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Hierarchical Proteinosomes for Programmed Release of Multiple Components

Xiaoman Liu, Pei Zhou, Yudong Huang, Mei Li, Xin Huang* and Stephen Mann*

*Dr. X. M. Liu, P. Zhou, Prof. Y. D. Huang, Prof. X. Huang, School of Chemical Engineering and Technology; State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin, 150001, China; *E-mail: xinquang@hit.edu.cn.*

*Dr. M. Li, Prof. S. Mann, Centre for Protolife Research and Centre for Organized Matter Chemistry, School of Chemistry, University of Bristol, Bristol, BS8 1TS, United Kingdom; *E-mail: s.mann@bristol.ac.uk.*

A facile route to hierarchically organized multi-compartmentalized proteinosomes based on a recursive Pickering emulsion procedure using amphiphilic protein-polymer nanoconjugate building blocks is described. The number of incarcerated guest proteinosomes within a single host proteinosome is controlled, and enzymes and genetic polymers encapsulated within targeted sub-compartments to produce chemically organized multi-tiered structures. Three types of spatiotemporal response – retarded concomitant release, synchronous release or hierarchical release of dextran and DNA – are demonstrated based on the sequential response of the host and guest membranes to attack by protease, or through variations in the positioning of disulfide-containing cross-links in either the host or guest proteinosomes integrated into the nested architectures. Overall, our studies provide a step towards the construction of hierarchically structured synthetic protocells with chemically and spatially integrated proto-organelles.

Compartmentalization is a fundamental requirement for many biochemical processes, critical to the evolution of eukaryotic cells, and instrumental in the origin of life.^[1] Many cellular organizations owe their complexity and efficiency to multi-compartmentalized structures, which in the form of membrane-bounded intracellular organelles provide specialized functions such as energy transduction, positional assembly and protein trafficking that collectively provide the cell with spatiotemporal control over informational and metabolic processing.^[2] In recent years, new approaches have been developed to understand cellular function by mimicking or reconstructing artificial protocell models based on bottom-up strategies.^[3] In particular, a series of single-compartment models of synthetic cellularity have been designed to investigate the chemical and physical basis of cell-free gene expression,^[4] artificial cytoskeletal assembly,^[5] enhanced or membrane-gated enzyme-mediated transformations,^[6] RNA catalysis,^[7] membrane growth and division^[8] and motility.^[5b,9] Increasing the complexity of these protocell models will inevitably require the design and

construction of colloidal-scale architectures with hierarchical organization and proto-organelle functions. In this regard, a number of different types of multi-compartmentalized structures have been recently reported,^[10] with potential applications in drug delivery, microreactor technology and biomaterials engineering.^[11] Typical examples include: vesosomes (liposome-in-liposome vesicles);^[12] polymersome-in-polymersome structures;^[13] dendrimersomes formed by the self-assembly of Janus dendrimers in solution;^[14] capsosomes prepared by layer-by-layer assembly to encapsulate sub-compartments in the shell or core of a polymeric capsule;^[11a,15] and polymer capsules-in-polymer capsule constructs fabricated by self-assembly,^[16] surface-initiated polymerization,^[17] double-emulsion,^[18] or microfluidic^[19] methodologies.

Previously, we developed an approach to the construction of a new type of single-compartmentalized microarchitecture based on the interfacial assembly of globular protein-polymer amphiphilic nanoconjugates at the surface of water droplets dispersed as a Pickering emulsion in oil (2-ethyl-1-hexanol).^[20] The building blocks were synthesized by covalently attaching approximately three molecules of the temperature-responsive polymer poly(N-isopropylacrylamide) (PNIPAAm; $M_n = 8800 \text{ g mol}^{-1}$, PDI = 1.19, monomer repeat units ≈ 75) to the surface primary amine groups of a cationized form of bovine serum albumin (BSA-NH₂). Spontaneous assembly of the BSA-NH₂/PNIPAAm nanoconjugates gave rise to spherical single microcompartments (proteinosomes) that were delineated by a closely packed monolayer with an outer and inner surface of PNIPAAm and protein-rich domains, respectively. Significantly, the membrane could be cross-linked with PEG-bis(N-succinimidyl succinate) ester (NHS-PEG9 ester, $M_w = 2000$) in the continuous oil phase by reaction of free primary amines on the protein surface to produce structurally robust proteinosomes that could be transferred into aqueous solution and employed as a novel type of synthetic protocell capable of high encapsulation efficiency, selective permeability, guest molecule encapsulation, gene-directed protein synthesis, spatially confined membrane-gated enzyme activity, and membrane mediated tandem catalysis.^[20,21]

Herein, we show that modulation of the concentration of the protein-polymer building blocks in combination with an applied shear stress can be used to control the size of the proteinosomes such that multi-compartmentalized proteinosomes can be produced by a recursive Pickering emulsion procedure (**Figure 1a**). By regulating the encapsulation sequence, we demonstrate that hierarchically structured proteinosomes comprising two or three levels of organization and controllable numbers of incarcerated guest proteinosomes can be prepared by a facile procedure. A range of enzymes and genetic polymers can be loaded into targeted sub-compartments to produce chemically organized multi-tier micro-architectures. We demonstrate that engineering of the protein-polymer membrane cross-links using judicious combinations of NHS-PEG9 ester

and PEG-bis(N-succinimidyl succinate) disulfide ester (NHS-PEG16-DS) gives rise to multi-compartmentalized proteinosomes that exhibit modulated programmed release behavior when exposed to an aqueous protease solution or when subjected to stepwise addition of a reducing agent (tris(2-carboxyethyl)phosphine (TCEP)) followed by protease. Using this strategy, we show three types of spatiotemporal response based on retarded concomitant release, synchronous release or hierarchical release of dextran and DNA from within the nested proteinosome architectures. In general, the fabrication of multi-tiered micro-compartmentalized colloidal-scale objects provides a step towards the construction of hierarchically structured synthetic protocells with chemically and spatially integrated proto-organelles.

Single chambered BSA-NH₂/PNIPAAm proteinosomes were produced according to previous methods.^[20] The average diameter of the proteinosomes could be systematically controlled between 10 and 200 μm by changing the concentration of the BSA-NH₂/PNIPAAm conjugate at a constant aqueous/oil volume fraction value ($\phi_w = 0.06$), and the amount of shear stress applied during formation of the Pickering emulsions (**Figure 1b**). In general, the proteinosomes decreased in size as the concentration of the building blocks increased from 0.2 to 8 mg/mL, and a further 2 to 3-fold decrease was observed in the mean diameter when the samples were vigorously shaken compared with gentle shaking. The above procedures were used to fabricate various types of multi-compartmentalized proteinosome. For example, single-chambered proteinosomes with a mean size of approximately 15 μm were self-assembled by vigorous shaking of a water/2-ethyl-1-hexanol mixture containing 8.0 mg/mL of the protein-polymer nanoconjugate (**Figure S1**, Supporting Information), followed by cross-linking with NHS-PEG9, and transfer into aqueous solution. An aliquot of this aqueous dispersion was added to an aqueous solution of BSA-NH₂/PNIPAAm (2.0mg/mL) and then mixed by vigorous shaking with 2-ethyl-1-hexanol at $\phi_w = 0.06$, and the resulting Pickering emulsions (**Figure S2**, Supporting Information) cross-linked with NHS-PEG9 and transferred into water to produce two-tier proteinosomes with low optical contrast (**Figure 1c**). The hierarchical structure of the proteinosomes was clearly visible in transmission electron microscopy (TEM) images, which showed a collapsed but structurally intact nested micro-architecture (**Figure 1d**). Similar procedures were employed to generate a three-tiered nested architecture. In this case, an aliquot of the aqueous suspension of the bi-compartmentalized structures was added to a 0.5 mg/mL aqueous solution of BSA-NH₂/PNIPAAm, mixed with 2-ethyl-1-hexanol at $\phi_w = 0.06$, cross-linked with NHS-PEG9, and transferred into water.

The number of proteinosomes entrapped within each two-tiered nested structure was modulated as a statistical distribution by controlling the mass ratio of the preformed (guest) proteinosomes and protein-polymer nanoconjugates used to generate the host proteinosome. To elucidate this, a batch of single compartmentalized proteinosomes

with average size of 20-25 μm was prepared, and different amounts of this aqueous suspension then mixed with a 1.0 mg/mL aqueous solution of BSA-NH₂/PNIPAAm protein-polymer nanoconjugates at a total aqueous volume of 60 μL . 1 mL of 2-ethyl-1-hexanol was then added and the mixture shaken by hand for 10 seconds to produce *ca.* 60 μm -sized proteinosomes with two levels of organization, which were subsequently cross-linked and transferred into water. As a consequence, the statistical number of encapsulated proteinosomes per host proteinosome could be modulated from 1 up to 6 by varying the guest proteinosome: BSA-NH₂/PNIPAAm nanoconjugate mass ratio used in the preparation from 4 : 10 to 30 :10 (**Figure 2** and **Figure S3-6**, Supporting Information). Increasing numbers of encapsulated proteinosomes could be obtained by decreasing the size of the guest proteinosomes by appropriate adjustments in the preparation procedure as summarized in Figure 1. For example, two-tiered structures prepared using 8-15 μm -sized proteinosomes as guest components in host structures 80-100 μm in diameter gave rise to the incarceration of tens of sub-compartments (**Figure S7, S8**, Supporting Information).

As a step towards preparing complex proteinosomes with hierarchically dependent properties we encapsulated multiple distinct components into targeted sub-compartments of the nested proteinosomes. In addition, we engineered the cross-links of the host and guest protein-polymer membranes to program the spatial and temporal release of the incarcerated macromolecules when exposed to certain chemical cues in the surrounding environment. First, we showed that different biological macromolecules could be readily loaded into targeted sub-compartments of the multi-tiered proteinosomes as a step towards the construction of hierarchically structured synthetic protocells with chemically and spatially integrated proto-organelles. For example, a recursive Pickering emulsion procedure was used to prepare three-tiered proteinosomes comprising three spatially separated enzymes with the inner, middle and outer chambers containing fluorescein isothiocyanate-labeled glucose oxidase (FITC-GO, green), rhodamine B isothiocyanate-labeled glucose amylase, (RBITC-GA, red), and DyLight 405-labeled horseradish peroxidase (DL405-HRP, blue) respectively (**Figure 3a-d**). No cross-contamination between the different levels of organization was observed. In addition, we encapsulated on a statistical basis two different types of similarly sized guest proteinosomes containing different macromolecular payloads (FITC-labelled dextran or RBITC-GA) into the host proteinosomes (**Figure 3e**).

Selective disintegration of either the host or guest proteinosomes within the hierarchical structures dispersed in aqueous solution was achieved by systematically engineering the protein-polymer membrane cross-links using the non-disulfide NHS-PEG9 ester or disulfide-containing NHS-PEG16-DS reagent, and then exposing the microstructures to the reducing agent TCEP. Thus, preparing two-tiered

proteinosomes with the host and guest membranes cross-linked by NHS-PEG16-DS or NHS-PEG9 ester, respectively, resulted in selective disintegration of the host proteinosome membrane and release of intact guest proteinosomes in the presence of the reducing agent (**Figure 4a,b** and **Figure S9**, Supporting Information). In contrast, addition of TCEP (2.5 mM, pH 8.0) to the nested structures, in which the guest and host proteinosomes were cross-linked by NHS-PEG16-DS or NHS-PEG9 ester, respectively, gave rise to membrane disassembly of the incarcerated proteinosomes and redistribution of their components within the compartmentalized space of the intact host microcapsule (**Figure 4c,d** and **Figure S9**, Supporting Information).

Given these observations, we established a strategy for the programmed spatial and temporal release of incarcerated macromolecules from different chambers of the multi-compartmentalized proteinosomes. For this, we prepared two-tiered micro-architectures with ds-DNA (2000 bp) and rhodamine B isothiocyanate-labeled dextran (RBITC-dextran) entrapped within the guest and host proteinosomes, respectively. To regulate the response to chemical cues, three variants of this organization were prepared. Firstly, we cross-linked the host and guest proteinosomes with the NHS-PEG9 ester so that both membranes were insensitive to TCEP but responsive to the presence of protease in the external solution. As a consequence, addition of protease (0.01 mg/mL) immediately triggered lysis of the exposed membrane of the host proteinosome to release RBITC-dextran followed by disassembly of the guest proteinosome and concomitant release of DNA after a delay of approximately 2 min (**Figure 4e**). Secondly, we prepared a similar two-tiered system but cross-linked the guest and host membranes with NHS-PEG16-DS or NHS-PEG9 ester, respectively. Addition of TCEP (2.5 mM, pH 8.0) triggered the selective disassembly of the incarcerated proteinosomes and release of DNA into the RBITC-dextran-containing host proteinosomes. As a consequence, no macromolecular release was observed in the bulk solution until a protease solution was introduced, after which both payloads were synchronously released (**Figure 4f**). Thirdly, we cross-linked the guest and host proteinosome membranes with NHS-PEG9 ester or NHS-PEG16-DS, respectively, to produce hierarchical release behavior in which RBITC-dextran was selectively and fully released on addition of TCEP whilst DNA was retained in the guest proteinosomes; the latter was subsequently released as a discrete pulse when protease was added to the dispersion (**Figure 4g**).

In conclusion, a facile route to hierarchically organized multi-compartmentalized proteinosomes based on a recursive Pickering emulsion procedure using amphiphilic protein-polymer nanoconjugate building blocks has been demonstrated. The statistical number of incarcerated guest proteinosomes within a single host proteinosome is controllable, although use of other procedures such as microfluidic processing could provide more precise regulation of the proteinosome diameters if required for example

for technological development. Functional macromolecules such as enzymes and genetic polymers can be encapsulated within the targeted sub-compartments to produce chemically organized multi-tiered structures. In addition, three types of spatiotemporal response – retarded concomitant release, synchronous release or hierarchical release of dextran and DNA – are demonstrated based on the sequential response of the host and guest membranes to attack by protease, or through variations in the positioning of disulfide-containing cross-links in either the host or guest proteinosomes integrated into the nested architectures.

More generally, our work provides a step towards the design and construction of a next generation of hierarchically structured protocell models based on multi-tiered micro-compartmentalized colloidal-scale objects. Our results indicate that nested proteinosomes have the potential to organize diverse functional components into spatial arrangements that are membrane-bounded and integrated locally without chemical interference. They offer therefore a possible route to the development of hierarchical protocells with specialized sub-compartments (proto-organelles) that increase the functional complexity and processing ability of these synthetic micro-ensembles. For example, it seems feasible that internal signaling of enzyme cascade reactions or switching of cell-free gene networks could be embedded within the hierarchical organization. And spatiotemporal release of the functional payloads and their reaction products as a response to certain chemical cues in the external environment could provide a mechanism for generating protocells with adaptive behavior or selective advantage within synthetic communities of these micro-compartmentalized objects.

ACKNOWLEDGMENTS

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FIGURES AND LEGENDS

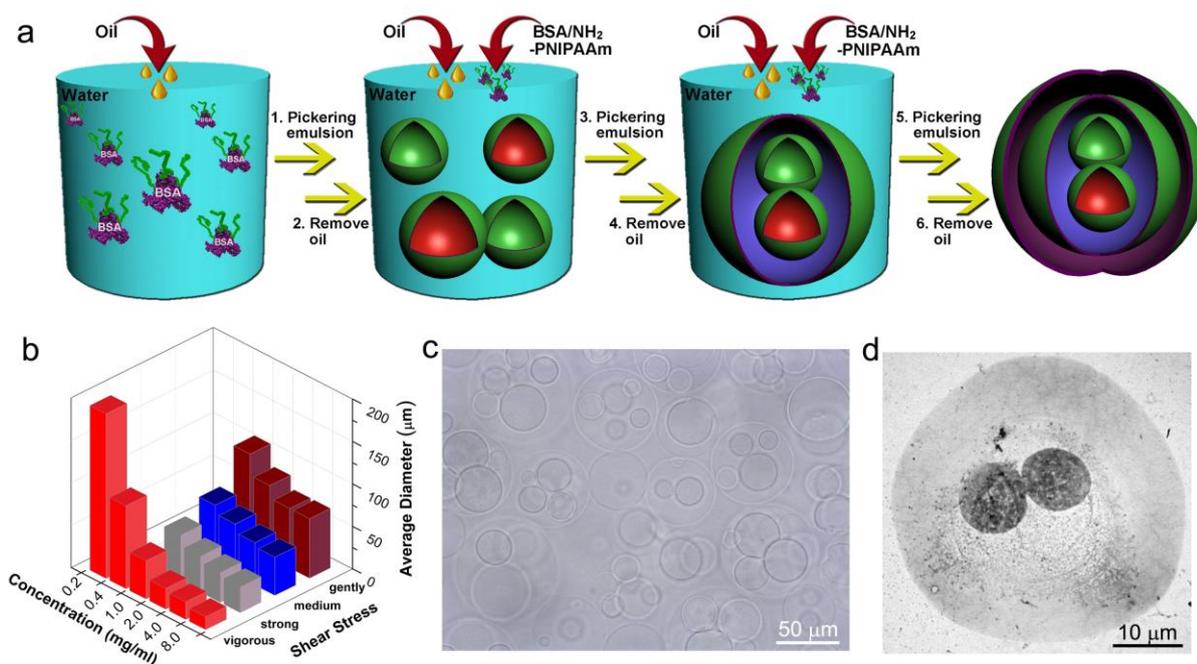


Figure 1. (a) Schematic illustration showing the general procedure for fabricating a hierarchical proteinosome micro-architecture comprising three nested layers of compartmentalization and different types of spatially occluded components. (b) Plot showing mean size of single-chambered proteinosomes dispersed in 2-ethyl-1-hexanol and prepared at $\phi_w = 0.06$ with different concentrations of BSA-NH₂/PNIPAAm and different shear forces. “Vigorous”, “strong”, “medium” and “gently” correspond to approximate shear forces of 8000, 6000, 4000 and 2000 rpm/min, respectively.” (c) Optical microscopy image of two-tiered BSA-NH₂/PNIPAAm proteinosomes dispersed in aqueous solution showing intact nested proteinosomes with low optical contrast. (d) TEM image of a two-tiered BSA-NH₂/PNIPAAm proteinosome structure comprising two entrapped sub-compartments.

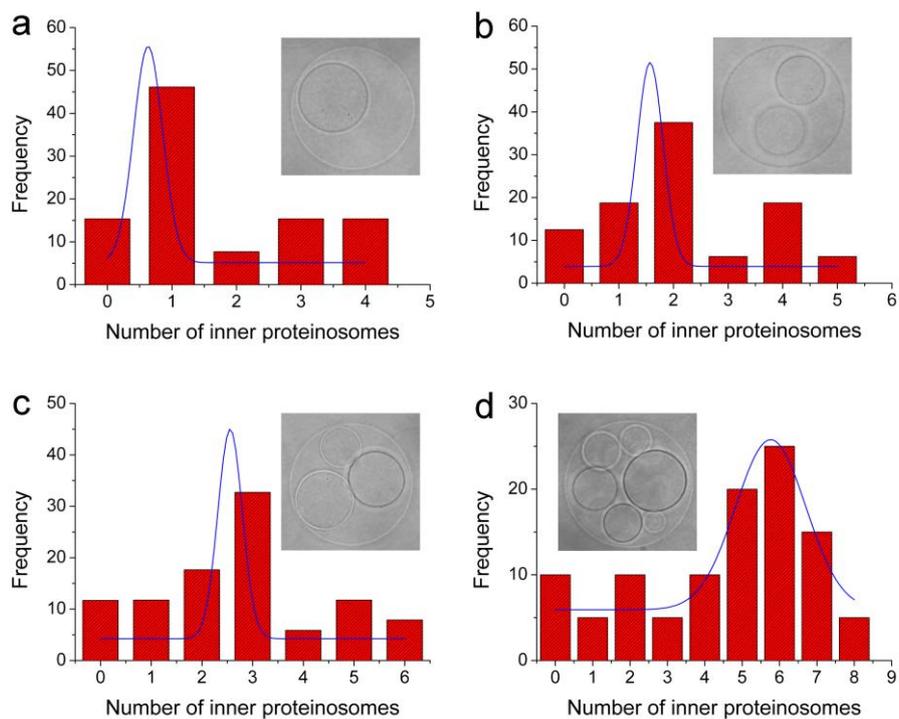


Figure 2. Statistical distributions of number of inner (guest) BSA-NH₂/PNIPAAm proteinosomes encapsulated within individual host BSA-NH₂/PNIPAAm proteinosomes determined from optical microscopy images by manual counting. The two-tiered proteinosomes were prepared under constant conditions but at different guest proteinosome: BSA-NH₂/PNIPAAm nanoconjugate mass ratios of (a) 4 : 10, (b) 8 : 10, (c) 12 : 10 and (d) 30 : 10. Insets show optical micrographs of typical multi-compartmentalized proteinosomes produced under the different mass ratios.

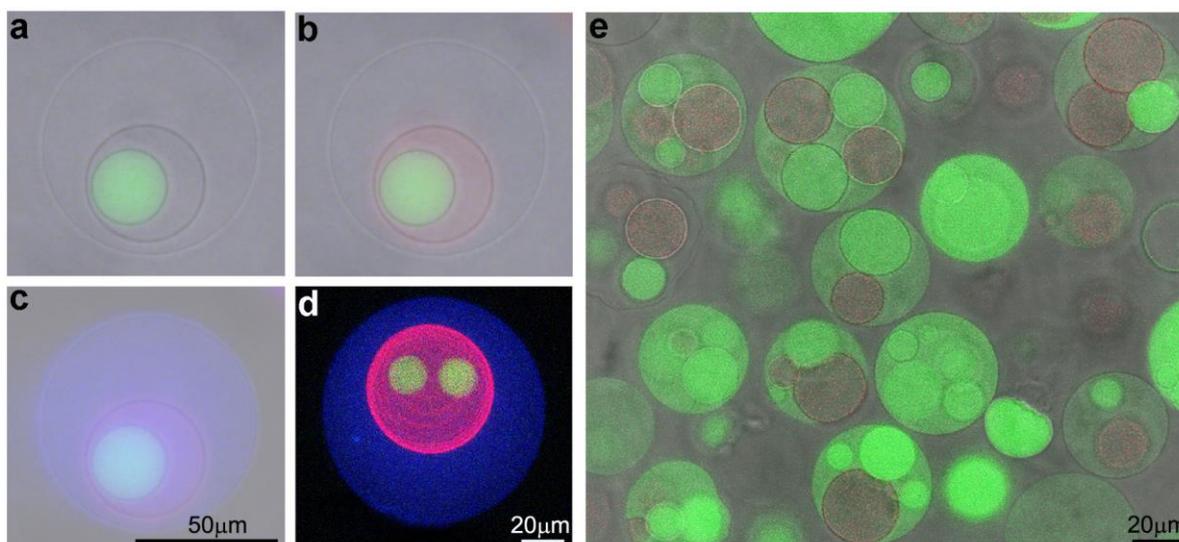


Figure 3. (a-c) Fluorescence microscopy images showing spatial separation of FITC-GO (green) within the inner chamber (a), FITC-GO (green) and RBITC-GA, (red) in the inner and middle chambers, respectively (b), and FITC-GO (green), RBITC-GA, (red)DL405-HRP (blue) within the inner, middle and outer chambers, respectively, of a three-tiered BSA-NH₂/PNIPAAm proteinosome. Images in (a), (b) and (c) are recorded using 450-490 nm excitation/510 nm emission, 515-560 nm excitation/580 nm emission, and 355-425 nm excitation/455 nm emission filters, respectively. (d) 3-channel confocal fluorescence microscopy image of a three-tiered proteinosome with a 1 : 1 : 2 configuration showing spatial separation of encapsulated FITC-GO (green), RBITC-GA (red) and DL405-HRP (blue). (e) Fluorescence microscopy image showing encapsulation of both FITC-labelled dextran- (green) and RBITC-GA (red)-containing guest proteinosomes into single host proteinosomes loaded with FITC-labelled dextran (green).

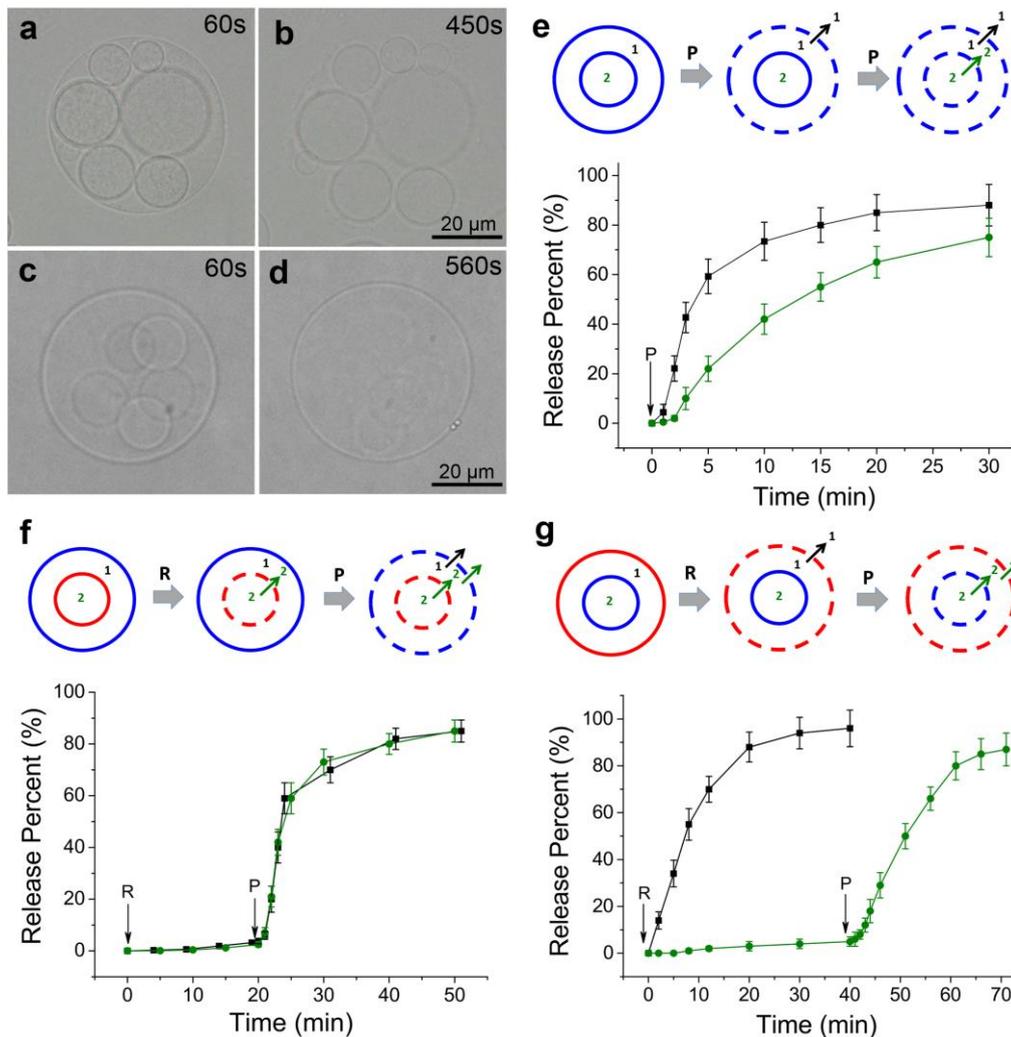


Figure 4. (a,b) Optical microscopy images showing a two-tiered proteinosome with host and guest membranes cross-linked by NHS-PEG16-DS or NHS-PEG9 ester, respectively, after addition of TCEP (5 mM, pH 8.0). The multi-tiered architecture is intact after 60 s (a) but the host proteinosome is disassembled after 450 s (b), releasing the guest microcapsules. (c,d) Optical microscopy images showing a two-tiered proteinosome with guest and host membranes cross-linked by NHS-PEG16-DS or NHS-PEG9 ester, respectively, after addition of TCEP (5 mM, pH 8.0). The multi-tiered architecture is intact after 60 s (c), but the guest proteinosomes are disassembled and their components redistributed within the host microcapsules after 560 s (d). (e-f) Plots and associated schemes showing programmed release behavior of RBITC-dextran (black plots) and DNA (green plots) in three variants of a two-tiered proteinosome structure. In each case, RBITC-dextran (1) and DNA (2) are initially encapsulated within the host and guest proteinosomes, respectively. Blue and red circles represent proteinosome membranes cross-linked by NHS-PEG9 ester or NHS-PEG16-DS, respectively; circles with full or dashed lines represent intact or disassembled membranes, respectively. (e) Retarded concomitant release; both host and guest proteinosome membranes are cross-linked with NHS-PEG9 ester and are lysed in sequence in the presence of protease (P) to release both components but with a 2 min delay for DNA. (f) Synchronized release; guest and host membranes are cross-linked with NHS-PEG16-DS or NHS-PEG9 ester, respectively, such that DNA

is released from the guest into the host proteinosome on addition of the reducing agent TCEP (R) but no macromolecules are released into the bulk solution. Subsequent addition of protease, releases both RBITC-dextran and DNA concurrently. (g) Hierarchical release; guest and host proteinosome membranes are cross-linked with NHS-PEG9 ester or NHS-PEG16-DS, respectively, such that RBITC-dextran is selectively and fully released on addition of TCEP (R) but DNA is retained in the no longer incarcerated guest proteinosomes. Addition of protease (P) after 40 min gives rise to a pulsed release of the DNA payload.