

## Supporting Information for

### Single-step fabrication of multi-compartmentalized biphasic proteinosomes

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#### Materials and Methods

##### Chemicals

Bovine serum albumin (BSA), N-isopropylacrylamide (NIPAAm), 4-(dimethylamino) pyridine (DMAP), N,N'-dicyclohexylcarbodiimide (DCC), 4-formylbenzoic acid, 2-ethyl-1-hexanol (EH), and dimethyl sulfoxide (DMSO) were all purchased from Sigma (China) and used as received. Dextran (Mw 70 kDa), sodium alginate, Ca<sub>2</sub>, 1,6-diaminohexane, fluorescein isothiocyanate-dextran (FITC-Dextran), triglyceride lipase, 4-methylumbelliferyl butyrate (MLBB), Na<sub>2</sub>CO<sub>3</sub>, HCl, and calcein were all obtained from Aladdin Industrial Corporation and used as received. All aqueous solutions were prepared using Milli-Q water.

##### Synthesis of BSA-NH<sub>2</sub> and BSA-NH<sub>2</sub>/PNIPAAm conjugates

Synthesis of BSA-NH<sub>2</sub> and BSA-NH<sub>2</sub>/PNIPAAm nanoconjugates followed the same procedures as reported previously.<sup>1-3</sup> Briefly, the cationized protein (BSA-NH<sub>2</sub>), was obtained by carbodiimide-activated conjugation of 1,6-diaminohexane with aspartic and glutamic acid residues on the external surface of BSA. BSA-NH<sub>2</sub>/PNIPAAm conjugates were prepared from BSA-NH<sub>2</sub> by adding end-capped mercaptothiazoline-activated PNIPAAm to a stirred solution of BSA-NH<sub>2</sub> to give a polymer : protein molar ratio of  $\approx$  9:1. The mixed solution was stirred overnight, followed by purification using a centrifugal filter (M<sub>w</sub> 50 kDa) to remove any unreacted PNIPAAm and salts.

##### Preparation of multi-compartmentalized proteinosomes (MCPs)

Multi-compartmentalized proteinosomes with different sizes and oil/water compositions were prepared by a double Pickering emulsion method. In brief, an aqueous solution was prepared using sodium carbonate buffer (pH 8.5) containing BSA-NH<sub>2</sub>/PNIPAAm (10.0 mg/mL), and then added to the oil 2-ethyl-1-hexanol typically at a water-to-oil volume ratio ( $\phi$ ) values of 1.0. In general, MCPs were produced in significant yields at  $\phi$  values of between 0.48 and 2.0. In each case, the emulsions were left to stand for 2 h, and then shaken by hand for 30 s to produce the MCPs.

## **Influence of BSA-NH<sub>2</sub>/PNIPAAm nanoconjugate concentration on MCP yield**

The influence of the initial concentration of BSA-NH<sub>2</sub>/PNIPAAm was investigated using a double Pickering emulsion method. In brief, an aqueous solution was prepared using sodium carbonate buffer (pH 8.5) containing BSA-NH<sub>2</sub>/PNIPAAm at different concentrations (1.25, 2.5, 5.0, 10.0, and 20.0 mg/mL), and then added to 2-ethyl-1-hexanol typically at a water-to-oil volume ratio of  $\phi=0.48$ . The emulsions were left to stand for 2 h, and then shaken by hand for 30 s to produce the MCPs

## **Membrane wall formation in MCPs**

Targeted assembly of a Ca-alginate shell on the external (host) and/or internal (guest) BSA-PNIPAAm membrane surfaces of MCPs was achieved by interfacial complexation arising from counter-diffusion of Ca<sup>2+</sup> and alginate ions. Simultaneous formation of Ca-alginate on both the host and guest membranes of w/o/w MCPs was achieved as follows. A water phase (0.2 mL) containing sodium carbonate buffer (pH 8.5), sodium alginate (0.2-1.0% w/w) and BSA-NH<sub>2</sub>/PNIPAAm (10 mg/mL) was prepared and added to 2-ethyl-1-hexanol (0.05 mL). After standing for 2 h, 2-ethyl-1-hexanol (0.05 mL) containing CaI<sub>2</sub> (0.5-1.5% w/w) was added and mixed gently in the upper oil phase, followed by shaking the mixture by hand for 30 s to produce w/o/w MCPs ( $\phi = 2$ ) with Ca-alginate shells deposited on both host and guest membranes.

Ca-alginate wall formation solely on the internal membranes of w/o/w MCPs was achieved by preparing an aqueous phase (0.2 mL) containing sodium carbonate buffer (pH 8.5), BSA-NH<sub>2</sub>/PNIPAAm (10 mg/mL) and sodium alginate (0.2-1.0% w/w), and adding this mixture to 2-ethyl-1-hexanol (0.1 mL) containing CaI<sub>2</sub> (0.5-1.5% w/w). The mixture was left to stand for 2 h, and then shaken by hand for 30 s. The generated w/o/w MCPs ( $\phi = 2.0$ ) dispersed in the upper layer were then immediately transferred to a vial containing an aqueous phase (0.2 mL) containing sodium carbonate buffer (50 mM, pH 8.5), such that only the internalized guest proteinosomes became coated in a Ca-alginate shell.

Ca-alginate wall formation specifically at the external surface of the BSA-NH<sub>2</sub>/PNIPAAm membrane of the host proteinosome of w/o/w MCPs was achieved by adding water (0.2 mL) containing sodium carbonate buffer (pH 8.5) and BSA-NH<sub>2</sub>/PNIPAAm (10 mg/mL) to 2-ethyl-1-hexanol (0.1 mL) containing CaI<sub>2</sub> (0.5-1.5% w/w). The mixture was left to stand for 2 h, and then shaken by hand for 30 s to produce w/o/w MCPs ( $\phi = 2.0$ ). The MCPs were transferred to a vial containing an aqueous solution of sodium alginate (0.2-1.0% w/w) to initiate counter-diffusion and formation of Ca-alginate specifically at the membrane surface of the host proteinosome.

Deposition of a Ca-alginate shell on the internal membranes of o/w/o MCPs was achieved by preparing an aqueous phase (0.048 mL) containing sodium carbonate buffer (pH 8.5), BSA-NH<sub>2</sub>/PNIPAAm (10 mg/mL) and sodium alginate (0.2-1.0% w/w), and adding this mixture to 2-ethyl-1-hexanol (0.1 mL) containing CaI<sub>2</sub> (0.5-1.5% w/w). The mixture was left to stand for 2 h, and then shaken by hand for 30 s. The o/w/o MCPs ( $\phi = 0.48$ ) were then immediately transferred to a vial containing pure 2-ethyl-1-hexanol (0.1 mL), such that only the internalized guest proteinosomes were coated in Ca-alginate.

Formation of a Ca-alginate shell at the external surface of the host proteinosome membrane of o/w/o MCPs was achieved by adding water (0.048 mL) containing sodium carbonate buffer (pH 8.5), sodium alginate (0.2-1.0% w/w) and BSA-NH<sub>2</sub>/PNIPAAm (10 mg/mL) to pure 2-ethyl-1-hexanol (0.1 mL). The mixture was left to stand for 2 h, and then shaken by hand for 30 s to produce o/w/o MCPs ( $\phi = 0.48$ ), which were immediately transferred to a vial containing 2-ethyl-1-hexanol (0.1 mL) with CaI<sub>2</sub> (0.5-1.5% w/w).

## Enzyme catalysis in MCPs

An aqueous solution (0.1 mL) containing BSA-NH<sub>2</sub>/PNIPAAm (10 mg/mL), sodium carbonate buffer (pH 8.5) and triglyceride lipase (2 mg/mL) was mixed with 2-ethyl-1-hexanol (0.05 mL) containing 4-methylumbelliferyl butyrate (MLBB; 5.0 mg/mL) in a vial and left to stand for 2 h. The mixture was then shaken by hand for 30 s to produce w/o/w MCPs ( $\phi = 2$ ). Fluorescence intensities arising from lipase-mediated hydrolysis of MLBB to butyric acid and blue fluorescent 4-methylumbelliferone were determined from time-dependent captured fluorescence images of MCPs using Image J software (Version 1.44p, USA), and normalized.<sup>4</sup> Control experiments were undertaken using mixtures of aqueous lipase and MLBB in 2-ethyl-1-hexanol that were prepared in the absence of BSA-NH<sub>2</sub>/PNIPAAm nanoconjugates. The water and oil phases were shaken by hand for 30s, and then left unstirred to produce bulk oil and water phase-separated layers within 2 min. Formation of the product in the oil layer was monitored by fluorescence microscopy.

## Synthesis of benzyl aldehyde-functionalized dextran (dex-CHO)<sup>5</sup>

Dextran (2 g, 0.0285 mmol, Mw 70 kDa), 4-formylbenzoic acid (0.96 g, 6.4 mmol), and DMAP (0.12 g, 0.984 mmol) were dissolved in 40 mL of DMSO, followed by the addition of DCC (1.2 g, 5.83 mmol). The system was stirred at room temperature for 18 h and then the impurities were removed by filtration. The dex-CHO product was obtained as a white solid after precipitation in a mixture of ethyl acetate and petroleum ether with a volume ratio of 9:1. The white solid was dissolved in deionized water, and any insoluble impurities were removed by filtration, followed by freeze-drying.

## Preparation of BSA-NH<sub>2</sub>/dex-CHO hydrogel

Typically, 100  $\mu$ L of 100 mg/mL BSA-NH<sub>2</sub> aqueous solution, 100  $\mu$ L of 100 mg/mL dex-CHO aqueous solution and 100  $\mu$ L of 0.05 M sodium phosphate buffer (pH=7.5) were mixed together by vortex. The time required for hydrogelation was determined by the test-tube inversion method: typically, no flow of the solution was observed for inverted samples.

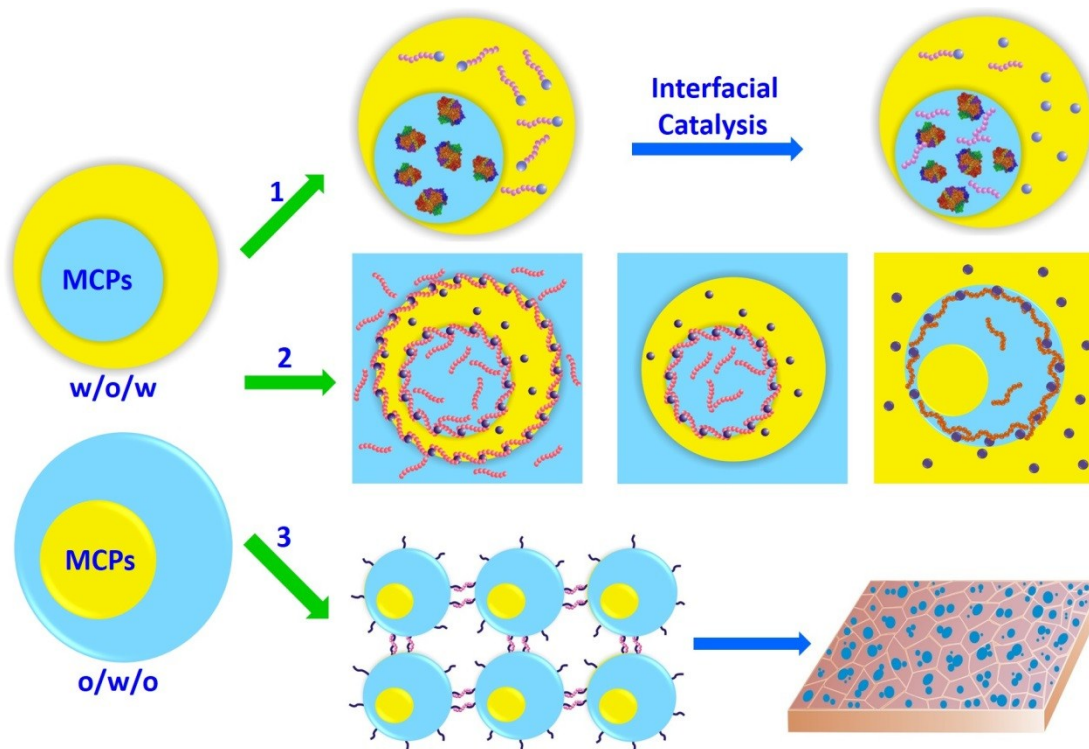
## Preparation of BSA-NH<sub>2</sub>/dex-CHO hydrogel films with embedded MCPs

80  $\mu$ L of a sediment of MCPs extracted from the bottom of the continuous aqueous phase of a w/o/w emulsion (or 50  $\mu$ L of a MCP sediment taken from the continuous oil phase of a o/w/o emulsion) and 100  $\mu$ L of dex-CHO (100 mg/mL) and BSA-NH<sub>2</sub> (100 mg/mL) in sodium phosphate buffer (0.05 M, pH=7.5) were mixed by gently shaking, and then poured into a petri dish and subjected to gentle pressure using a cover slide to produce within *ca.* 3 min a thin hydrogel film with embedded MCPs.

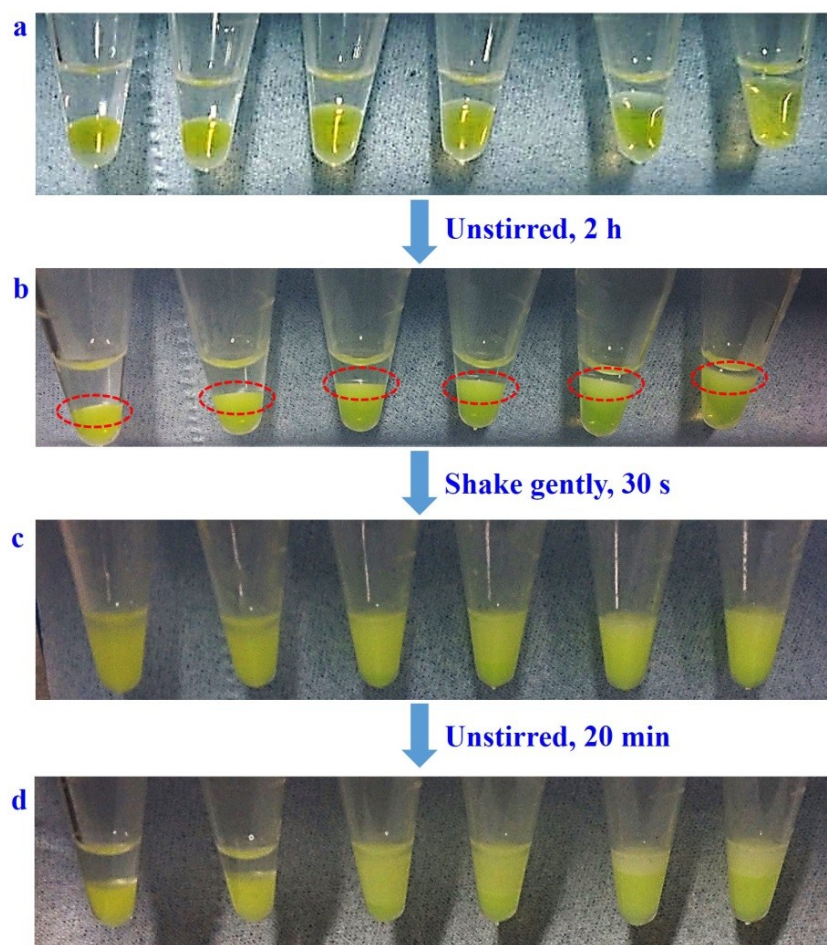
## Glucose-triggered release of guest proteinosomes from BSA-NH<sub>2</sub>/dex-CHO hydrogel-embedded MCPs

O/w/o MCPs were prepared as above but in the presence of glucose (sodium carbonate buffer, (pH 8.5), BSA-NH<sub>2</sub>/PNIPAAm (10 mg/mL), glucose (200 mg/mL), 2-ethyl-1-hexanol (48  $\mu$ L),  $\phi = 0.48$ ) and the MCP sediment added to 100  $\mu$ L of dex-CHO (100 mg/mL) and BSA-NH<sub>2</sub> (100 mg/mL)

in sodium phosphate buffer (0.05 M, pH=8.5) containing glucose oxidase (5 mg/mL). The mixture was then poured into a petri dish and subjected to gentle pressure using a cover slide to produce within *ca.* 3 min a thin enzymatically active hydrogel film with embedded MCPs. An inverted microscope was employed to observe the glucose-triggered release of the guest proteinosomes from the hydrogel matrix.

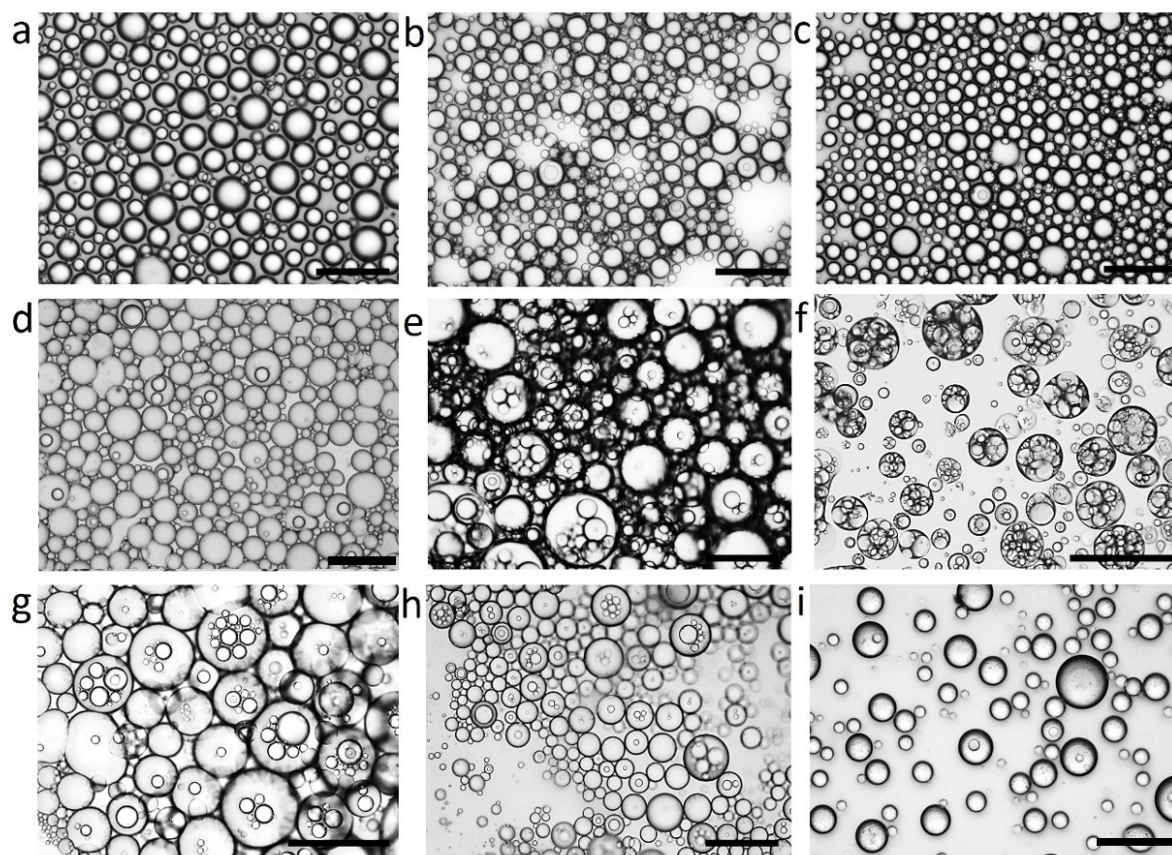


**Scheme S1.** Schematic illustration of MCPs structures utilized in three aspects of this work. **Pathway 1:** use of w/o/w MCPs ( $\phi = 2$ ) for enhanced interfacial catalytic reactions by encapsulation of lipase and 4-methylumbelliferyl butyrate (substrate) within the internalized water (blue) and oil (yellow) phases, respectively. **Pathway 2:** use of w/o/w or o/w/o MCPs for the targeted assembly of a Ca-alginate shell on the various proteinosome membranes within the nested micro-architectures; (left) w/o/w MCP ( $\phi = 2$ ) and Ca-alginate wall formation on both the external (host) and internal (guest) BSA-NH<sub>2</sub>/PNIPAAm proteinosome membranes; (centre) w/o/w MCP ( $\phi = 2$ ) with Ca-alginate deposition specifically on the internal membrane of the encapsulated proteinosome; (right) o/w/o MCP ( $\phi = 0.48$ ) with Ca-alginate wall formation specifically on the external surface of the BSA-NH<sub>2</sub>/PNIPAAm membrane of the host proteinosome. In each case, wall formation occurs by interfacial complexation arising from counter-diffusion of Ca<sup>2+</sup> (small spheres) present in the oil phase as CaI<sub>2</sub>, and alginate polyanions (small curved lines) dissolved in the aqueous phase (Na alginate). **Pathway 3:** embedding of o/w/o MCPs ( $\phi = 0.48$ ) in BSA-NH<sub>2</sub>/dextran-CHO hydrogel films as a step towards the immobilization of synthetic protocells in self-healing biomimetic matrices.

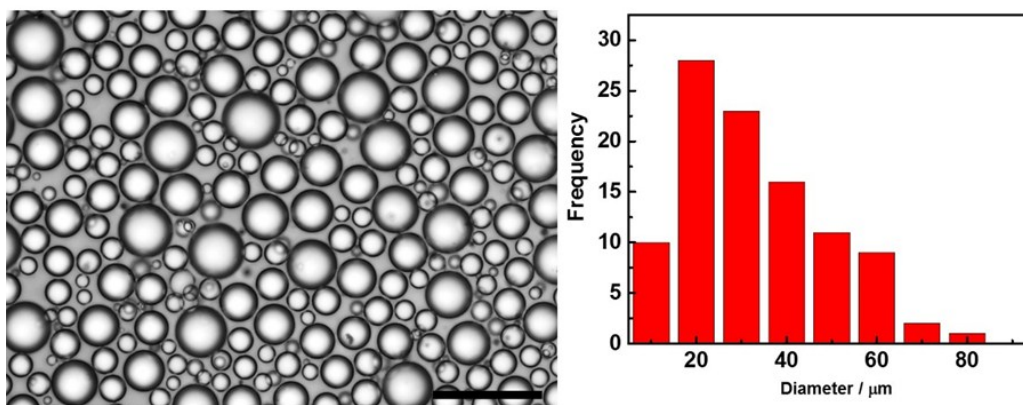


**Figure S1.** Preparation of MCP dispersions at  $\phi$  values of (from left to right) 0.24, 0.36, 0.48, 1.0, 2.0 and 3.0. (a) Samples showing phase-separated mixture consisting of an aqueous lower layer containing BSA-NH<sub>2</sub>/PNIPAAm (10 mg/mL), sodium carbonate buffer (pH 8.5), and FITC-Dextran (0.1 mg/mL; green coloration), along with an upper layer of 2-ethyl-1-hexanol immediately after addition, and after being left unstirred for 2 h (b). The red dashed lines highlight the formation of an interfacial layer of the BSA-NH<sub>2</sub>/PNIPAAm conjugates. (c) Samples after shaking by hand for 30 s showing formation of a dispersion of MCPs, which in some cases sedimented after being left unstirred for 20 min (d).

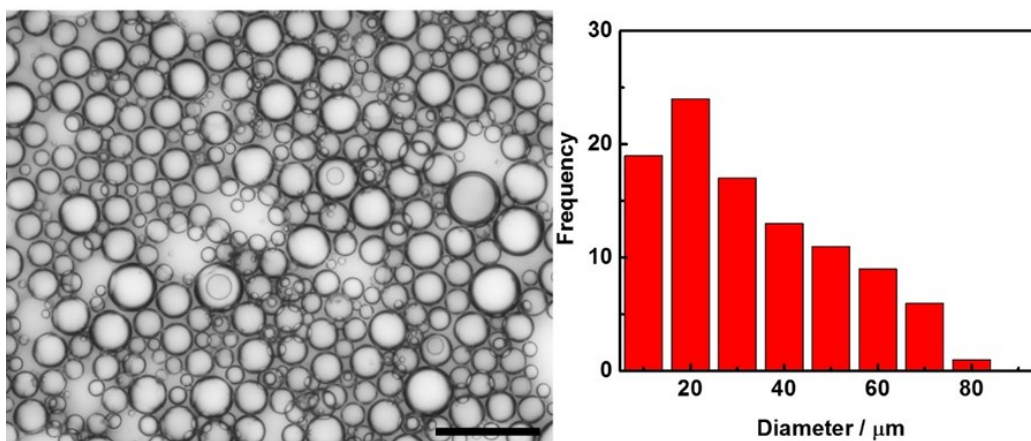




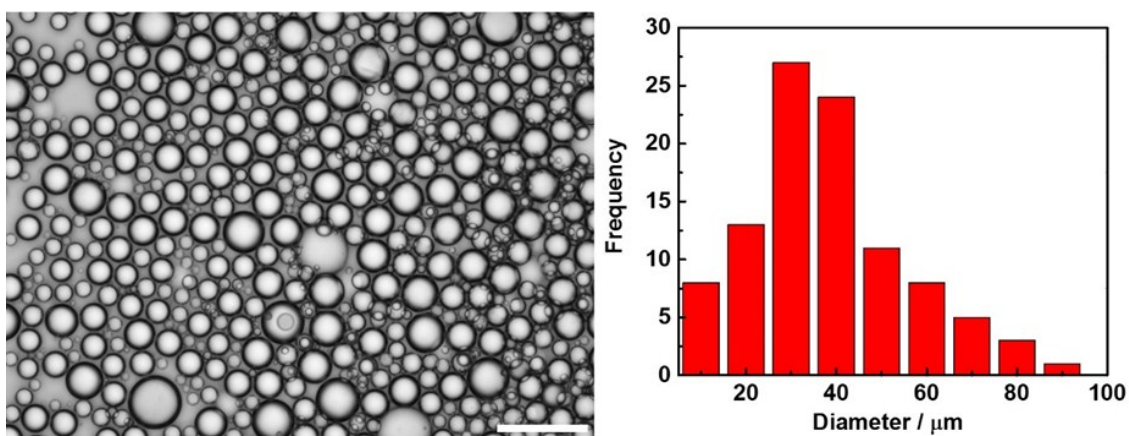
**Figure S2.** Optical microscopy images of proteinosomes obtained at different  $\phi$  values; 0.06 (a), 0.12 (b), 0.24 (c), 0.36 (d), 0.48 (e), 1.0 (f), 2.0 (g), 3.0 (h), and 4.0 (i). Scale bars are 100  $\mu\text{m}$ .



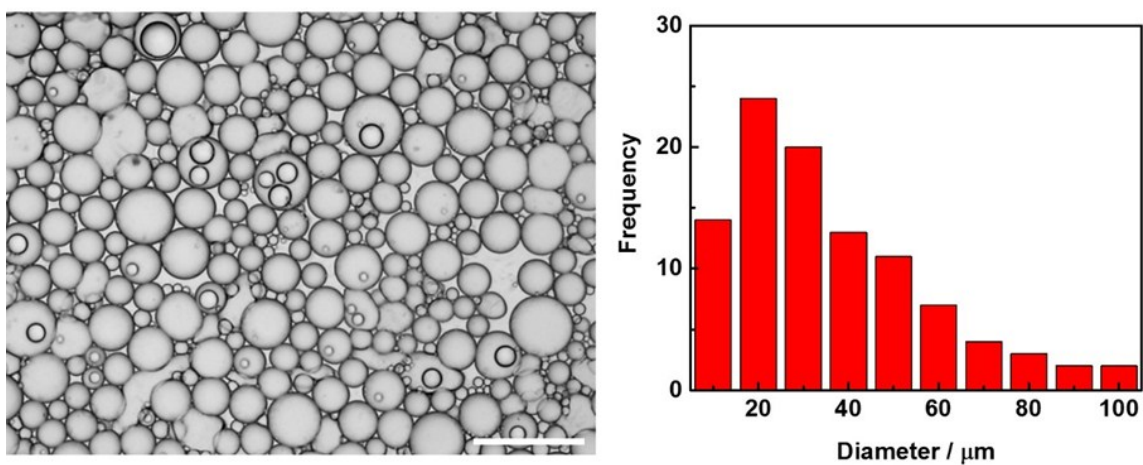
**Figure S3.** Optical microscopy image (left) of single-compartment water-filled proteinosomes in oil obtained at  $\phi = 0.06$ , and corresponding size distribution plot (right); scale bar = 100  $\mu\text{m}$ .



**Figure S4.** Optical microscopy image (left) of single-compartment proteinosomes and occasional o/w/o MCPs obtained at  $\phi = 0.12$ , and corresponding size distribution plot (right); scale bar = 100  $\mu\text{m}$ .

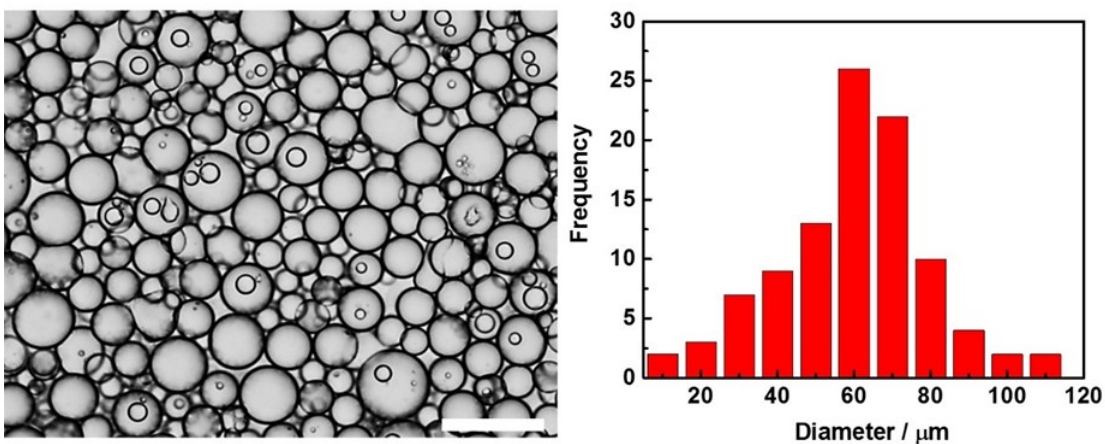


**Figure S5.** Optical microscopy image (left) of single-compartment proteinosomes and occasional o/w/o MCPs obtained at  $\phi = 0.24$ , and corresponding size distribution plot (right); scale bar = 100  $\mu\text{m}$ .

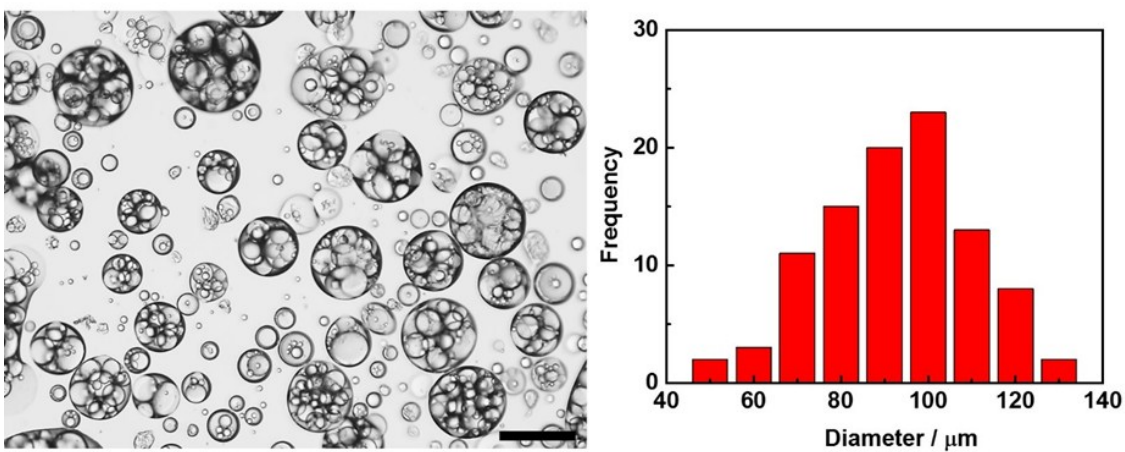


**Figure S6.** Optical microscopy image (left) of single-compartment proteinosomes and increasing numbers of o/w/o MCPs obtained at  $\phi = 0.36$ , and corresponding size distribution plot (right); scale bar = 100  $\mu\text{m}$ .

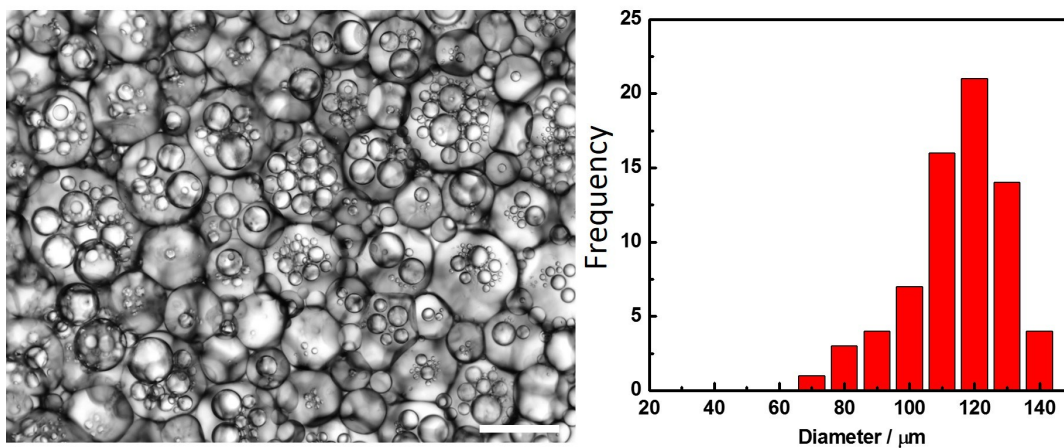




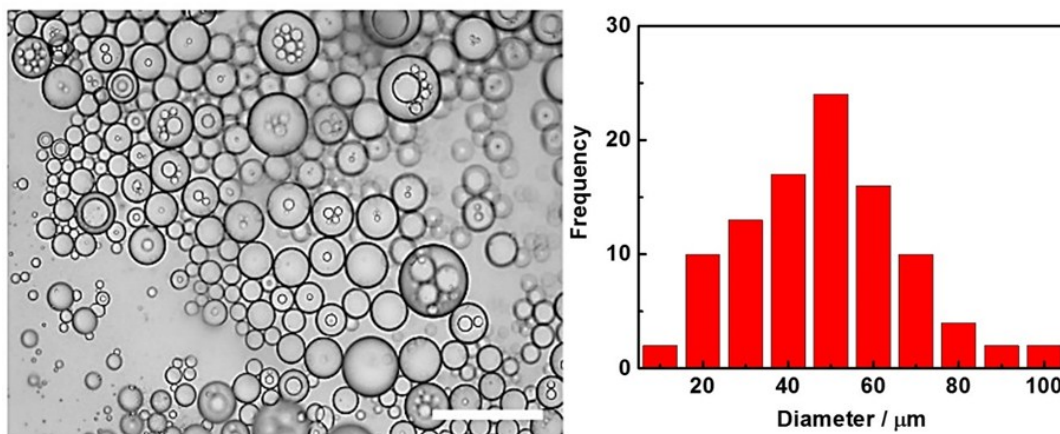
**Figure S7.** Optical microscopy image (left) showing mixture of single-compartment proteinosomes and o/w/o MCPs obtained at  $\phi = 0.48$ , and corresponding size distribution plot (right); scale bar = 100  $\mu\text{m}$ .



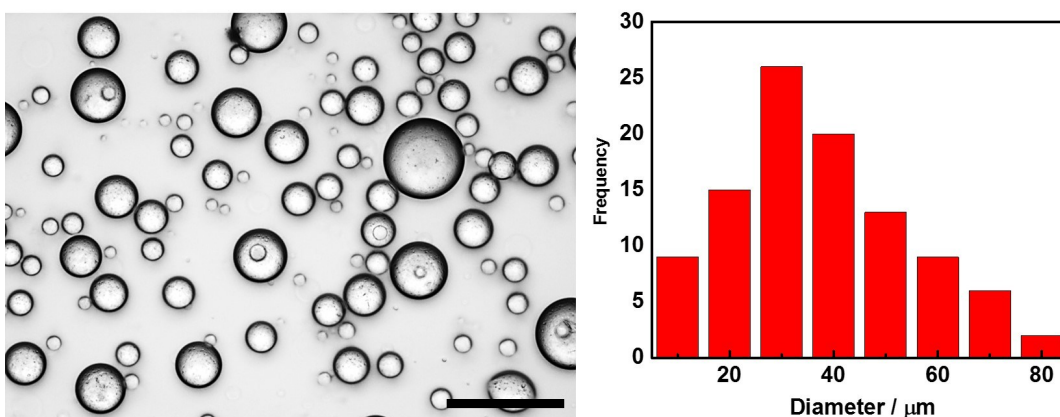
**Figure S8.** Optical microscopy image (left) showing large population of o/w/o MCPs obtained at  $\phi = 1.0$ , and corresponding size distribution plot (right); scale bar = 100  $\mu\text{m}$ .



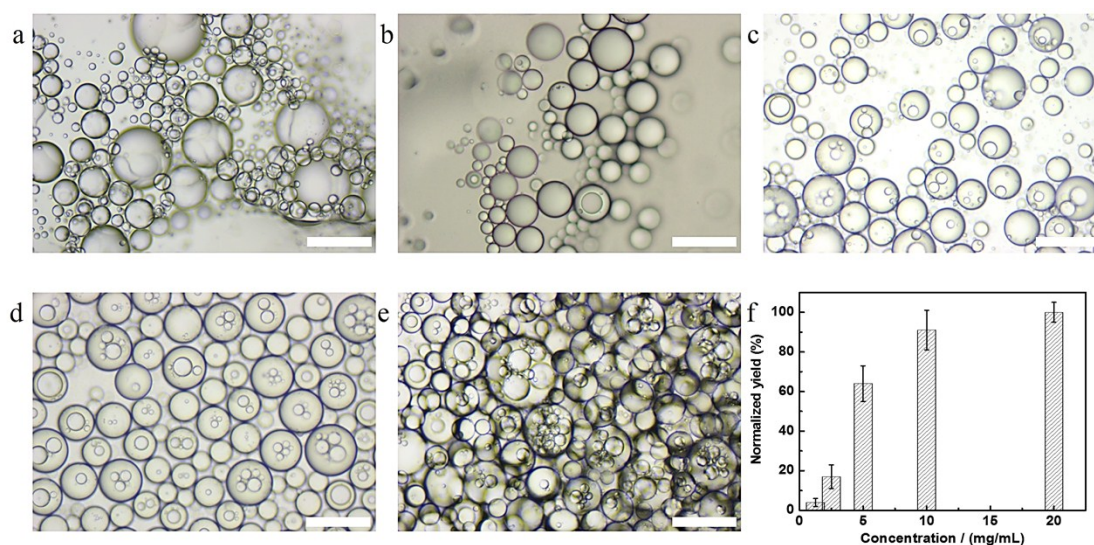
**Figure S9.** Optical microscopy image (left) showing population of w/o/w MCPs obtained at  $\phi = 2.0$ , and corresponding size distribution plot (right); scale bar = 100  $\mu\text{m}$ .



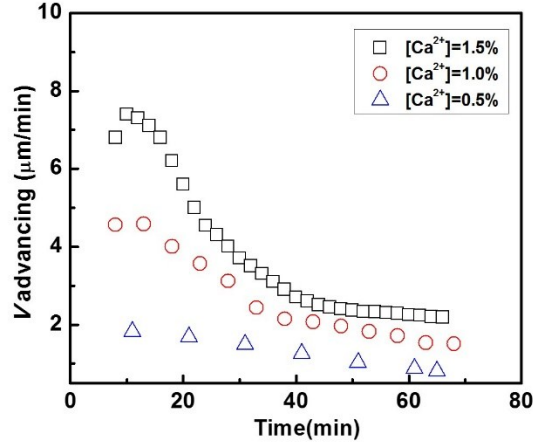
**Figure S10.** Optical microscopy image (left) showing decreasing population of w/o/w MCPs obtained at  $\phi = 3.0$ , and corresponding size distribution plot (right); scale bar = 100  $\mu\text{m}$ .



**Figure S11.** Optical microscopy image (left) showing mainly single-compartment oil-filled proteinosomes in water obtained at  $\phi = 4.0$ , and corresponding size distribution plot (right); scale bar = 100  $\mu\text{m}$ .



**Figure S12.** Optical microscopy images (a-e) showing mixtures of single-compartment proteinosomes and o/w/o MCPs obtained at  $\phi = 0.48$ , and BSA-NH<sub>2</sub>/PNIPAAm concentrations of 2.5 (a), 5 (b), 10 (c) and 20 mg/mL (d); scale bar = 100  $\mu\text{m}$ . (f) Normalized plot showing yields of <20, 65, 90 and 100% for samples prepared at 2.5, 5, 10 and 20 mg/mL, respectively. Similar trends of concentration influence could be observed at other  $\phi$  values.



**Figure S13.** Plot showing the time-dependent change in the rate of growth of a Ca alginate shell ( $v_{\text{advancing}}$ ) formed by interfacial complexation at the surface of a large o/w single-compartment proteinosome containing different concentrations of encapsulated  $\text{CaI}_2$  and dispersed in a continuous aqueous phase containing Na alginate (1% w/w).  $v_{\text{advancing}}$  was determined from optical microscopy images. The plots were used to optimize the  $\text{Ca}^{2+}$  and Na alginate concentrations required to produce MCPs with well-defined Ca alginate shells. Theoretically,  $v_{\text{advancing}}$  was also derived from the diffusion and reaction stoichiometry using Fick's first law.<sup>6,7</sup> The fluxes of alginate ( $J_a$ ) and  $\text{Ca}^{2+}$  ( $J_c$ ) consumed by the reaction zone in the vicinity of the hydrogel front are given by:

$$J_c = -D_c \cdot \frac{\partial C}{\partial x} \quad (2)$$

$$J_a = v_{\text{advancing}} \cdot A_0 \quad (3)$$

where,  $D_c$  denotes the diffusion coefficient of  $\text{Ca}^{2+}$  ions through the alginate gel,  $C$  represents the concentration of  $\text{Ca}^{2+}$  ions at position  $x$ , and  $A_0$  is the initial concentration of bulk sodium alginate. In the traveling wave approximation, stoichiometrically equivalent amounts of alginate must be converted to the hydrogel by  $\text{Ca}^{2+}$  ions reaching the reaction zone, such that:

$$J_c = N_c \cdot J_a \quad (4)$$

where  $N_c$  is the stoichiometric coefficient of the  $\text{Ca}^{2+}$ -alginate reaction. Therefore:

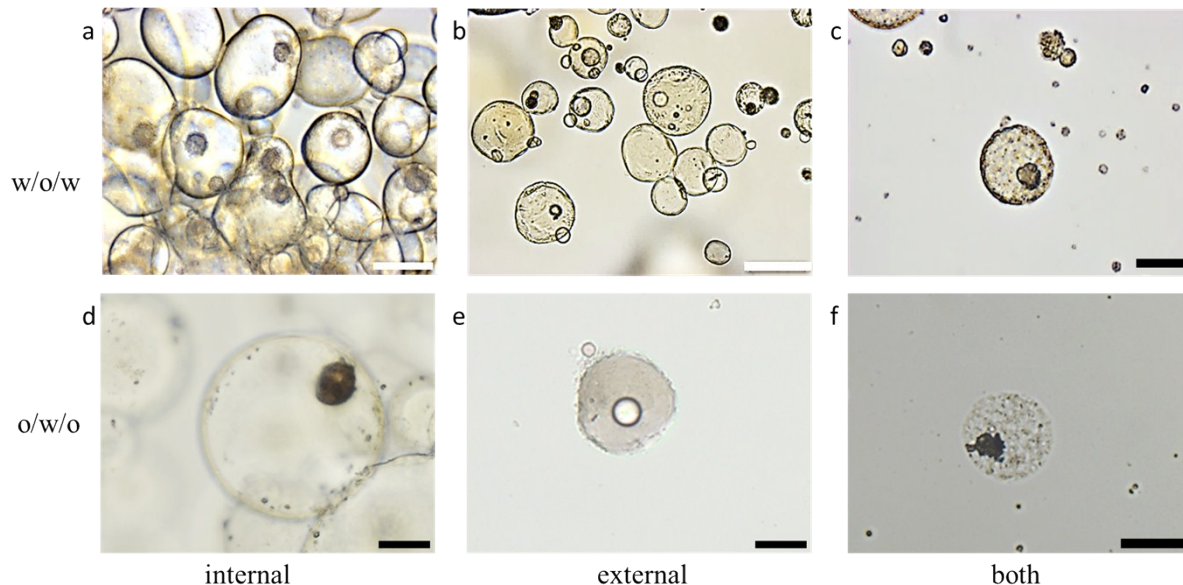
$$-D_c \cdot \frac{\partial C}{\partial x} = N_c \cdot v_{\text{advancing}} \cdot A_0 \quad (5)$$

and solving of the boundary conditions of (5) yields the expression:

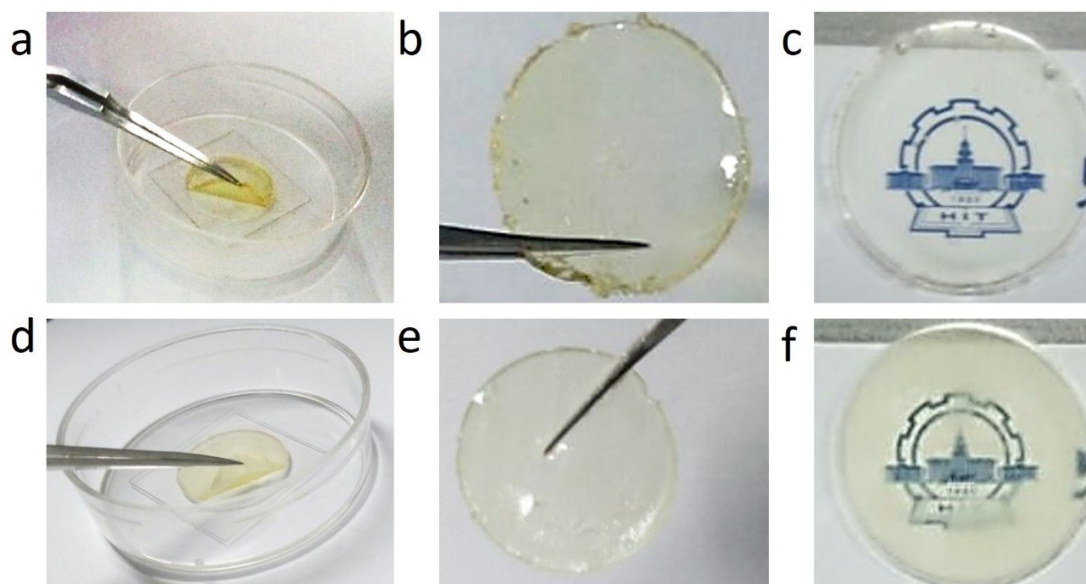
$$v_{\text{advancing}} = \frac{1}{2} \left( \frac{2D_c \cdot C_0}{N_c \cdot A_0 \cdot t} \right)^{1/2} \quad (6)$$

where  $C_0$  is the initial concentration of  $\text{Ca}^{2+}$  ions in the oil phase. Given this relationship, the concentration of  $\text{Ca}^{2+}$  ions was optimised at around 1.5% w/w to produce polysaccharide shells with sufficiently thickness to stabilize the proteinosome membranes. Similarly, considering the viscosity and reaction rate, the concentration of sodium alginate used was optimized at 1% w/w, to ensure that the hydrogel was formed in a limited time.



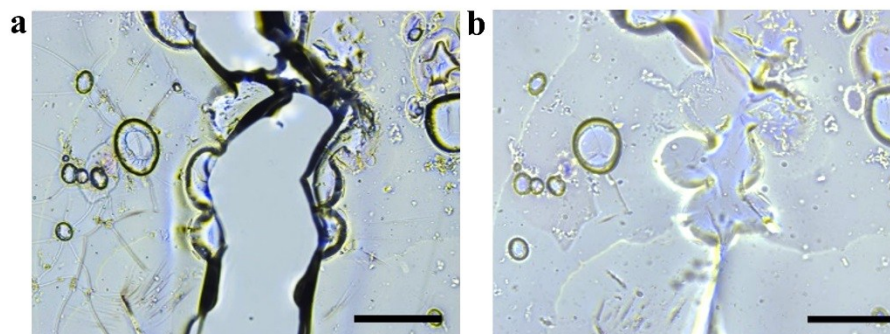


**Figure S14.** Optical microscopy images showing interfacial deposition of Ca-alginate shells; deposition on the internal membranes of guest proteinosomes in (a) w/o/w MCPs ( $\phi = 2$ ) and (d) o/w/o MCPs ( $\phi = 0.48$ ), (b) deposition on the external membrane of the host proteinosomes in (b) w/o/w MCPs ( $\phi = 2$ ) and (e) o/w/o MCPs ( $\phi = 0.48$ ), and (c) deposition on both the internal and external membranes in (c) w/o/w MCPs ( $\phi = 2$ ) and (f) o/w/o MCPs ( $\phi = 0.48$ ). Scale bars. 30  $\mu\text{m}$ .

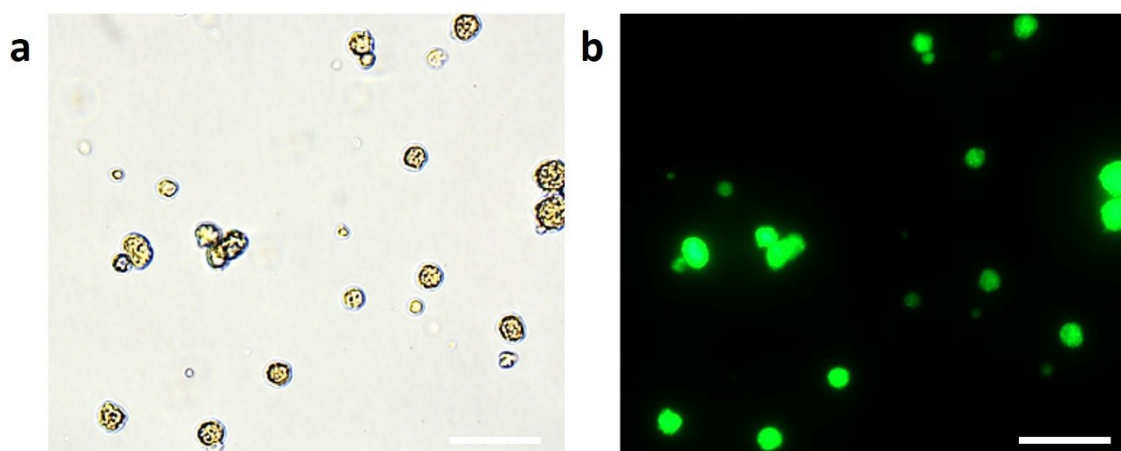


**Figure S15.** Photographs showing BSA-NH<sub>2</sub>/dex-CHO hydrogel films. (a,b,c) without and (d,e,f) with embedded MCPs (o/w/o,  $\phi = 0.48$ , no Ca alginate modification). Images show as-prepared hydrogels in a petri dish (a,d); self-supporting hydrogel films in air (b,e); and transparent hydrogel films placed over the surface of the University Badge of Harbin Institute of Technology (c,f).





**Figure S16.** Optical microscopy images of MCP-embedded BSA-NH<sub>2</sub>/dex-CHO hydrogel films (o/w/o MCPs,  $\phi = 0.48$ , no Ca alginate modification) before (a) and 10 min after (b) self-healing. The MCP-embedded hydrogel was coated on a glass surface, and then subjected to a mechanical scratch to produce a crack with width of *ca.* 150  $\mu\text{m}$ . In a moist environment, the crack sealed within 10 min, indicating that the presence of the embedded MCPs had minimal effect on the self-healing properties of the bulk hydrogel. Scale bars are 100  $\mu\text{m}$ .



**Figure S17.** Optical (a) and fluorescence (b) microscopy images of intact oil-filled proteinosome sub-compartments released from Ca alginate-modified o/w/o MCPs ( $\phi = 0.48$ ) embedded in a BSA-NH<sub>2</sub>/dex-CHO hydrogel. Only the internal membranes of the o/w/o MCPs ( $\phi = 0.48$ ) were coated by Ca alginate. Released proteinosomes were collected by centrifugation. Fluorescence originates from FITC-dextran encapsulated in the inner sub-compartments. Scale bars, 100  $\mu\text{m}$ .

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