SUPPLEMENTARY INFORMATION

for

Microgel Core/Shell Architectures as Targeted Agents for Fibrinolysis

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EXPERIMENTAL METHODS

Materials. All materials were purchased from Sigma-Aldrich unless specified otherwise. The primary monomer *N*-isopropylmethacrylamide (NIPMAm) was purified via recrystallization from *n*-hexane (J.T. Baker). The following were all used as received: crosslinker *N,N′*-methylenebis(acrylamide) (BIS), comonomer used for shell synthesis acrylic acid (AAc, Fluka), methacryloxyethyl thiocarbamoyl rhodamine B (mRhoB, Poly Fluor 570, Polysciences Inc.), $CaCl_2$, KCl, NaCl, anionic initiator ammonium persulfate (APS), buffer preparation materials

sodium dihydrogen phosphate, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), 4-morpholineethanesulfonic acid (MES) and formic acid (EMD Millipore), 3-aminopropyltrimethoxysilane (APTMS) for substrate functionalization for AFM imaging, coupling reagents *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide sodium salt (NHSS), 2-maleimidoethylamine trifluoroacetate salt (AEM), human fibrinogen (FIB3, Enzyme Research Laboratories), human α-thrombin (Enzyme Research Laboratories, HT 1002a), peptides from GenScript GPRPFPAC (GPRP), GPSPFPAC (GPSP) and AHRPYAAC (AHRP), ethanol, isopropanol and acetone. The solutions were prepared using distilled water, deionized to a resistance of 18 MΩ (Barnstead E-Pure system). Solutions were filtered through a 0.2 μm Acrodisc syringe filter before use.

Microgel core synthesis. Microgel cores were synthesized by free radical precipitation polymerization. In a typical synthesis, NIPMAm and BIS in molar compositions of 98% and 2% respectively were dissolved in 48.5 mL distilled, deionized water, to a total monomer concentration of 140 mM, along with SDS at the relevant concentration (refer to Table 1). The resulting solution was filtered through a 0.8 μm Acrodisc syringe filter and introduced into a 100 mL three necked, round bottom flask along with a magnetic stirrer. The flask was fitted with a thermometer, condenser and N_2 inlet and introduced into an oil bath, which was heated at 100 °C/h. Stirring was kept constant at 400 rpm and the solution was purged with N_2. Once the temperature was stable at 70 °C, 0.5 mL of a solution of mRhoB in DMSO (final concentration 0.1 mM) was introduced into the flask, following which 1 mL of the initiator APS was added after filtration through a 0.2 μm Acrodisc syringe filter. The reaction was allowed to proceed for 4 h under a N_2 blanket, after which it was cooled down to room temperature. The solution was then filtered through glass wool.

Microgel shell synthesis. Core/shell microgels were synthesized using a 'seed-and-feed' method. The shell monomer solution was prepared by dissolving NIPMAm, BIS and AAc in molar feed ratios of 93%, 2% and 5% respectively, in 39.5 mL distilled, deionized water, to a total monomer concentration of 50 mM. Following the addition of SDS (final concentration: 2 mM), the resulting solution was filtered through a 0.8 μm Acrodisc syringe filter. This monomer shell solution along with 10 mL of the respective core solution was heated to 70 °C in a manner similar to the core synthesis and was initiated with 0.5 mL APS once temperature stability was achieved. The synthesis was then allowed to proceed for 4 h under a N_2 blanket, with constant stirring at 400 rpm. It was then cooled down to room temperature and the solution was filtered through glass wool. The microgels were then purified by pelleting via ultracentrifugation at 104000–417000 × *g* for 20–70 min, depending on the microgel type. Every run of ultracentrifugation was followed by removal of the supernatant and resuspension in DI water. This process was repeated six times, after which the microgels were lyophilized prior to characterization.

Dynamic Light Scattering. Hydrodynamic radii (R_H) of the microgels were determined via dynamic light scattering (DLS, DynaPro, Protein Solutions). Measurements were performed in the following buffers: pH 7 phosphate (50 mM ionic strength), pH 7.4 HEPES (6 mM ionic strength), pH 3 formate (50 mM ionic strength) and pH 3 formate (6 mM ionic strength). Microgel samples in the respective buffers were thermally equilibrated for 20 min, following which scattering intensity fluctuations based on Brownian motion of the microgels were detected at a scattering angle of 90°. Twenty-five (25) acquisitions of 20 s each were obtained. These were used to generate intensity-time correlation functions, and cumulants analysis was used in order to

attain diffusion coefficients. The Stokes-Einstein equation was then utilized to calculate R_H values. This procedure was repeated 3 more times on the same sample in order to generate a total of 100 R_H values for each microgel sample and averages of these values along with standard deviations are presented.

Atomic Force Microscopy. Atomic Force Microscopy (AFM) was performed using an MFP-3D AFM (Asylum Research). Images were acquired in air and under ambient conditions in the tapping mode, using pyramidal cantilevers (Nanoworld, Force Constant 42 N/m) made of heavily doped silicon. Image processing was performed using software written in an IgorPro environment (Wavemetrics, Inc.)

Samples were prepared on 22 mm × 22 mm glass coverslips (VWR). The coverslips were cleaned by sequential sonication in Alconox solution, DI water, acetone, 95% ethanol, and isopropanol for 20 min each. They were then functionalized in a 1% (v/v) APTMS/absolute ethanol solution on a shaker table for 2 h, following which they were washed with DI water. The microgel solutions prepared in phosphate buffer (pH 7, 50 mM ionic strength) were then used for submonolayer deposition onto the glass substrates by centrifugation at 2250 × *g* for 10–25 min (depending on the microgel type) at 25 °C using a plate rotor. The coverslips were rinsed well with DI water and dried with nitrogen before imaging.

Production and purification of +6 GFP. Plasmids with genes encoding for +6 GFP[1] from Prof. David Liu's research group (Howard Hughes Medical Institute, Harvard University) were used for transformation of *E.coli*, following which the production and purification of the protein was performed in a manner similar to that utilized by Cronican et al.[2] with slight modifications. Briefly, transformed *E.coli* were grown to an optical density of 0.6 in LB broth

(Lennox, Alfa Aesar), following which they were induced with 1 mM IPTG (Isopropyl β-D-thiogalactopyranoside, ThermoFisher) at 30 °C for 4 h. The cells were then harvested and preserved as pellets at −80 °C. These pellets were thawed in a solution of 1X PBS with 2 M NaCl and sonication was employed to lyse them. Following centrifugation at 10000 × *g* for 8 min, the supernatant was mixed with Ni-NTA resin (Fisher Scientific) for 30 min at 4 °C. The removal of resin by centrifugation was followed by multiple washes with 2 M NaCl and 20 mM imidazole (Fisher Scientific) and then a 2 M NaCl + 500 mM imidazole solution was used for detachment of the protein from the resin. Dialysis against 1X PBS for 18 h at 4 °C was then carried out, followed by SDS-PAGE staining with Simply Blue (ThermoFisher).

+6 GFP encapsulation studies. Lyophilized core/shell microgels were weighed and resuspended in 0.20 μM +6 GFP solution in the respective buffer (6 mM ionic strength HEPES or 150 mM ionic strength HEPES). After a 20 h equilibration, the microgel solutions were centrifuged at 104000–417000 × *g* for 20–70 min, depending on the microgel type. The supernatants were analyzed for presence of +6 GFP by fluorescence measurements at λ_{ex} = 475 nm and λ_{em} = 520 nm. Normalization was performed against stock +6 GFP solutions in the respective buffer conditions.

Fibrin clot formation. Solutions of fibrinogen (13.51 mg/mL) and thrombin (10 U/mL) were thawed to room temperature. The fibrinogen was mixed with $CaCl_2$ (final concentration 5 mM), HEPES (final concentration 25 mM HEPES, 150 mM NaCl) and DI water, following which clot formation was initiated by introducing thrombin at a final concentration of 1 U/mL. The final concentration of fibrinogen was either 3 mg/mL (size-based localization studies) or 2 mg/mL (clot disruption studies). The clots were formed in plastic capillary tubes (Globe

Scientific, Inc., I.D. = 0.85 mm), and were allowed to polymerize overnight in a humid environment, to avoid abnormalities due to drying. For the perfusion experiments, they were cut into 1.5 cm pieces for utilization with each sample solution.

Permeability measurements and perfusion studies. Flow studies were performed using the experimental setup represented in Scheme 1. 1X HEPES buffer (same as that used for clot preparation) was first perfused through the 1.5 cm clots. Following 15 min equilibration, flow rates of buffer post traversal through the fibrin clots were measured for 3–5 min (depending on the clot formation conditions), from a solution reservoir height of 18 in. Three measurements were made for every clot and clot permeability values were calculated using Darcy's law,

$$Q = \frac{k}{\mu} A \frac{\Delta P}{L}$$

where Q is the volumetric flow (m^3/s), k is Darcy's constant or the Darcy permeability (m^2), μ is the liquid viscosity (kg/ms), A is the cross-sectional area of the clot (m^2), ΔP is the pressure gradient across the clot (kg/ms^2) and L is the length of the cylindrical clot (m).

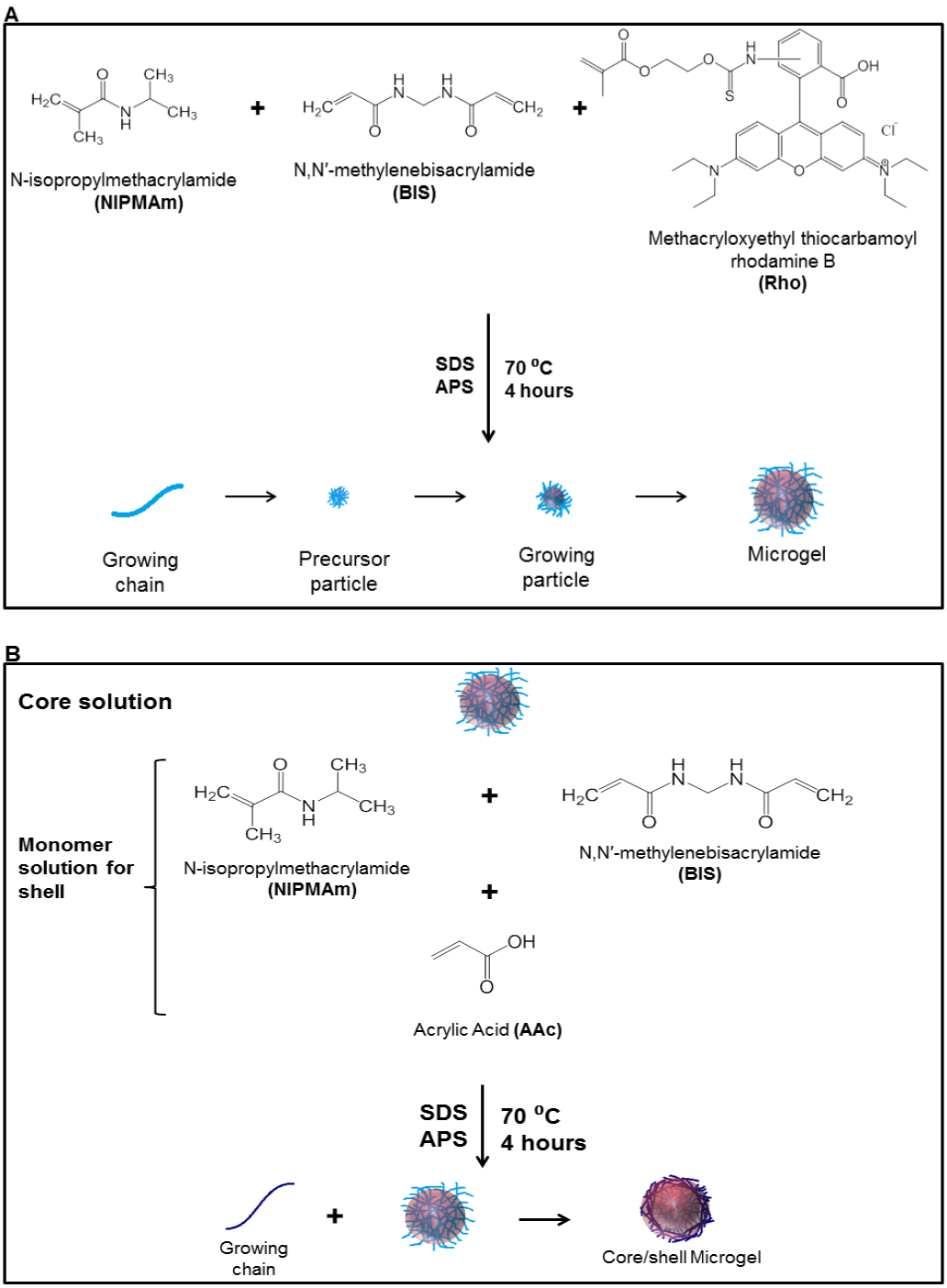
Following confirmation of consistency in clot permeabilities, buffer solutions in the reservoirs were replaced by microgel solutions at a concentration of 0.1 mg/mL and these were allowed to flow through the clots. After equilibration for 15 min, eluent collection was begun and was performed at 30 min intervals. The eluents were analyzed for their fluorescence using a plate reader (Infinite® 200 PRO NanoQuant™, Tecan Group LTD., San Jose, CA) at excitation and emission wavelengths of 540 nm and 575 nm, respectively. Following background corrections for the buffer, fluorescence intensities normalized to those of the respective reservoir microgel solutions (0.1 mg/mL) were calculated. Three trials were performed and values of normalized

fluorescence intensities are presented as averages of values obtained at the respective time points from these trials. Standard deviations were also obtained from these values and are presented.

Conjugation of peptides to microgels. L C/S microgels were divided into three sections and resuspended in MES buffer (20 mM ionic strength). They were then conjugated to AEM through carbodiimide coupling, using a 2X molar excess of EDC and NHSS and a 1:1 –COOH:AEM molar ratio, based on the theoretical number of AAc moieties incorporated in the microgel shells. This conjugation was allowed to proceed for 2 h, following which dialyses for all three solutions were performed against MES buffer for 15 h. The MES buffer was then replaced with phosphate buffer (pH 7, 140 mM ionic strength) and following equilibration for ~8 h, peptide conjugations to GPRP, GPSP and AHRP (1:0.2 molar ratio of theoretical –COOH:peptide) were performed overnight through thiol-maleimide coupling. The resulting solutions were dialyzed against DI water for one week and placed on a shaker for homogeneity. A small portion of each solution was lyophilized in order to determine concentration of the stock solutions.

Clot disruption studies under flow. Clot disruption analyses were performed using a setup similar to the one utilized for permeability measurements. 1.5 cm fibrin clots were first analyzed for their consistency in permeability by conducting flow rate measurements using 1X HEPES buffer. The Darcy constant k was determined for each clot, following which reservoir buffer solutions (at an 18 in height) were replaced with the respective peptide-conjugated L C/S microgel solutions (0.1 mg/mL). After equilibration for 10 min, flow rate measurements (3 min each) were conducted for each microgel solution, after passage through the clot. This was repeated every 10 min until clot disruption was observed. Pictures of clots were also taken at

various time points during the experiment. Two separate trials were performed and the results from these are presented.



Scheme S1. General representation of a two-step core (A) and core/shell (B) microgel synthesis

Microgel characterization

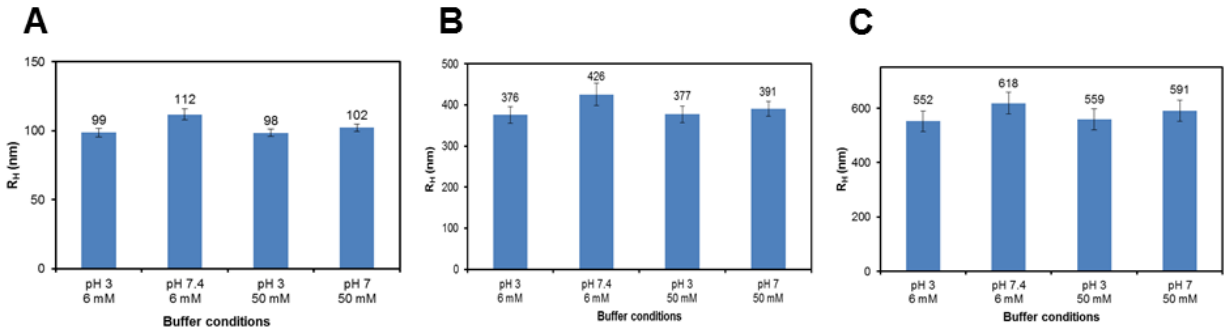


Figure S1. DLS measurements performed on S C/S (A), I C/S (B) and L C/S (C) microgels generated R_H values that demonstrated a slightly greater magnitude of pH responsivity at 6 mM ionic strength, than at 50 mM ionic strength, due to partial screening of charges in the presence of a higher concentration of ionic species in solution.

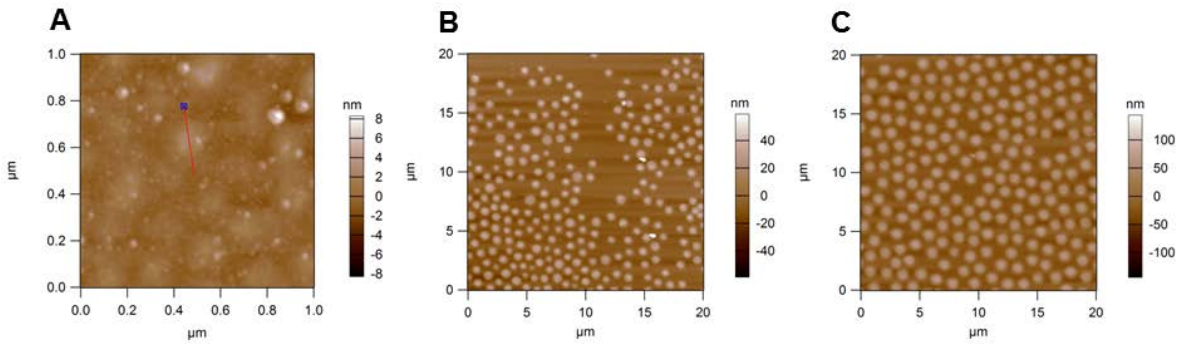


Figure S2. AFM height traces of S C/S, I C/S and L C/S microgels deposited on APTMS-functionalized glass by centrifugal deposition at 2250 × *g* for 10–25 min at 25 °C, depending on the microgel type. Particles in all three size ranges were spherical and the differences in microgel size were evident from the images and height profiles.

Production of +6 GFP

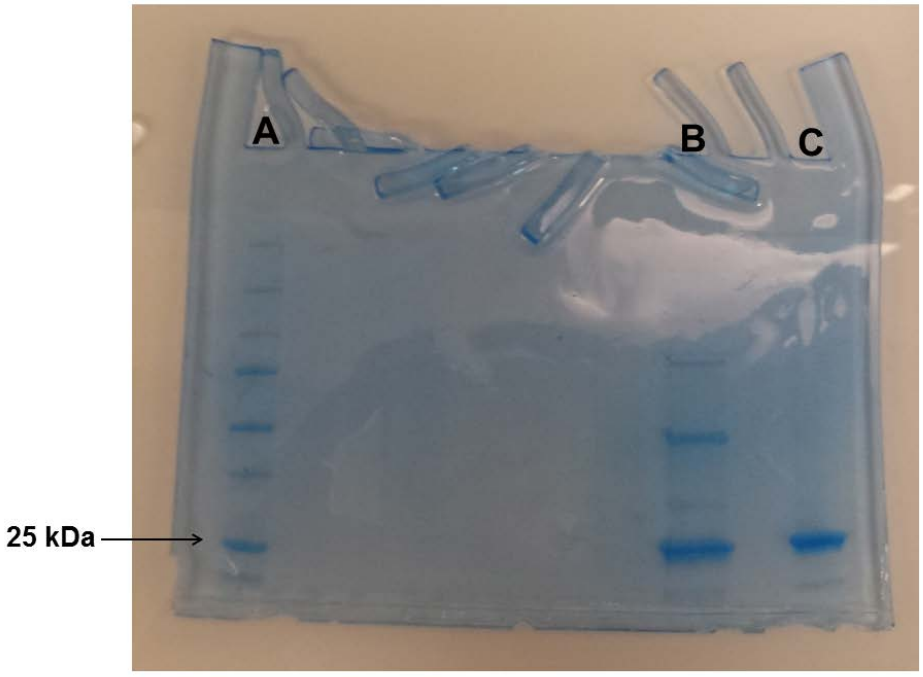


Figure S3. SDS-PAGE of purified +6 GFP. Based on the molecular weight standards (A), the bands represent the presence of +6 GFP (molecular weight of GFP is 27 kDa) [3]. B and C represent the +6 GFP solutions before and after dialysis, respectively.

Analysis of +6 GFP encapsulation in core/shell microgels

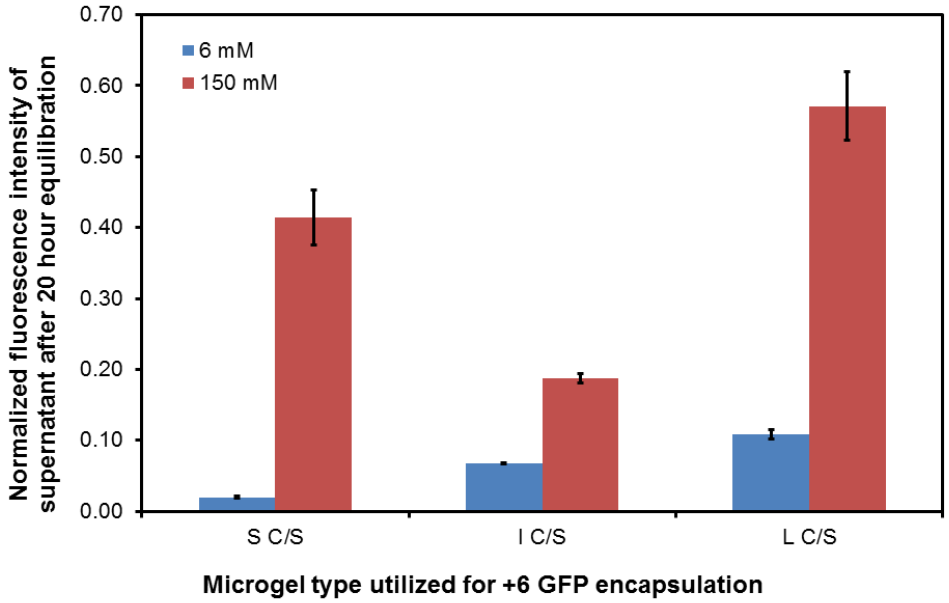
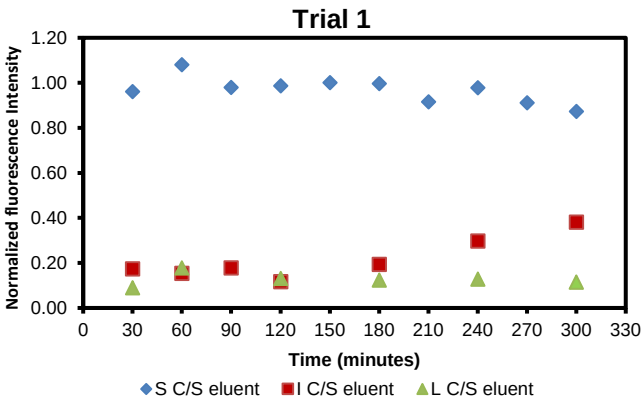
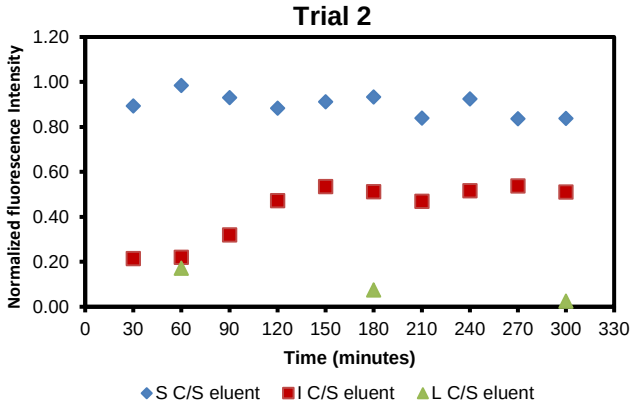


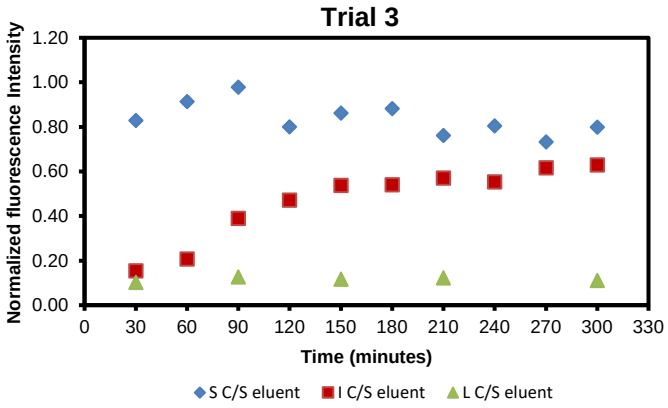
Figure S4. Analysis of encapsulation of +6 GFP by S C/C, I C/S and L C/S microgels. Fluorescence intensities of supernatants were normalized to those of +6 GFP solutions in the respective buffers. Charge screening at higher (150 mM) ionic strength was found to be effective in causing differential encapsulation *(Values presented as averages of 3 measurements on single samples, error bars represent standard deviations in these three values).*

This analysis revealed that the size of +6 GFP (~ 27 kDa) is in a range that allows passage into microgels with the crosslinking density used in these experiments. Thus, encapsulation of other proteins with dimensions similar to +6 GFP into microgels may be feasible. Additionally, these results also indicate that charge interactions play an important role in this encapsulation.

Raw data (Fig. 1)







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