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Biomimetic assembly of proteins into microcapsules on oil-in-water droplets with structural reinforcement via biomolecular recognition-based cross-linking of surface peptides

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Biomimetics; protein microcapsules; oil/water interface; biomolecular recognition; collagen peptides; self-assembly

In nature, many bacteria are capped by monolayers of S-layer proteins, which self-assemble into closely packed 2D crystal at the surface of wide range of bacteria¹, to enhance mechanical stabilization of membranes, protection against viruses, and resistance against pH changes.² To build artificial cellular structures, molecular assembly on oil/water interfaces has been used to generate hollow particles whose shells can be chosen from molecules to metal nanoparticles.³ Since the organizations of molecules and particles at the interface of immiscible fluids are driven by the reduction of interfacial energy^{3–6}, this assembly processes of both symmetric and asymmetric molecules are very robust, and therefore this technique has been widely applied for the assembly of micro-/ nano-particles at flat and curved interfaces.⁷ Although this approach produced a variety of functional microcapsules by using micro-/ nano-particles, self-assembly of proteins on oil/water interfaces to create stable microcapsules were rarely reported⁸, and the stabilities of as-prepared protein microcapsules are not well studied. To create mechanically stable artificial cellular structures composed of proteins, we mimic the S-layer proteins to reinforce the molecular interface by capping with proteins via molecular recognition. This type of protein super-structure is a promising scaffold for the developments of multi-functionalized hierarchical materials with the eases of biomolecular conjugation and bio-compatibility.

Herein, we report a simple and versatile method for the fabrication of protein microcapsules on oil-in-water droplet. In this strategy, protein is biotinylated by using biotin ligase,^{9, 10} and this genetically-engineering protein (Figure 1-a) can be assembled at the oil/water interface, followed by cross-linking of the proteins with streptavidin via the biotin-streptavidin biomolecular recognition (Figure 1-b). Previously, the preparation of protein capsules can be achieved in a one-step through various chemical approaches.¹¹ The advantage in our approach is to use streptavidin, a readily accessible biomaterial with high binding specificity to biotin, for the cross-linker of the microsphere assembly and reinforcement without conventional catalysts¹² and chemical cross-linkers¹³. The specific biotinylation site on the collagen nanowire can be genetically engineered at the specific position by the biotin ligase technique, which is difficult to match by other chemical assembly methods. As a result, the cross-linking by using molecular recognition becomes also site-specific.¹¹ Previously,

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chemical coupling agents such as glutaraldehyde have been used to stabilize the biomolecular assembly on the fluid-fluid interfaces,¹¹ but this cross-linking tends to poison the active sites of functional proteins and this chemical treatment results in the loss of activity even though biomolecules were successfully assembled at the interfaces.¹⁴ The biotinylation sites of our genetically-modified proteins were restricted at only N- and C-terminal ends by using the biotin ligase method, which enables assembling the desired protein microcapsules without scarifying the protein functions with high mechanical stability. The stability of resulting microcapsules under the harsh conditions such as extreme temperature and pH is gained due to strong biotin-streptavidin interaction ($K_d \sim 10^{-15}$)¹⁵ at the head-to-head N-terminal ends and the tail-to-tail C-terminal ends.

To demonstrate the proof of concept of this protein assembly and molecular recognition-based stabilization at fluid-fluid interfaces, we engineered collagen-mimetic peptide B877B, a triple helical molecular wire containing a segment of the $\alpha 1$ chain of type I collagen.^{16, 17} To accomplish the site-specific biotinylation of B877B, biotin acceptor peptides (BAP) were fused at N- and C-terminus, and biotin ligase was co-expressed with B877B in *E. coli* BL21(DE3) (methods and materials are described in detail in the Supporting Information).^{9, 10} Since biotin ligase attaches a biotin moiety on the unique lysine residue in BAP, each triple helical peptide nanowire composed of three B877B displays up to 6 biotins (Figure 1-a). After overexpressed B877B was purified by using Ni-NTA column and HPLC (Figure S1, S2), circular dichroism (CD) spectra show that the resulting engineered peptides maintain the triple helical conformation with a small positive peak at about 225 nm and a deep negative peak at about 197 nm (Figure S3).^{16, 17} To achieve the proposed stable interfacial assembly of the B877B triple helix peptides at the molecular interface, both ends of B877B triple helix need to be biotinylated for molecular recognition-based cross-linking. If both ends of B877B triple helix are fully displayed, there should be six biotinylation sites (Figure 1-a). It means that if four biotin moieties are probed on the triple helix, both ends are assured to be biotinylated. Time-of-flight mass spectrometry (TOF-MS) revealed that full biotinylation of B877B can be accomplished by the co-expression of biotin ligase in *E. coli* during ligation (Figure S4-a, S5-a).¹⁸ It should be noted that adding the excess amount of biotin in growth media is not enough to display four biotins.

Protein microcapsule can be prepared by adding octane with aqueous solution containing B877B peptide (1:10 volume ratio), followed by vigorously stirring. In this step, B877B self-assembles on the oil-in-water droplets. The microcapsule structure can be subsequently locked by mixing with streptavidin solution (PBS, pH7.4) with the formation of biotin-streptavidin linkages on the peptide shell (Figure 1-c). Resulting microcapsules are spherical (Figure 2) with the sizes ranging from 4 to 170 μm (Figure S6) and we could still observe the microcapsules on microscope after leaving the microcapsules for at least half a month at room temperature. The broad size distribution of microcapsules is due to the polydispersity of oil droplet templates⁶ and the uniformity can be improved by controlling the size of oil droplets with the microfluidic devices^{19, 20}. Figure 2-a shows fluorescence image of protein microcapsules that the B877B assembly on the shell is locked by the Cy2-labeled streptavidin binding. The intense fluorescence at the droplet boundaries indicates that the protein assembly occurs at the octane/water interface. In addition, SDS-PAGE further confirmed the binding of streptavidin with biotinylated B877B peptides on the microcapsules (Figure S7). Few stable microcapsules were observed in the absence of B877B or streptavidin after 10 min. To probe whether streptavidin binds at the biotin site of the B877B peptides to construct microcapsules, we examined another control experiment to mix F877 (not-biotinylated collagen peptide)¹⁶ with streptavidin. This control experiment shows that without the biotin the collagen peptide and streptavidin could not generate microcapsules (Figure 3-a, S8-a). Because B877B peptide and F877 peptide have the same amino acid sequence except the biotinylated BAPs fused at N- and C-terminals, this

observation indirectly supports that the cross-linking of biotin at the ends of collagen peptides with streptavidin is necessary for the formation of microcapsules⁸.

When protein microcapsules were analyzed by scanning electron microscope (SEM), the spherical-shaped structure was observed (Figure S9-a), which indicates that the robust assemblies of protein shells on the oil droplet template are strong and elastic enough to sustain the spherical shape without complete collapsing after drying. Previously, the step height of microcapsules composed of inorganic nanoparticles was analyzed by using atomic force microscope (AFM) to confirm the monolayer assembly of nanoparticles at the oil/water interfaces.⁶ We also analyzed the step height of the flattened protein microcapsule after drying. Based on Figure S9-b and S9-c, the step height is measured as 30~90 nm and the flattening seems to occur in certain degrees but the complete collapsing does not occur even after drying, which is probably due to the structural reinforcement with the protein capping. The upper limit of the step height matches the thickness of bilayer of B877B ($\sim 40 \text{ nm} \times 2$)^{16, 17} and two streptavidin molecules ($\sim 5 \text{ nm} \times 2$)²¹, however complex factors such as protein denaturation due to the drying effect⁴ make it difficult to determine the exact arrangement of B877B peptide at the oil/water interface on oil droplets.

In the applications of microreactors, inorganic material templates and high density catalyst, it is desirable for the protein microcapsules to be stable in unnatural environments such as extreme pHs.^{22, 23} When the protein microcapsules were incubated at pH 2.2, 5.2, 7.4, 9.4 and 11.5 overnight, the microcapsules structures were stable in this wide pH range (Figure 3-b, S8-b). Since the stability of protein microcapsules is consistent with the pH-stability of biotin-streptavidin interaction,²⁴ this excellent rigidity of microcapsules is likely due to the streptavidin capping on the biotinylated B877B peptide shells. The excellent stability of protein-capped microcapsules can also be observed under temperature changes. Figure 3-c shows that $\sim 70\%$ of B877B-assembled microcapsules capped by streptavidin were stable at 60 °C, and this thermostability is better than most of widely-used liposomes.²⁵ Interestingly, the thermostability of the microcapsule assembled from B877B was higher than the one assembled from B877 peptide (Figure 3-c, S8-c), the same collagen-mimetic peptide but biotinylated at only single terminus (Figure S4-b, S5-b).¹⁷ The double side streptavidin-capped microcapsule from B877B peptides could have better thermal stability due to the larger number of biotin-streptavidin interactions on the surface of the peptide assemblies.

This as-prepared protein microcapsule is a versatile scaffold, and can be used as a template to develop functionalized hierarchical colloidosomes. For example, streptavidin-labeled CdSe/ZnS core/shell quantum dots (QDs) (Invitrogen) can be easily incorporated onto the microcapsules. TEM micrograph in Figure 2-c shows that QDs in the diameter of 10 nm are assembled at the biotin sites of B877B peptide assembly on the microcapsule. When these microspheres were stained by uranyl acetate to locate B877B peptides on the microspheres in TEM, the protein layers become visible in the TEM image in Figure S10.

In summary, robust assembly of protein microcapsule was demonstrated at fluid-fluid interface. The biotinylated collagen peptides mimic S-layer proteins to be aligned on the oil-in-water droplets and stabilized the microcapsules by capping and cross-linking biotin sites of the peptide with streptavidin. Due to the strong biotin-streptavidin interaction, as-prepared protein microcapsules were stable over wide range of pH (pH 2.2–11.5) and at high temperature. The surface of the protein microcapsules can be easily modified by functional materials such as QDs as these external materials are conjugated with streptavidin. By using the same strategy, numerous functional materials will be displayed on the protein microcapsules for a variety of biotechnological applications.

Experimental Section

Microcapsule formation on octane/water interface

Protein concentration was measured with UV-Vis absorptiometer. B877B was dissolved in water (2 μ M, 100 μ L) and octane (10 μ L) was added slowly on the water phase. Plastic test tube containing B877B solution and octane was vigorously stirred by hand for 5 min. Then streptavidin dissolved in PBS (2 μ M, 100 μ L, pH7.4) was added to the oil-in water emulsion, and mixed for another 5min.

Stability test of microcapsul

To test pH-stability, the protein microcapsules were incubated in glycine-HCl buffer (pH2.2), HEPES (pH5.2), PBS (pH7.4, 9.4) or Tris-HCl buffer (pH11.5) overnight. To test thermostability, the protein microcapsules were prepared in PCR tubes and were incubated at 20–90 °C for 30 min on a thermal cycler. After each treatment, stable microcapsules were washed with 100 μ L of PBS 3 times to remove excess proteins and broken microcapsules. Washed microcapsules were suspended in 100 μ L of PBS and 5 μ L of the samples were observed at 50-fold magnification. The number of the stable microcapsules in the field of view ($2 \times 1.6 \times 0.1$ mm³) were counted by a hemacytometer. The remnant of the samples were dried in vacuum chamber. Then, dried proteins were dissolved in 50 μ L of water and applied to UV-Vis absorptiometer to measure the absorbance at 280 nm for the estimation of the protein amount. Thermostability of the protein microcapsules was shown as percentage of the number of stable microcapsules or Abs_{280nm}, where one of 20 °C was shown as 100%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Structure of the collagen-like triple helical B877B peptide with the site-specific biotinylation at N- and C-terminus. The green circles depict the displayed biotins in the final step of the expression of fusion protein, (b) Procedure of protein microcapsule formation on octane/water interface, whose structure is reinforced by biotin-streptavidin interaction. (c) Detailed illustration of assembled structure of the B877B-streptavidin hybrid microcapsules.

Figure 2.

(a) Fluorescence micrograph of the protein microcapsules composed of B877B peptide and Cy2-labeled streptavidin. Outlines of microcapsules where streptavidin molecules are assembled have higher contrast. (b) TEM images of QD-conjugated streptavidin assembled on the protein microcapsule (inset) Magnified image of the QD-assembled protein microcapsule.

Figure 3.

Stability test for protein microcapsules based on the microcapsule counting. (a) Comparison of microcapsules composed of B877B peptide (biotinylated on both ends), streptavidin, [F877 peptide (non-biotinylated) +streptavidin], and [B877B peptide +streptavidin]. (b) pH-stability of protein microcapsules composed of B877B peptide+streptavidin at pH 2.2–11.5. (c) Thermostability of protein microcapsules composed of streptavidin and B877B peptide (biotinylated on both ends, closed circle) or B877 peptide (biotinylated on one end, open triangle) at 20–90°C.