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Secretion and reversible assembly of extracellular-like matrix by enzyme-active colloidosome-based protocells

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ABSTRACT

The secretion and reversible assembly of an extracellular-like matrix by enzyme-active inorganic protocells (colloidosomes) is described. Addition of N-fluorenylmethoxy carbonyl-tyrosine-(O)-phosphate to an aqueous suspension of alkaline phosphatase-containing colloidosomes results in molecular uptake and dephosphorylation to produce a time-dependent sequence of supramolecular hydrogel motifs (outer membrane wall, cytoskeletal-like interior and extra-protocellular matrix) that are integrated and remodelled within the microcapsule architecture and surrounding environment. Heat-induced disassembly of the extra-protocellular matrix followed by cooling produces colloidosomes with a densely packed hydrogel interior. These procedures are exploited for the fabrication of nested colloidosomes with spatially delineated regions of hydrogelation.

INTRODUCTION

Although the functioning of living cells is very much dependent on the reversible supramolecular assembly of extended non-covalent structures such as cytoskeletal scaffolds and extracellular matrices, the biomimetic construction of synthetic cell-like entities has generally overlooked these soft matter/materials chemistry design concepts. Recent studies have focused on the co-reconstitution of cytoskeletal and membrane components, as well as the use of small-molecule amino acid precursors such as N-fluorenylmethylcarbonyl-tyrosine-(O)-phosphate (Fmoc-TyrP) to investigate the influence of reversible hydrogel assembly on vesicle shape deformation and motility, outer wall formation in proteinosomes, and enzyme catalysis in water-in-oil colloidosomes comprising a semi-permeable cross-linked membrane of silica nanoparticles. Peptide hydrogels have also been self-assembled within microparticles using microfluidics, and at oil/water interfaces.

The spontaneous assembly of colloidal particles at oil/water interfaces to produce Pickering emulsion droplets that can be subsequently cross-linked into shell-like micro-architectures (colloidosomes) provides a versatile method for the preparation of mechanically robust, semi-permeable microcapsules with uses in micro-encapsulation, micro-reactor technology and controlled delivery. Moreover, functionalized nanoparticles have been used to prepare colloidosomes capable of emulsion-based interfacial catalysis and type I microporosity. Given that water-in-water colloidosomes can be exploited as inorganic-based protocell models for integrating various biomimetic or biological processes, including capsule growth and division, membrane gating and signalling of encapsulated enzyme
In reactions, herein we investigate the possibility of developing the first example of an artificial protocell capable of internally directing the synthesis of a spatially extensive external network of soft nanofilaments analogous to the extracellular matrix of living cells. For this, we prepare alkaline phosphatase (ALP)-containing water-in-water silica colloidosomes and add Fmoc-TyrP to the aqueous continuous phase so that diffusion of the derivatized amino acid through the semi-permeable membrane results in ALP-mediated dephosphorylation of the substrate, followed by production of Fmoc-TyrOH and spontaneous self-assembly into nanofilaments. We demonstrate that this simple procedure produces a time-dependent sequence of supramolecular hydrogel motifs that proceeds through three stages: initial formation of an outer wall on the external surface of the colloidosome membrane; construction of a cytoskeletal-like matrix within the interior of the microcapsules; and secretion of the hydrogel into the surrounding environment to generate an extended matrix along with physically embedded intact colloidosomes. We show that the extra-protocellular matrix is spatially remodelled by heat-induced disassembly followed by cooling to produce colloidosomes with a densely packed hydrogel interior and no external hydrogel phase. Finally, we demonstrate how the above procedures can be exploited for the fabrication of nested colloidosomes comprising spatially delineated regions of hydrogelation.

**EXPERIMENTAL SECTION**

**Preparation of Colloidosomes and Hydrogel Formation.** Enzyme-encapsulated nanoparticle-stabilized water-in-oil Pickering emulsions were prepared using previous methods. Typically, calf intestine alkaline phosphatase (ALP, 1 µL, Calbiochem, 34800 U mL⁻¹) was dissolved in 100 µL of aqueous solution of alkaline buffer containing 50 mM tris(hydroxymethyl)aminomethane HCl (Sigma), 50 mM sodium carbonate (Sigma), and 1 mM magnesium chloride (Sigma). The enzyme solution was added to 30 mg of partially hydrophobic 30 nm-sized silica nanoparticles (prepared as described previously), followed by addition of dodecane (3 mL) to give an aqueous/oil volume fraction of 0.03. The mixture was then mixed in a homogenizer at 10k r.p.m for 30 seconds to form a Pickering emulsion.

Transfer of the colloidosomes from the oil phase into a continuous aqueous phase was achieved by modification of the protocol reported previously. Crosslinking of the silica nanoparticle membrane was undertaken by addition of tetramethyldichlorosilane (TMOS) (0.27 mmol, 40 µL) to the Pickering emulsion containing the preformed colloidosomes. The dispersion was gently rotated for 3 days, after which the cross-linked colloidosomes were transferred from the oil into an aqueous phase by slowly changing the polarity of continuous phase. For this, the Pickering emulsion was carefully placed on top of a solution of 70 vol% ethanol in
distilled water, and then centrifuged at 5000 r.p.m for 5 min. The upper supernatant was removed and replaced by 50 vol% ethanol in water prior to centrifugation at 5000 r.p.m for 5 min. The colloidosomes were then redispersed in alkaline buffer and centrifuged as above to ensure complete removal of ethanol.

Colloidosomes were also prepared as above but using fluorescein isothiocyanate (FITC)-labelled silica nanoparticles and rhodamine B isothiocyanate (RBITC)-tagged ALP. 30 mg of the partially hydrophobic silica nanoparticles was dispersed in 6 mL of ethanol, sonicated for 5 min, and 12 µL of an ethanolic solution of 3-aminopropyltriethoxysilane (APTES) (1 µL/100 µL) added and the mixture stirred at room temperature for 2 h. An ethanolic solution of FITC (1 mg/mL, 30 µL) was then added and the mixture left stirring at room temperature overnight. The FITC-labelled silica nanoparticles were transferred from ethanol to dodecane by centrifugation/redispersion using a sequence of ethanol/dodecane solvents with volume ratios of 80:20, 50:50 and 0:100. FITC-silica nanoparticles dispersed in dodecane were stored in the dark prior to use. RBITC-ALP was prepared by adding 1 µL of a DMSO solution of RBITC (1.0 mg/20 mL) to 2 µL of aqueous ALP. The labelled enzyme was stored in the fridge.

Protocell-mediated, enzyme-directed hydrogel formation was induced by addition of a buffered suspension of the ALP-containing cross-linked colloidosomes (100 µL) to 500 µL of alkaline buffer containing 12 mmol of N-fluorenylmethoxycarbonyl-tyrosine-(O)-phosphate (Fmoc-TryP) at pH 9.2. The reaction mixture was left unstirred at room temperature for at least 20 h (final pH = 8.5). Similar experiments were undertaken at 12 mmol Fmoc-TyrP but with higher or lower loadings of the ALP-containing colloidosomes (200 or 50 µL, respectively).

**Hydrogel Disassembly/reassembly in Single-tiered Colloidosomes.** Non-covalent disassembly/reassembly was induced in the colloidosome/hydrogel matrix by heating the samples above the gel-sol transition temperature (53°C), followed by slow cooling to room temperature. Typically, a colloidosome/hydrogel matrix was aged for 20 h, heated to 60°C for up to 2 h, and then cooled and left at room temperature for up to 20 h. Samples were studied at various times by confocal optical and fluorescence microscopy (Hoechst 33258 blue fluorescence), and SEM. Monitoring of enzyme reactivity in the protocells after heating and cooling was undertaken by addition of p-nitrophenylphosphate (pNPP) as a substrate. The supernatant was replaced by an aqueous solution of pNPP (0.25 M, 500 µL) and formation of p-nitrophenyl (pNP) over time assessed by observing changes in the colour of the reaction solution.

Control experiments were undertaken as follows; (a) addition of water-filled colloidosomes (no ALP) to a preformed Fmoc-TyrOH hydrogel produced by enzyme-mediated dephosphorylation in bulk solution, followed by heating and cooling the mixture as above, and (b) as for (a) but using colloidosomes containing EDTA-
deactivated ALP molecules. Deactivation of ALP was undertaken by addition of ethylenediaminetetraacetic acid (EDTA), which inhibited enzyme activity by extracting \( \text{Zn}^{2+} \) ions from the metalloprotein. Deactivation of the enzyme was confirmed by adding pNPP, which produced no pNP product.

**Hydrogelation in Nested Colloidosomes.** Enzymatically active colloidosomes prepared as above were incarcerated as guest components within larger silica nanoparticle-stabilized colloidosomes to produce protocells with nested micro-architectures. For this, 100 µL of a Pickering emulsion of cross-linked ALP-containing colloidosomes was transferred into 500 µL buffered aqueous phase, and 100 µL of the sedimented colloidosomes used to prepare a second water-in-dodecane Pickering emulsion in combination with partially hydrophobic 30 nm-sized silica nanoparticles. TMOS (20 µL) was then added to the dispersion to cross-link the host colloidosome membrane, and the samples left rotating overnight. The colloidosome-in-colloidosome microcapsules were transferred into a buffered aqueous phase containing Fmoc-TyrP (12 mmol, 500 µL) using the same method as described above. The dispersion was left at room temperature for at least 1 day to ensure hydrogelation within the colloidosome-in-colloidosome micro-architectures, and investigated by confocal fluorescence microscopy using Hoechst 33258 blue fluorescence as a stain for nanofilament (hydrogel) formation.

In other experiments, a 3-day aged sample of the nested colloidosomes was heated at 60°C for up to 2 h to disassemble the hydrogel, and then cooled and left at room temperature for up to 3 days. The spatial location of the resulting matrix of reassembled hydrogel in the nested colloidosomes was studied by confocal fluorescence microscopy.

**Characterization Methods.** Staining with Hoechst 33258 was used to monitor formation of the Fmoc-TyrOH nanofilaments in the presence of the ALP-containing colloidosomes. In general, 7 µL of 1 mM Hoechst 33258 fluorescence dye was added to the mixture at the beginning of the reaction. Time-dependent increases in the intensity of Hoechst 33258 blue fluorescence were monitored by optical microscopy over a period of 7 h. Samples extracted at various time periods (typically, 1, 7 and 20 h) for confocal fluorescence microscopy imaging were also stained by addition of a second aliquot of Hoechst 33258.

Optical and fluorescence microscopies were performed on a Leica DMI3000 B manual inverted fluorescence microscope at 20x and 40x magnification. A fluorescence filter with excitation at 340-380 nm and an emission cut off at 400 nm was used. Confocal microscopy images were obtained on a Leica SP5-II confocal laser scanning microscope attached to Leica DMI 6000 inverted epifluorescence microscope, equipped with 150 mW Ar laser (458, 476, 488, 514 nm lines) and 405
nm diode laser used for UV dyes. The 3D images were processed using Volocity 6.0 software (PerkinElmer, USA).

Time-dependent monitoring of Fmoc-TyrP concentration in the reaction mixtures was undertaken using UV-vis spectroscopy (Perkin Elmer Lambda II). Background spectra of alkaline buffer were recorded prior to the start of the measurements. An aqueous dispersion of ALP-containing colloidosomes (100 µL) was added to 500 µL of alkaline buffer containing 12 mmol Fmoc-TyrP at pH 9.2, and a 350 µL aliquot loaded into a 500 µL quartz cuvette (path length, 10 mm). The colloidosomes were allowed to sediment, and absorbance spectra (200-400 nm) were then collected at room temperature from the supernatant every hour for a period of 20 h.

Rheology measurements were performed at room temperature using a Malvern Kinexus Pro+ geometry parallel plate rheometer. The gap between the rheometer and base plate was set at 0.1 mm for all experiments. Oscillatory amplitude sweeps at 1 rads⁻¹ were initially performed to determine the percentage strain from the linear viscoelastic region for frequency sweep measurements. Storage modulus (G') and loss modulus (G'') were then plotted as a function of frequency to determine the viscoelastic properties of a protocell/hydrogel composite aged for 20 h. The data were compared with similar investigations on a control sample of a Fmoc-TyrOH hydrogel prepared at the same substrate/ enzyme concentrations in bulk aqueous solution. Rheological measurements were also made for a sample of cross-linked colloidosomes prepared at the same wt/wt% but in the absence of a hydrogel.

Scanning electron microscopy (SEM; JSM IT300, 15 kV) and transmission electron microscopy (TEM; Jeol 1200 Mk2, 120 kV) were used to study the structure and morphology of the colloidosome/hydrogel composites. For SEM, samples were mounted on sticky carbon pads placed on top of a SEM stub, and left overnight to air dry. A 15 nm-thick coating of Ag was sputtered onto the surface of the mounted samples prior to investigation. Samples for TEM were prepared by transferring 3 µL of the colloidosome-containing hydrogel to 20 µL of buffer, followed by vigorous shaking. 3 µL of the dispersed material was placed onto a carbon-coated TEM grid and left overnight to air dry.

RESULTS AND DISCUSSION

Enzymatically active, semi-permeable inorganic colloidosomes were prepared in the form of silica nanoparticle-stabilized ALP-containing aqueous micro-droplets using a procedure involving the formation of a water-in-oil Pickering emulsion,29-31 followed by cross-linking of the colloidosome membrane, and stepwise transfer from oil into water.27 Optical microscopy images showed well-defined hollow spherical microstructures that were between 40 and 70 µm in diameter (Figure S1). Under the
conditions employed, approximately 50 million ALP molecules, equivalent to a potential activity of $2.0 \times 10^{-3}$ U, were encapsulated in a volume of 60 pL ($d = 50 \mu m$). To generate a protocell-mediated cytoskeletal-like matrix, an aqueous solution of Fmoc-TyrP was added to the external phase, and the reaction mixture left unstirred at room temperature such that the colloidosomes settled under gravity to produce a distinct lower layer consisting of closely packed microcapsules. Significantly, aging the mixture for 2 days transformed the sedimented layer of colloidosomes into a macroscopic Fmoc-TyrOH hydrogel (Figure 1a), which remained intact when the sample tube was inverted (Figure 1b). Thus, even though enzyme-mediated dephosphorylation of the Fmoc-TyrP substrate was initiated within the interior of the colloidosomes, growth of the Fmoc-TyrOH nanofilaments appeared to be unimpeded by the presence of the semi-permeable cross-linked silica nanoparticle membrane. Non-covalent self-assembly of Fmoc-TyrOH nanofilaments within the hydrogel was confirmed by staining the samples with the fluorescent dye Hoechst 33258, and imaging the materials under UV irradiation (Figure 1c). The samples could be readily removed to produce a self-supporting colloidosome/hydrogel matrix that remained macroscopically stable when hydrated (Figure 1d). Similar materials were also prepared using higher or lower loadings of the ALP-containing colloidosomes, although the hydrogel yield after 2 days of aging was increased or decreased, respectively (Figure S2).

Time-dependent studies on the enzyme-mediated synthesis of the Fmoc-TyrOH hydrogel matrix showed significant Hoechst 33258 fluorescence intensity in the macroscopic composites 6 h after initiation, followed by a progressive increase in blue fluorescence up to around 20 h (Figure 1e-h). Corresponding UV-vis spectra associated with residual substrate molecules in the upper supernatant layer of the reaction system showed a slow decrease in the intensity of the Fmoc-TyrP fluorenyl/phenol $n \rightarrow \pi^*$ absorption peak at 299 nm over the first 6 h of the reaction, followed by a rapid reduction in absorption between 6 and 8 h (Figure 1i, Figure S3). Interestingly, the concentration of Fmoc-TyrP in the supernatant did not decrease considerably after 8 h even though the blue fluorescence associated with staining of the hydrogel increased during these later stages of the reaction.

The viscoelastic properties associated with the formation of the soft composites were investigated by measuring the storage modulus ($G'$) and loss modulus ($G''$) as a function of frequency for a sample aged for 20 h (Figure 1j). Values of $G'$ were greater than those for $G''$ across the frequency range investigated, and both moduli decreased considerably as the frequency increased, indicating that the colloidosome/hydrogel matrix was susceptible to deformation under conditions of high shear. Significantly, at low frequency values, the values of both $G'$ and $G''$ were tenfold higher than those measured for a Fmoc-TyrOH hydrogel prepared under the same conditions but in bulk solution, and much higher (particularly for the $G''$ values) than for a sample of colloidosomes alone (no hydrogel) (Figure 1j). In
contrast, although $G'$ was greater than $G''$ across the entire frequency range for the bulk hydrogel, both the storage and loss moduli remained stable at higher frequency.

Given the complex viscoelastic properties exhibited by the colloidosome-containing hydrogels, we undertook a series of experiments using scanning electron microscopy (SEM) and confocal fluorescence microscopy to determine the microstructure of the composites. SEM images of samples aged for 20 h showed an integrated network of intact spherical microcapsules that were coated with and embedded within a viscoelastic matrix of entangled hydrogel filaments (Figure 2a), confirming that an extra-protocellular matrix was self-generated by the enzyme-active colloidosomes. Dilution of the samples fifty-fold was used to study individual components of the colloidosome/hydrogel matrix aged for 20 h; the images showed discrete long nanofilaments that were 400-600 nm in width, and which emanated in large numbers from single microcapsules (Figure 2b). In contrast, discrete filaments were hardly ever imaged for hydrogels prepared in bulk solution at the same enzyme/substrate concentrations (Figure S4), suggesting that nucleation and growth of the protocell-mediated nanofilaments was spatially constrained by encapsulation of ALP within the colloidosomes. Discrete nanofilaments were also observed for samples aged for 20h and subsequently imaged under aqueous conditions using confocal fluorescence microscopy and Hoechst 33258 staining (Figure S5).

Secretion and assembly of the colloidosome-mediated extra-protocellular matrix were investigated using confocal fluorescence microscopy. To confirm the spatial localization of the components, colloidosomes were prepared using fluorescein isothiocyanate (FITC)-labelled silica nanoparticles and rhodamine B isothiocyanate (RBITC)-tagged ALP. The corresponding 3D confocal fluorescence images recorded at the onset of enzyme-mediated dephosphorylation showed spatially segregated areas of red and green fluorescence within or localized on the surface of the individual micro-compartment(s), respectively (Figure 3). Red fluorescence was also observed at the colloidosome surface, suggesting that ALP molecules were adsorbed on the silica membrane as well as encapsulated within the aqueous interior. Significantly, staining with Hoechst 33258 showed an intense blue fluorescence at the membrane surface, indicating that the initial stages of hydrogel growth occurred within 15 min of Fmoc-TyrP addition, and were preferentially localized at the protocell membrane (Figure 3b). This was confirmed by high magnification SEM images of individual colloidosomes extracted from the reaction mixture after 15 min, which showed a delicate “hairy” texture on the outer surface of the spherical microarchitecture (Figure 3c,f). Nucleation of Fmoc-TyrOH nanofilaments at the membrane surface was consistent with the generation of a supersaturated interfacial region due to the high substrate concentration gradient and enzyme localization associated with the initial stages of the dephosphorylation reaction.
Confocal fluorescence microscopy images recorded after 1 h indicated that growth of the amino acid hydrogel continued on the surface of the colloidosomes to produce a relatively smooth wall that enveloped the cross-linked silica nanoparticle membrane. In addition, Hoechst 33258 blue fluorescence was also apparent within the interior of the colloidosomes, indicating that the encapsulated, non-membrane bounded ALP molecules remained active and accessible to the Fmoc-TyrP substrate in the presence of the external hydrogel wall (Figure 3d,e). Progressive infilling of the internal volume occurred over a period of 7 h to produce colloidosomes with a hydrogel interior and roughened outer surface comprising a tangled array of Fmoc-TyrOH nanofilaments, some of which extended into the surrounding solution (Figure 3g,h). After 20 h, the colloidosomes were completely embedded in a continuous extra-protocellular matrix, and interestingly, appeared devoid of the internalized hydrogel (Figure 3j,k). As shown in Figure 1i, Fmoc-TyrP dephosphorylation was essentially arrested after a period of 8 h even though blue fluorescence associated with staining of the macroscopic hydrogel continued to increase (Figure 1 f,g), suggesting that the marked reduction in blue fluorescence observed within individual colloidosomes at 20 h was associated with in situ disassembly of the internalized hydrogel and redistribution of the solubilized Fmoc-TyrOH monomers into the extra-protocellular environment via a dissolution-reprecipitation process. This interpretation was consistent with changes in the optical texture of individual protocells viewed at 7 and 20 h by optical microscopy (Figure 3i,l), as well as transmission electron microscopy (TEM) images that revealed a corresponding marked transformation from an amorphous hydrogel matrix within the colloidosomes after 5 h to an extra-protocellular network of discrete, morphologically distinct Fmoc-TyrOH nanofilaments at 20 h (Figure S6).

The ability of the Fmoc-TyrOH hydrogel to spontaneously undergo slow structural reconstruction inspired us to undertake experiments in which a cycle of reversible non-covalent disassembly/reassembly was induced in the colloidosome/hydrogel matrix by heating the samples above the gel-sol transition temperature (53°C, DSC results, Figure S7), followed by slow cooling to room temperature. Typically, a colloidosome/hydrogel matrix was aged for 20 h, heated to 60°C for up to 2 h, and then cooled and left at room temperature for up to 20 h. Disassembly of the hydrogel matrix at 60°C was confirmed by confocal optical and fluorescence microscopy that showed a progressive decrease in Hoechst 33258 fluorescence intensity due to nanofilament disintegration and concomitant release of the intercalated dye molecules. Thus, the colloidosomes imaged 2 h after heating showed only residual blue fluorescence at the surface of the silica nanoparticle membrane, and appeared transparent when imaged optically (Figure 4a-c). As expected, cooling the samples to room temperature reassembled the Fmoc-TyrOH hydrogel but surprisingly this occurred almost exclusively within the colloidosome micro-compartment (Figure 4d-f). Nucleation of the hydrogel was optically visible
after 3 h at room temperature (Figure S8), and resulted in a closely packed aggregate of bundled fibres throughout the interior of the colloidsomes (Figure S9). As a consequence, very few nanofilaments were observed in the external medium and a self-supporting macroscopic hydrogel composite was therefore not produced when the mixture was aged for 2 days. Repeating the heating and cooling cycle resulted respectively in the disassembly and reassembly of the hydrogel specifically within the protocell interior (Figure S10), indicating that annealing the initially produced supramolecular hydrogel did not influence the mechanism responsible for preferential nucleation within the colloidsome interior.

Based on control experiments, we attributed the localized reassembly of the hydrogel to enzyme-mediated chemical seeding of the supersaturated Fmoc-TyrOH solution produced on slow cooling. In particular, addition of colloidsomes containing EDTA-deactivated ALP molecules to a preformed Fmoc-TyrOH hydrogel produced by enzyme-mediated dephosphorylation in bulk solution, followed by heating and cooling the mixture as described above, resulted in minimal reassembly of the matrix inside the protocells (Figure S11a,b). A similar result was obtained when enzyme-free buffer-filled colloidsomes were added to a preformed hydrogel matrix and the mixture heated and cooled (Figure S11c,d). Significantly, the heating and cooling protocol did not compromise the dephosphorylation activity of the ALP molecules trapped within the hydrogelated interior of the reconstituted protocells (Figure S12), indicating that the retained enzymatic capacity was responsible for preferential nucleation and growth of the reassembled hydrogel after cooling from 60°C. Thus, we propose that ALP-mediated dephosphorylation within the colloidsomes of residual Fmoc-TyrP (see Figure 1i) present in the cooled supersaturated solution generates a population of activated clusters that lower the activation energy required for reassembly to occur from the supersaturated solution.

We also undertook proof-of-concept studies on enzyme-mediated hydrogel assembly in multi-compartmentalized colloidsomes. For this, ALP-containing colloidsomes were used as catalytically active “proto-organelles” that were housed within enzyme-free host colloidsomes, and exploited for the fabrication of inorganic protocells with hierarchical organization and spatially delineated hydrogel interiors. Diffusion of Fmoc-TyrOH from the external phase into the two-tiered nested micro-architecture gave rise to dephosphorylation specifically within the incarcerated ALP-containing colloidsomes such that they became progressively filled with a hydrogel within 2 days of leaving the dispersion at room temperature (Figure 5a,b). Staining with Hoechst 33258 also showed limited amounts of blue fluorescence within the host protocell after 2 days, which increased substantially after 3 days (Figure 5c), indicating that enzyme activity within the entrapped guest colloidsomes gave rise to the secretion of an extended hydrogel matrix within the host colloidsome. Heating the nested microcapsules to 60°C disassembled the hydrogel in both the host and guest colloidsomes (Figure 5d,e), and subsequent
cooling to room temperature and aging for 3 days resulted in nucleation and growth of the Fmoc-TyrOH matrix specifically within the entrapped colloidosomes (Figure 5f). As a consequence, multi-compartmentalized protocells comprising hydrogel-filled ALP-containing guest colloidosomes dispersed within an enzyme- and matrix-free host micro-architecture were produced.

CONCLUSIONS
The above studies indicate that a series of supramolecular hydrogel motifs can be integrated into enzyme-active inorganic protocells by controlling the time course of ALP-mediated dephosphorylation of membrane-permeable substrate molecules added to the external environment. No hydrogel formation is observed when the colloidosomes are exposed to Fmoc-TyrP in the absence of ALP, indicating that the assembly process is fundamentally dependent on catalytic activity within the inorganic microcapsules (Figure S13). Colloidosomes comprising a thin fibrous shell of Fmoc-TyrOH nanofilaments are prepared at low reaction times (< 30 min), whilst internally hydrogel structured micro-compartments surrounded by a semi-permeable silica nanoparticle membrane encased within a secreted extra-protocellular wall are produced after 7 h or so. In contrast, reaction times of 20 h give rise to the embedding of the water-filled enzyme-containing colloidosomes within an extended hydrogel matrix, which transforms into a self-supporting bulk protocell/hydrogel composite within 2 days. Moreover, temperature-induced disassembly of the extra-protocellular matrix followed by slow cooling to room temperature results in hydrogelation specifically within the colloidosomes to produce inorganic micro-compartments with densely packed supramolecular interiors. Interestingly, ALP-containing colloidosomes can be employed as catalytically active “proto-organelles” that are incarcerated within single enzyme-free host colloidosomes, and used to fabricate inorganic protocells with hierarchical organization and spatially delineated hydrogel interiors.

In general, our results provide a first step towards a synthetic microcapsule capable of internally directing the synthesis of a spatially extensive external network of soft nanofilaments analogous to the extracellular matrix of living cells. As such, it should be possible to develop the system within a protocellular context; for example by establishing communities of colloidosomes and living cells embedded within the hydrogel matrix. As the hydrogel matrix is secreted specifically by the enzyme-containing microcapsules it should be possible to couple this compartmentalized enzyme-mediated assembly process with chemical signals originating from the metabolism of the co-located living cells. If so, this could provide a pathway towards the development of synthetic communities of non-living/living constructs with symbiotic properties.
ASSOCIATED CONTENT

Supporting Information
Additional Figures.

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Notes
The authors declare no competing interests.

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REFERENCES


Figure 1. (a-c) Photographs of Hoechst 33258-stained Fmoc-TyrOH colloidosome/hydrogel matrices observed under normal light (a,b) or UV irradiation (c). Samples were prepared by mixing 100 µL of an aqueous dispersion of ALP-containing colloidosomes with 25 µmol of Fmoc-TyrP in alkaline buffer solution and aging for 2 days. Samples in b and c are shown inverted in a plastic tube. (d) Optical microscope image of extracted sample showing self-supported colloidosome/hydrogel matrix; scale bar = 5 mm. (e-h) Photographs of samples recorded under UV light at (e) 0, (f) 7, (g) 20 and (h) 48 h. (i) Plot showing time-dependent decrease in concentration of Fmoc-TyrP associated with substrate dephosphorylation in the presence of ALP-containing colloidosomes. (j) Rheometry frequency sweep of the storage modulus (G’, closed symbols) and loss modulus (G”, open symbols) for a Fmoc-TyrOH colloidosome/hydrogel matrix (blue circles), Fmoc-TyrOH hydrogel prepared in bulk solution (black squares) and colloidosomes in the absence of a hydrogel (red triangles).
Figure 2. SEM images of (a) Fmoc-TyrOH colloidosome/hydrogel matrix after 20 h, and (b) diluted sample of (a) showing nanofilamentous matrix associated with the surface of the inorganic protocells. Scale bars = 50 µm.
Figure 3. (a,b) 3D confocal fluorescence microscopy images of individual ALP-containing colloidosomes recorded 15 min after addition of Fmoc-TyrP. (a) Single colloidosome showing green and red fluorescence originating from the FITC-SiO$_2$ nanoparticle membrane and encapsulated RBITC-ALP, respectively. Red fluorescence is also observed at the colloidosome surface. (b) Blue fluorescence from Hoechst 33258 staining of the colloidosome displayed in (a) showing nucleation of Fmoc-TyrOH nanofilaments on the protocell surface. (c,f) SEM images of a single colloidosome after 15 min showing secretion of a thin extra-protocell wall on the silica nanoparticle membrane; scale bars = 10 and 3 µm, respectively. (d,e) Blue fluorescence associated with individual ALP-containing colloidosomes recorded 1 h after addition of Fmoc-TyrP and viewed approximately side-on (d) or in cross-section (e); analogous images recorded at 7 and 20 h are shown in (g,h) and (j,k), respectively. Note the thickening of the membrane-associated hydrogel shell, extension of the matrix into bulk solution, and disappearance of hydrogel from the colloidosome interior at 20 h. Approximately 90% of the colloidosomes imaged were empty after the ageing process. (i,l) Bright field confocal images of single colloidosomes recorded after 7 h (i) or 20 h (l) showing presence and absence, respectively, of a hydrogel within the colloidosome interior; scale bars = 15 µm. Scale bars in all fluorescence microscopy images, 50 µm.
Figure 4. (a,b) Fluorescence confocal microscopy images of single ALP-containing colloidosomes stained with Hoechst 33258 and recorded 20 h after addition of Fmoc-TyrP at room temperature (a), and at 60°C (above the gel-sol transition temperature) after 2 h (b), showing temperature-induced disassembly of the Fmoc-TyrOH nanofilaments to produce hydrogel-free colloidosomes; scale bar = 20 µm. (c) Bright field image corresponding to sample shown in (b). (d-f) Cross-sectional (d) and 3D (e) fluorescence confocal microscopy images, and optical image (f) of individual colloidosomes after cooling from 60°C to room temperature and aged for 20 h showing reconstitution of the hydrogel specifically within the microcapsule interior. Scale bars for all images = 20 µm.
Figure 5. (a-c) Confocal fluorescence microscopy images of two-tier colloidosome microstructures showing a single ALP-containing guest colloidosome encapsulated within a single host colloidosome. The samples were stained with Hoechst 33258 and images recorded 1 (a), 2 (b) and 3 days (c) after addition of Fmoc-TyrP to the external aqueous phase. A hydrogel matrix is observed initially only within the guest colloidosome (b), and then within both the host and guest colloidosomes (c). The blue fluorescence ring associated with the host colloidosome after 1 day was attributed to interaction of Hoechst 33258 with aggregates of Fmoc-TyrP monomers bound at the silica membrane surface, rather than formation of a supramolecular Fmoc-TyrOH hydrogel. This was confirmed from control experiments using ALP-free colloidosomes, which also showed a blue fluorescence ring at the membrane surface in the presence but not absence of Fmoc-TyrP; scale bars = 15 µm. (d-e) Confocal fluorescence microscopy images of enzymatically active two-tiered colloidosomes recorded 3 days after addition of FmocTyrP; as-prepared at room temperature showing Fmoc-TyrOH matrix in both host and guest chambers (d), after heating to 60°C to induce disassembly of the hydrogel within the host and guest colloidosomes (e), and after heating to 60°C followed by aging at room temperature for 3 days showing hydrogel reassembly preferentially in the ALP-containing guest colloidosome (f); scale bar = 15 µm.
Secretion and reversible assembly of extracellular-like matrix by enzyme-active colloidosome-based protocells

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The secretion and reversible assembly of an extracellular-like matrix by enzyme-active inorganic protocells (colloidosomes) is described as a step towards the fabrication of nested colloidosomes with spatially delineated regions of hydrogelation.