITC-labeled BSA (FITC-BSA, 5.0 mg) was dissolved in 0.5 mL of distilled water, and FITC-BSA loaded hollow capsules were prepared by using a method described above. The hollow capsules were separated from the solution by centrifugation, and the supernatant was collected and diluted to 10 mL. The resulting supernatant was filtered using a 220 nm water phase filter, and the UV absorbance of FITC was measured on a UV-Vis spectrometer. The amounts of FITC-BSA encapsulated in the hollow capsules and in the solutions can be calculated based on the UV-Vis results.

Bioactivity of BSA in the Hollow Capsules

p-Nitrophenyl acetate (21 mg, 0.1 mmol) was dissolved in 1.0 mL of acetonitrile, and 30.0 μ L of the solution was mixed with 10.0 mL of phosphate buffer saline solution (PBS, 100 mmol/L, pH = 7.4) of BSA-loaded hollow capsules at 20 °C. To monitor the production of nitrophenol moiety, UV absorptions of the solution at 406 nm were recorded at different time intervals. The UV absorption curves of self-hydrolysis of *p*-nitrophenyl acetate and native BSA catalyzed hydrolysis of *p*-nitrophenyl acetate under the same conditions were measured for comparison.

Degradation of BSA-loaded Hollow Capsules by DTT

To 1.0 mL of BSA-loaded capsule solution, 9.2 mg (0.06 mmol) of DTT and 0.2 mL of ethanol were added. The final concentration of DTT was 0.05 mol/L. The solution was stirred at room temperature for 24 h under argon atmosphere.

Characterization

A Varian UNITY-plus 400 MHz nuclear magnetic resonance spectrometer was used in ¹H-NMR measurements. A size exclusion chromatograph (SEC) equipped with Hitachi L-2130 HPLC pump, Hitachi L-2350 column oven, three

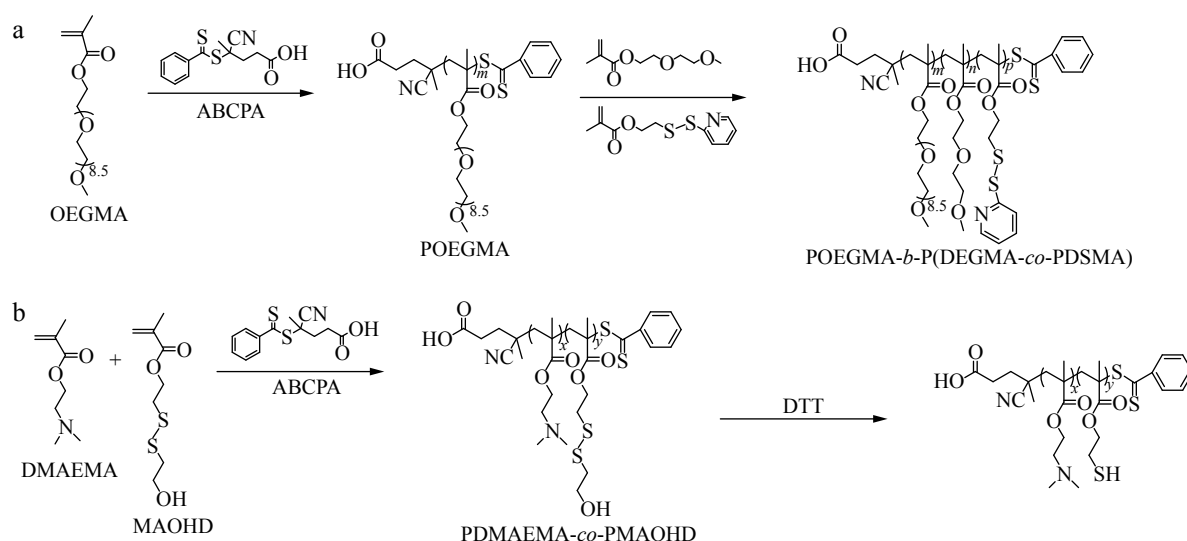
Shodex columns (5000-5 K, 400-0.5 K, and 5-0.15 K), and a Hitachi L-2490 refractive index detector was used in the measurements of apparent molecular weight and dispersity of the polymers. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images were obtained on a FEI Nova NanoSEM 450 and a Tecnai G2 20 S-TWIN electron microscope operated at a voltage of 200 kV. The TEM specimens were stained under OsO₄ atmosphere. Atomic force microscopy (AFM) images were collected on a Nanoscope IV atomic force microscope (Digital Instruments Inc.). Hydrodynamic diameters (*D_h*) of the capsules were collected on a Malvern Zetasizer Nano-ZS equipped with a 10 mW HeNe laser. UV-Vis spectra were collected on a Shimadzu 2450 UV-Vis spectrophotometer at room temperature. The ultrasonic condition was given by a KQ5200DB Ultrasonic Cleaner (power 200 W) with an operating frequency of 40 kHz.

RESULTS AND DISCUSSION

As shown in Scheme 2, POEGMA-*b*-P(DEGMA-*co*-PDSMA) was synthesized by two-step RAFT polymerization. POEGMA was synthesized by RAFT polymerization at 60 °C in 1,4-dioxane. By employing POEGMA macro-CTA, DEGMA was copolymerized with PDSMA yielding POEGMA-*b*-P(DEGMA-*co*-PDSMA) block copolymer. The POEGMA macro-CTA and POEGMA-*b*-P(DEGMA-*co*-PDSMA) block copolymers were analyzed by ¹H-NMR (Fig. S2, in ESI) and SEC (Fig. S3, in ESI).

¹H-NMR spectrum of POEGMA is shown in Fig. S2(a) (in ESI). The average degree of polymerization (*DP_n*) of POEGMA calculated based on ¹H-NMR is about 36 (POEGMA₃₆), and the number-average molecular weight (*M_n*) is 8.3×10^3 . SEC analysis result confirmed the low polydispersity index (PDI = 1.13) of the polymer. POEGMA-*b*-P(DEGMA-*co*-PDSMA) block copolymer was synthesized using POEGMA₃₆ as macro-CTA. ¹H-NMR spectrum of the block copolymer is shown in Fig. S2(b) (in ESI). Based on the ¹H-NMR results, the composition of the block copolymer was determined. Our calculation results indicate that the repeating unit numbers of DEGMA and PDSMA on the block copolymer chains are 49 and 9, respectively, so the block copolymer is assigned as POEGMA₃₆-*b*-P(DEGMA₄₉-*co*-PDSMA₉). SEC curves of POEGMA₃₆ (curve a) and POEGMA₃₆-*b*-P(DEGMA₄₉-*co*-PDSMA₉) (curve b) are presented in Fig. S3 (in ESI). According to the SEC results, apparent molecular weight (*M_n*) and PDI of the block copolymer are 15.3×10^3 and 1.14, respectively. After the second step RAFT polymerization, SEC curve of the block copolymer moves to the higher molecular weight region.

¹H-NMR spectrum of PDMAEMA-*co*-PMAOHD is shown in Fig. S2(c) (in ESI). In the spectrum, the resonance signals at 2.30 ppm representing the N-methyl protons (—N—CH₃), at 7.87, 7.52, and 7.37 ppm corresponding to the protons on the benzene rings of RAFT CTA, at 4.25 ppm corresponding to the methylene protons next to the ester groups of MAOHD units, and at 3.87 ppm corresponding to the methylene protons next to the hydroxyl groups, are ob-



Scheme 2 Outlines for the synthesis of (a) POEGMA₃₆-*b*-P(DEGMA₄₉-*co*-PDSMA₉) block copolymer and (b) PDMAEMA₄₅-*co*-PMAOHD₇

served. Based on ¹H-NMR result, the composition of the copolymer can be determined. Our calculation results indicate that the repeating unit numbers of PDMAEMA and MAOHD are about 45 and 7. The copolymer is thereby assigned as PDMAEMA₄₅-*co*-PMAOHD₇. SEC curve of PDMAEMA₄₅-*co*-PMAOHD₇ is presented in Fig. S3 (in ESI). The apparent molecular weight (M_n) of PDMAEMA₄₅-*co*-PMAOHD₇ is 5.8×10^3 and PDI is 1.18.

FITC-BSA was prepared by a reaction between FITC and BSA.^[35] The UV-Vis absorptions of FITC in water at different concentrations are shown in Fig. S4(a) (in ESI). Based on the standard curve and the absorption of FITC-labelled BSA (Figs. S4b and S4c, in ESI), the average number of FITC molecules on a BSA molecule was calculated to be 4.66. An emulsion containing toluene as the continuous phase and distilled water as the dispersed phase was used as a template and BSA-loaded hollow capsules were prepared by interfacial thiol-disulfide exchange reaction between POEGMA₃₆-*b*-P(DEGMA₄₉-*co*-PDSMA₉) and reduced PDMAEMA₄₅-*co*-PMAOHD₇. After centrifugation, the capsules with FITC-BSA were collected and the amount of FITC-BSA in the supernatant was determined by UV-Vis (Fig. S4d, in ESI). Our calculation result indicates that the encapsulation efficiency of FITC-BSA is about 80%.

The structures of BSA-free and BSA-loaded capsules were studied by direct observation with TEM, prior to which the PDMAEMA domains or/and BSA were stained under OsO₄ atmosphere. Fig. 1(a) shows a TEM image of BSA-free hollow capsules prepared from aqueous solution. The sizes of the hollow capsules are in the range of 70–170 nm. In order to demonstrate the cross-linked membranes on the capsules, the aqueous dispersion of hollow capsules was freeze-dried and the dried hollow capsules were dispersed in THF. THF is a good solvent for all the components in the capsules. Fig. 1(b) shows a TEM image of capsules prepared from THF dispersion. TEM result indicates that the hollow structures are maintained in THF, demonstrating the cross-linked structure of the capsules. It is noted that the sizes of the hol-

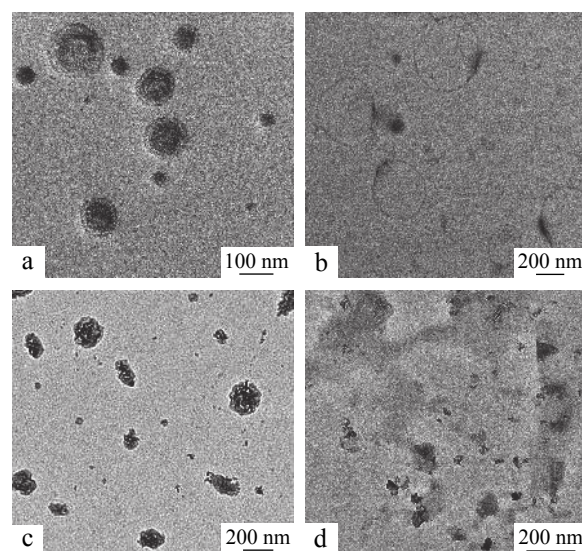


Fig. 1 TEM images of (a) BSA-free hollow capsules prepared from aqueous dispersion, (b) BSA-free hollow capsules prepared from THF dispersion, (c) BSA-loaded hollow capsules prepared from aqueous dispersion, and (d) BSA-loaded hollow capsules after treatment with DTT in aqueous dispersion

low capsules in THF are in the range of 300–500 nm, which are larger than those in aqueous solution due to the swelling of the membranes in THF. A TEM image of BSA-loaded hollow capsules is shown in Fig. 1(c), where deep dark domains representing encapsulated protein molecules can be observed in hollow capsules. Because of the encapsulated protein molecules inside the structures, the sizes of BSA-loaded capsules are bigger than those of the BSA-free ones. The hollow structures can be degraded upon cleavage of disulphide bonds on the membranes by DTT. Fig. 1(d) shows a TEM image of the hollow capsules after treatment with DTT. TEM result indicates that the hollow capsules are degraded, and structureless polymer aggregates and protein aggregates are formed. This result indicates that the BSA-loaded hollow capsules are degradable in the presence of DTT.

The morphology of BSA-loaded capsules was also characterized by SEM. The SEM samples were prepared by casting the aqueous dispersion of capsules onto silicon wafers and the silicon wafers were moved immediately to the surface of an iron block partly immersed in liquid nitrogen. Water was removed by freeze drying. Fig. 2(a) shows a SEM image of BSA-loaded capsules. BSA molecules in the hollow capsules are responsible for the creases and wrinkles on the surfaces of collapsed capsules. Similar to the TEM result, after treatment with DTT the hollow capsules are degraded, and polymer films and small polymer or protein aggregates are observed in the SEM image (Fig. 2b).

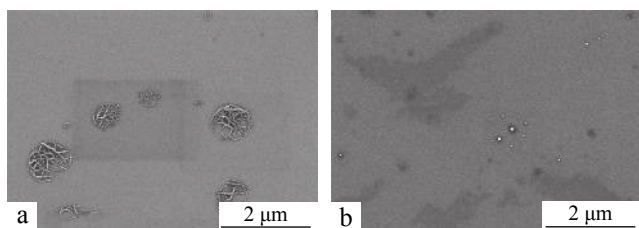


Fig. 2 SEM images of (a) BSA-loaded capsules and (b) BSA-loaded capsules after treatment with DTT

Confocal laser scanning microscopy (CLSM) is a powerful tool in the study of materials with fluorescence labels,^[34] and it allows direct visualization of the structures. Fig. 3(a) shows a CLSM image of FITC-BSA-loaded capsules in aqueous solution. A large number of green fluorescent particles are observed in the image, demonstrating the encapsulation of FITC-BSA inside the hollow capsules. However, the fluorescent particles disappear completely upon addition of DTT into the solution (Fig. 3b), which is due to the cleavage of disulfides and degradation of the hollow structures.

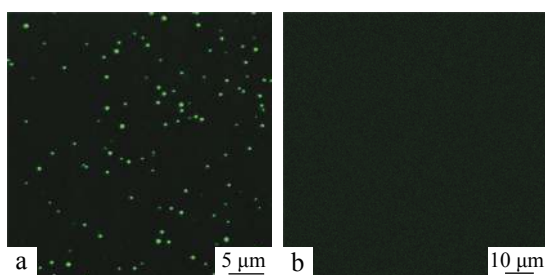


Fig. 3 CLSM images of (a) FITC-BSA-loaded hollow capsules and (b) FITC-BSA-loaded capsules after treatment with DTT

The hydrolysis of *p*-nitrophenyl acetate (NPA) to 4-nitrophenylacetic acid (NPAA) by native BSA and BSA in the capsules was tested to determine the esterase-like activity of the capsules.^[36] As shown in Fig. 4(a), the intensity of UV absorption of NPAA at 412 nm increases with time. Because NPA undergoes self-hydrolysis in the buffer solution, the UV-Vis spectra of NPA in buffer solution under the same experimental conditions were also recorded. The kinetics curves for NPA hydrolysis in the presence of native BSA and BSA-loaded capsules, and for the self-hydrolysis of NPA are shown in Fig. 4(b), which demonstrate that the

catalytic efficiency of BSA encapsulated in the hollow capsules is slightly higher than that of native BSA. There are hydrophobic PDSMA and reduced PMAOHD domains in the membranes of the capsules, and the hydrophobic NPA tended to accumulate in the hydrophobic domains when it was added into the solution, resulting in high local concentration of reactants in the system and higher catalytic efficiency of BSA encapsulated in the capsules.^[37,38]

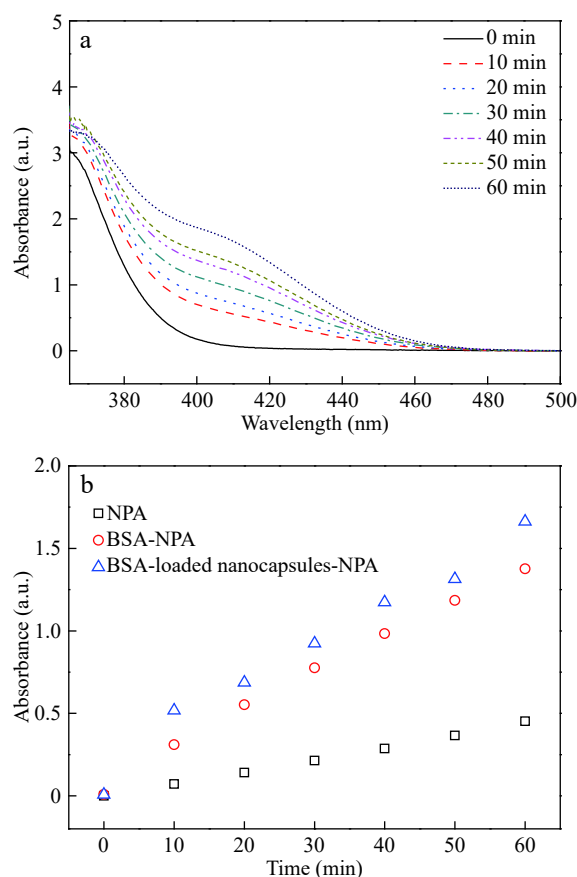


Fig. 4 (a) UV-Vis spectra of the BSA-loaded hollow capsules catalysing the hydrolysis of *p*-nitrophenyl acetate; (b) Kinetic comparison of the hydrolysis of *p*-nitrophenyl acetate catalyzed by BSA-loaded hollow capsules, native BSA, and PBS

The above bioactivity tests show that small molecules, such as *p*-nitrophenyl acetate, can diffuse into the hollow capsules through the hydrophobic membranes. However, it is necessary to determine whether macromolecules, especially biological macromolecules, can diffuse into the capsules. Protease is able to cleave peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, resulting in degradation of the proteins.^[39,40] Herein, we choose trypsin as a typical protease to investigate the effect of protease on the bioactivity of encapsulated protein molecules. Aqueous solution of trypsin was added into aqueous solution of BSA-loaded capsules at 37 °C. BSA hydrolysis by trypsin was monitored by comparing the bioactivities of “free” BSA and BSA in the hollow capsules both in the presence of trypsin. As shown in Fig. 5, the bioactivity of native BSA decreases significantly after treatment with trypsin

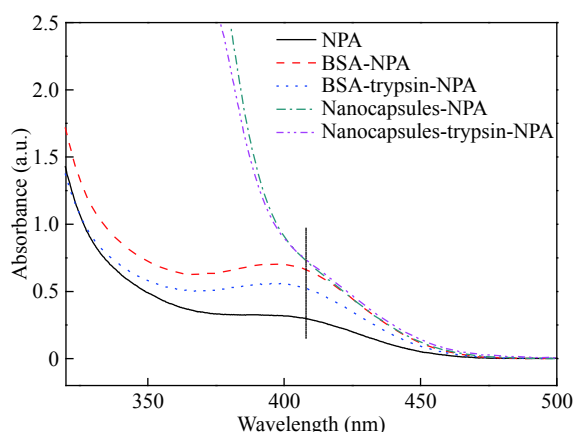


Fig. 5 Hydrolysis efficiency of *p*-nitrophenyl acetate catalyzed by native BSA and BSA-loaded hollow capsules before and after treatment with trypsin

due to the hydrolysis of BSA. In contrast, the bioactivity of BSA encapsulated in hollow capsules remains almost unchanged after treatment with trypsin, indicating that the membranes could prevent the trypsin from diffusing into the hollow capsules.

CONCLUSIONS

In conclusion, BSA-loaded hollow capsules were prepared by thiol-disulfide cross-linking reactions at liquid-liquid interface. The cross-linked membranes of the capsules can protect the encapsulated BSA from hydrolysis by trypsin, while small molecular reactants are able to diffuse into the hollow capsules. The cleavage of disulfides on membranes leads to the degradation of hollow structures and the release of encapsulated protein molecules. The hollow capsules may find potential applications in the biomedical fields of bioengineering, artificial cells, and protein delivery.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://dx.doi.org/10.1007/s10118-019-2253-9>.

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