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EXPERIMENTAL SECTION

Synthesis of BSA-NH₂/PNIPAAm conjugates

The BSA-NH₂/PNIPAAm conjugate was synthesized according to our previous methods [Huang, X.; et. al. *Nature Communications* **2013**, *4*, 2239]. First, cationized bovine serum albumin (BSA-NH₂) was synthesized by carbodiimide-activated conjugation of 1,6-diaminohexane to aspartic and glutamic acid residues on the external surface of the protein. For this, a solution of 1,6-diaminohexane (1.5 g, 12.9 mmol) was adjusted to pH 6.5 using 5 M HCl, and added dropwise to a stirred solution of the protein (200 mg, 2.98 μ mol). The coupling reaction was initiated by adding N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 100 mg) immediately, and again (50 mg) after 5 h. The pH value was maintained at 6.5 using dilute HCl, and the solution was stirred for a further 6 h. The solution was then centrifuged to remove any precipitate and the supernatant was dialyzed (dialysis tubing 12–14 kDa MWCO) extensively against Milli-Q water.

End-capped mercaptothiazoline-activated PNIPAAm ($M_n = 8,800 \text{ g mol}^{-1}$, 10 mg in 5 mL of water) was synthesized according to our previous reported methods [Huang, X.; et. al. *Nature Communications* **2013**, *4*, 2239], and added to a stirred solution of BSA-NH₂ (10 mg in 5 mL of PBS buffer at pH 8.0). The mixed solution was stirred for 12 h, and then purified by using a centrifugal filter (MWCO 50 kDa) to remove any unreacted PNIPAAm and salts. After freeze-drying, the BSA-NH₂/PNIPAAm conjugate was obtained. Based on MALDI-TOF and UV–vis spectroscopy measurements, there were ca. 3.3 PNIPAAm chains per BSA molecule.

Preparation of single compartmentalized proteinosomes

Proteinosomes were prepared by mixing an aqueous BSA-NH₂/PNIPAAm solution with 2-ethyl-1-hexanol followed by shaking the mixture by hand for 10 seconds. The samples were prepared at a constant aqueous/oil volume fraction (ϕ_w) of 0.06. Typically, 0.06 mL of aqueous BSA-NH₂/PNIPAAm in pH 8.5, sodium carbonate buffer were mixed with 1.0 mL of the oil. The

proteinosomes were then cross-linked in the continuous oil phase by addition of a NHS-PEG-containing cross-linker(PEG-bis(N-succinimidyl succinate (NHS-PEG9) ester or PEG-bis(N-succinimidyl succinate) disulfide ester (NHS-PEG16-DS)) for 12 h, which reacted with the free primary amine groups of BSA-NH₂.

Transfer of the cross-linked proteinosomes into water was achieved as follows. After 3 hours sedimentation (left to stand), the upper clear oil layer was discarded and 1 mL of 75% ethanol was added with a gentle shaking. Then the dispersion was dialyzed against 75% ethanol, and after 3 h dialysis, the dispersion was added dropwisely into 50 mL of Milli-Q water. Finally by dialyzing against water for at least 1 day to make sure all the oil was removed.

Preparation of multi-compartmentalized proteinosomes

Multi-compartmentalized proteinosomes were prepared based on a recursive Picking emulsion procedure. Typically, we used a 8.0 mg/mL aqueous dispersion of BSA-NH₂/PNIPAAm nanoconjugates to prepare a proteinosome stock solution comprising microcapsules with a mean size of ca. 15 μ m (proteinosome concentration; 1.0 mg/mL). We then mixed a certain amount of this stock solution with an aqueous solution of the BSA-NH₂/PNIPAAm nanoconjugates, keeping the total aqueous phase volume at 60 μ L with the concentration of BSA-NH₂/PNIPAAm nanoconjugates equal to 1.0 mg/mL. After adding 1mL of 2-ethyl-1-hexanol into the solution, the mixture was shaken by hand for 10 seconds, and a proteinosome-in-proteinosome structure obtained. The nested proteinosomes were cross-linked with a NHS-PEG-containing cross-linker, and the transferred into aqueous solution.

Encapsulating the two-tier proteinosome structure into a larger proteinosome to produce a three-tiered microarchitecture was undertaken by repeating the above procedures with appropriate modifications in the concentration of the BSA-NH₂/PNIPAAm nanoconjugates to generate appropriately sized host proteinosomes. Typically, the sequence order of concentrations of BSA-NH₂/PNIPAAm nanoconjugates used was 8.0 mg/mL, 1.0 mg/mL and 0.5 mg/mL.

Sub-compartment encapsulation of enzymes into hierarchically ordered

The above procedures were adopted except that the enzymes were added to the aqueous BSA-NH₂/PNIPAAm solution prior to mixing with the oil phase. For example, the targeted loading of a three-tier proteinosome with different fluorescence-labeled enzymes was undertaken by first encapsulating fluorescein isothiocyanate-labeled glucose oxidase (FITC-GO, green) into small proteinosomes, which were subsequently used as the first guest microcapsules. Rhodamine B isothiocyanate-labeled glucose amylase (RBITC-GA, red) together with the pre-formed FITC-GO-containing proteinosomes were added to an aqueous solution of the BSA-NH₂/PNIPAAm nanoconjugates (final concentration, 1.0 mg/mL) and the two-tier proteinosomes cross-linked and transferred to water as described above. Finally, the procedure was repeated by mixing the two-tier FITC-GO/RBITC-GA proteinosomes with DyLight 405-labeled horseradish peroxidase (DL405-HRP, blue) and a solution of BSA-NH₂/PNIPAAm nanoconjugates (0.5 mg/mL in the mixed solution) to produce a three-tier triple proteinosome with spatially organized enzymes.

Programmed release of RBITC-Dextran and DNA from two-tier proteinosomes

DNA (salmon testes, Sigma, ~ 2000b.p) was encapsulated into the interior of single-level proteinosomes by adding aqueous DNA (20 µL, 2.0 mg/mL) to 40 µL of an aqueous solution of BSA-NH₂/PNIPAAm nanoconjugates (12 mg/mL), followed by addition of 1.0 mL of 2-ethyl-1-hexanol. The proteinosomes were then cross-linked with NHS-PEG9 ester, and transferred into water as described above. The DNA loaded proteinosomes were mixed with an aqueous solution of RBITC-dextran and BSA-NH₂/PNIPAAm nanoconjugates (final concentration, 1.0 mg/mL), followed by addition of 1.0 mL of 2-ethyl-1-hexanol and NHS-PEG9 ester to cross-link the host proteinosome, and transfer into water. Under these conditions, the two-tiered micro-architecture consisted of DNA and dextran encapsulated specifically within the guest and in proteinosomes, respectively, and comprised

non-disulfide membranes throughout. Similar procedures were used to capture DNA and dextran in the guest and host compartments, but the membranes of the guest and host proteinosomes were cross-linked using different reagents (NHS-PEG9 ester or NHS-PEG16-DS) to produce two-tiered structures comprising different membrane chemistries.

For the two-tier proteinosomes cross-linked with only NHS-PEG9 ester, release of encapsulated DNA and RBITC-dextran was induced by protease. An aqueous dispersion of the DNA/RBITC-dextran-proteinosome-in-proteinosome was mixed with 20 mL of a buffered protease solution (*Streptomyces griseus*, Sigma, ≥ 3.5 units/mg solid, $M_w \sim 50$ kDa; pH 7.4 buffer) at a concentration of 0.01 mg/mL. Aliquots (2 mL) of the suspension were removed at various time intervals, filtered to remove any intact proteinosomes, and the concentration of released SYBR green I-stained DNA or RBITC-dextran in the bulk phase determined by measuring changes in fluorescence intensity at 522 nm for DNA, and 580 nm for RBITC-dextran. This procedure was also used to determine the release of encapsulated components from the NHS-PEG9/NHS-PEG16-DS cross-linked proteinosome-in-proteinosomes. In this case, release was first triggered by addition of tris(2-carboxyethyl)phosphine (TCEP) (2.5 mM, in pH 8.0 PBS) for 20-40 minutes, followed by addition of 1 mg/mL of protease.

Synthesis of fluorescence-labeled proteins

Fluorescein isothiocyanate (FITC)-labeled glucose oxidase (FITC-GO) was synthesized by dissolving a dried powder of the protein (5.0 mg) in 2.0 mL of pH 8.5 sodium carbonate buffer solution (100 mM), followed by dropwise addition of 50 μ L DMSO solution of FITC (1.0 mg/mL). The solution was stirred at room temperature for 5 h, purified by dialyzing against Milli-Q water, and freeze-dried. Rhodamine B isothiocyanate (RBITC)-labeled glucose amylase, and DyLight 405-labeled horseradish peroxidase (DL405-HRP) were prepared by the same procedure using a DMSO solution of RBITC (1.0 mg/mL) or aqueous solution of DL405 (1.0 mg/mL).

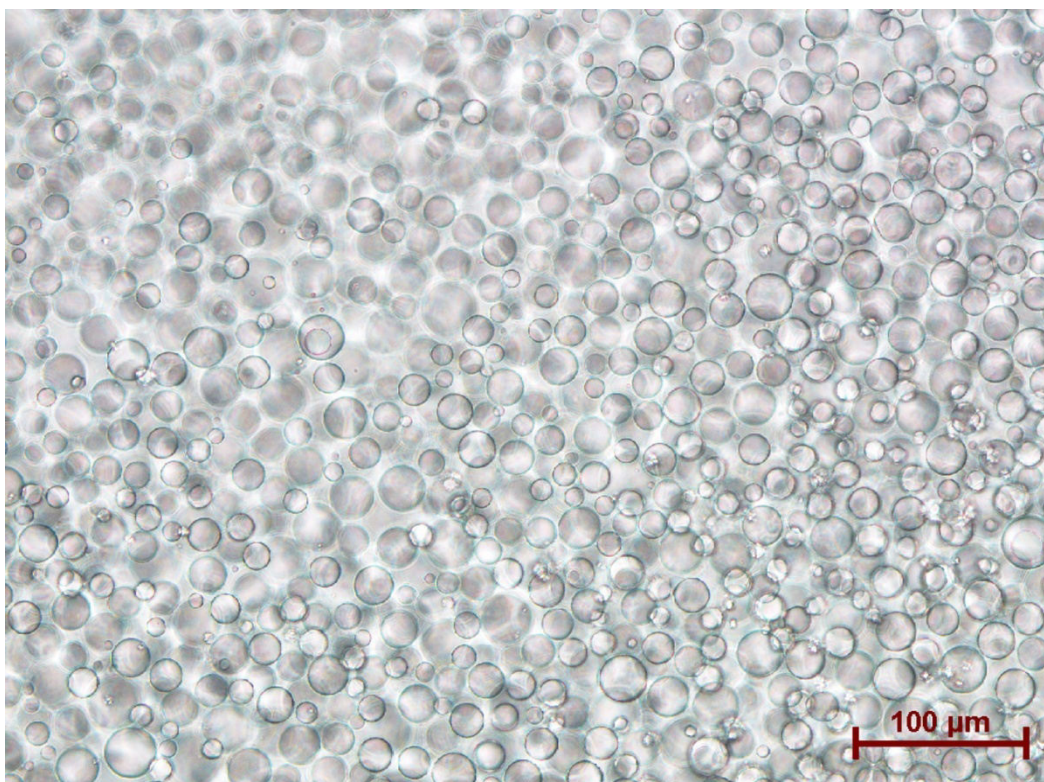


Figure S1. Optical microscopy image of single-chambered BSA-NH₂/PNIPAAm proteinosomes dispersed in 2-ethyl-1-hexanol. The sample was prepared under vigorous shaking at a ϕ_w value of 0.06, and using a BSA-NH₂/PNIPAAm concentration of 8.0 mg/mL.

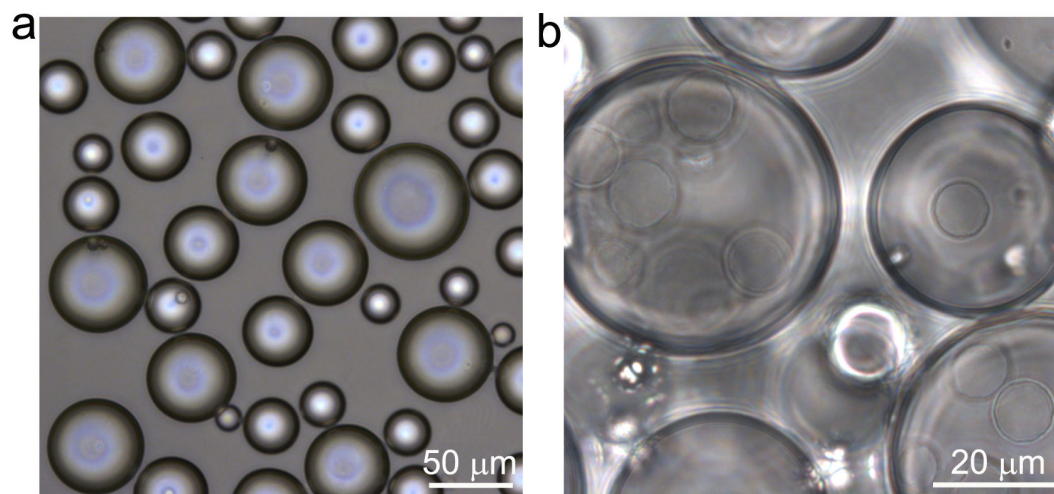


Figure S2. Optical microscopy images of two-tiered BSA-NH₂/PNIPAAm proteinosomes dispersed in oil (a), and after partial drying in air (b).

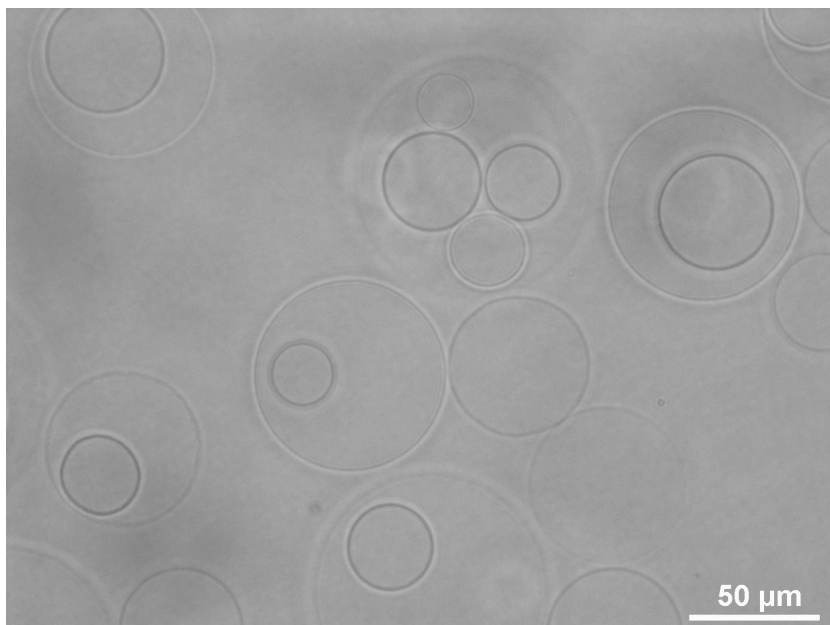


Figure S3. Optical microscopy image of two-level BSA-NH₂/PNIPAAm proteinosomes prepared at a guest proteinosome: protein-polymer nanoconjugate mass ratios of 4 : 10.

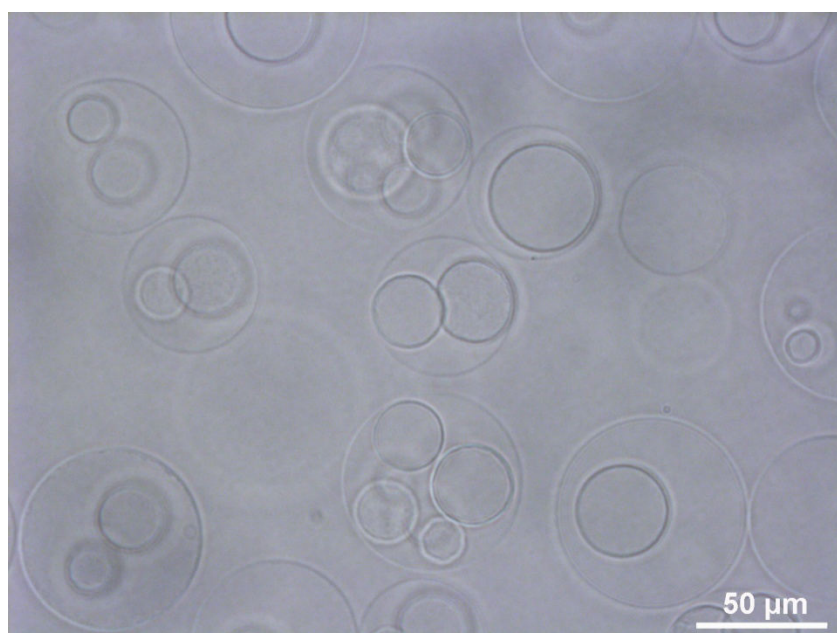


Figure S4. Optical microscopy image of two-level BSA-NH₂/PNIPAAm proteinosomes prepared at a guest proteinosome: protein-polymer nanoconjugate mass ratios of 8 : 10.

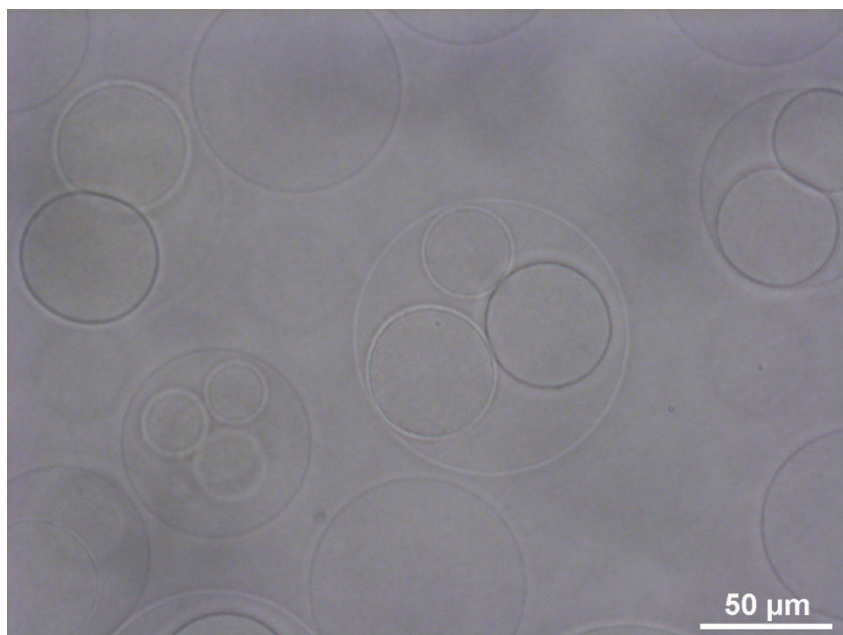


Figure S5. Optical microscopy image of two-level BSA-NH₂/PNIPAAm proteinosomes prepared at a guest proteinosome: protein-polymer nanoconjugate mass ratios of 12 : 10.

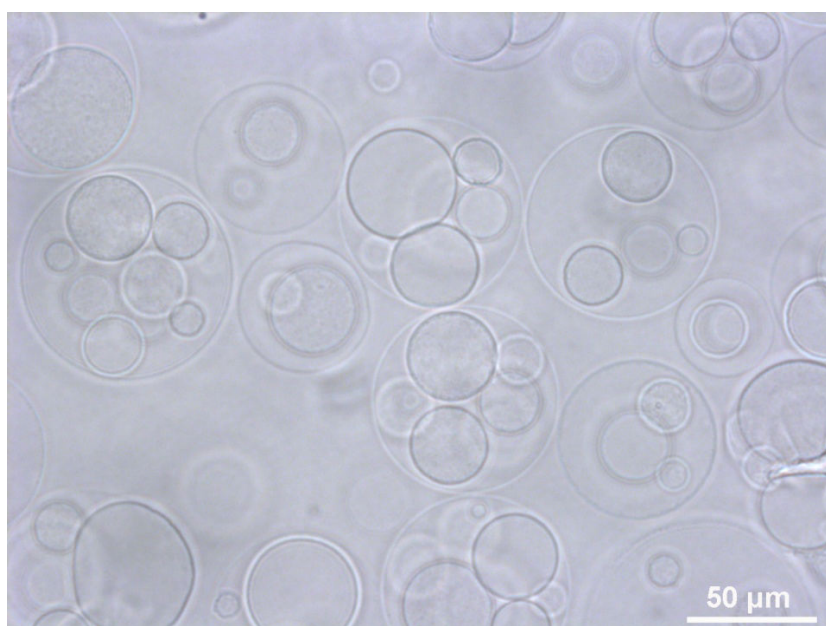


Figure S6. Optical microscopy image of two-level BSA-NH₂/PNIPAAm proteinosomes prepared at a guest proteinosome: protein-polymer nanoconjugate mass ratios of 30 : 10.

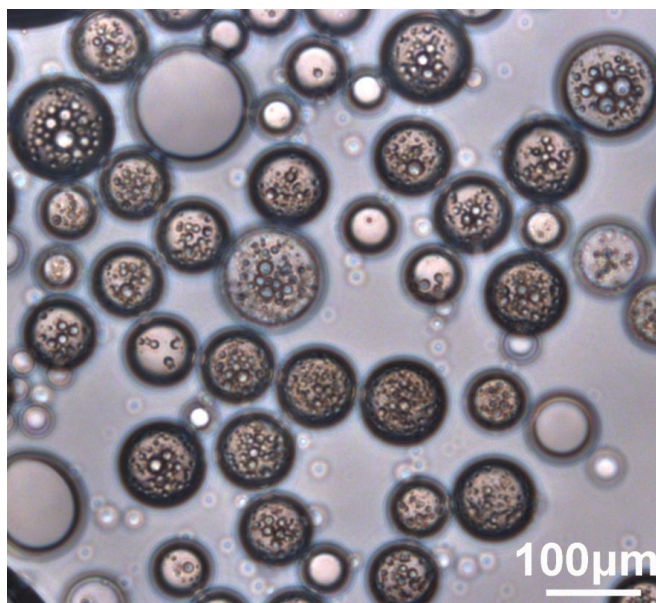


Figure S7. Optical microscopy image showing multi-compartmentalized BSA-NH₂/PNIPAAm proteinosomes in 2-ethyl-1-hexanol prepared by encapsulating 8-15 μm-sized guest proteinosomes into individual host proteinosome 80-100 μm in diameter. Tens of proteinosomes are encapsulated within each host microstructure.

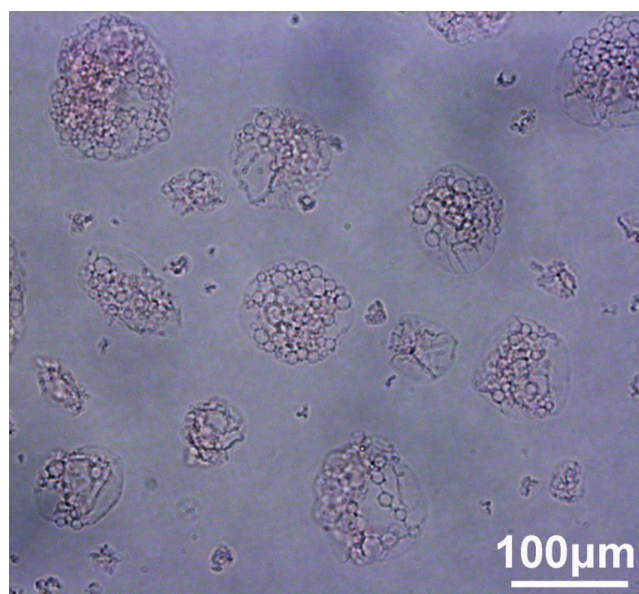


Figure S8. Optical microscopy image of multi-compartmentalized BSA-NH₂/PNIPAAm proteinosomes in 2-ethyl-1-hexanol phase after drying. The samples were prepared by encapsulating 8-15 μm-sized guest proteinosomes into individual host proteinosome 80-100 μm in diameter. Tens of proteinosomes are encapsulated within each host microstructure.

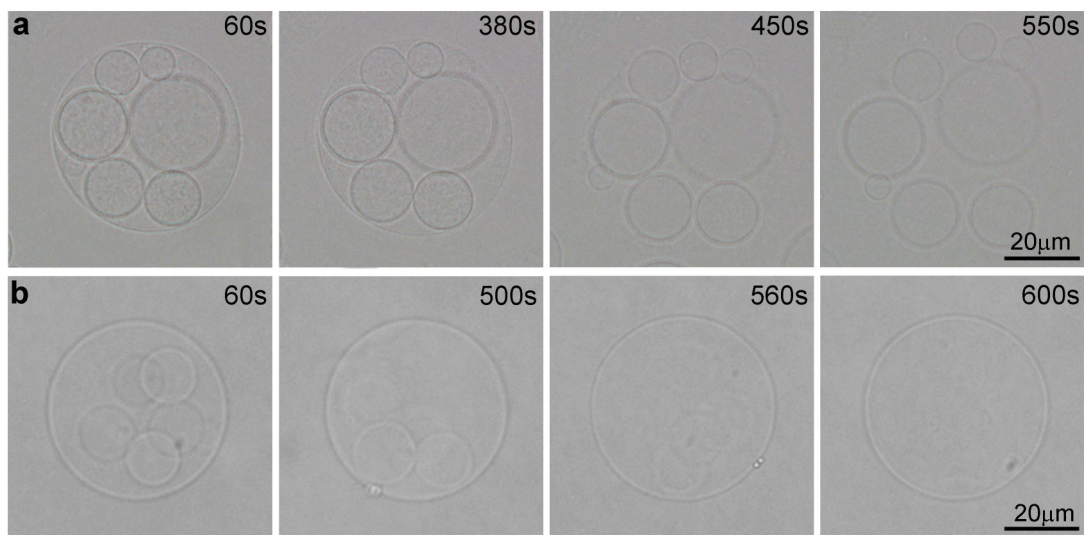


Figure S9. (a,b) Time-dependent series showing optical microscopy images of two-tiered proteinosomes with host and guest membranes cross-linked respectively by NHS-PEG16-DS or NHS-PEG9 ester (a), or NHS-PEG9 ester or NHS-PEG16-DS, respectively (b), and after addition of TCEP (5 mM, pH 8.0). (a) Complete disassembly of the host proteinosome occurs after 550 s whilst the guest proteinosomes remain intact and slowly disperse into the external solution. (b) Complete disassembly of the guest proteinosome membranes occurs after 600 s leaving an intact host proteinosome.