Supporting Information

Arbitrarily-Shaped Microgels Composed of Chemically Unmodified Biopolymers

*Yadu N. Vakkipurath Kodakkadan, Kristyna Idzakovicova, Josef Sepitka, Daniël ten Napel, Eric Safai, Petr Cigler, Frantisek Stepanek and Ivan Rehor**

Contents

1. Materials and Methods

Materials. Sodium alginate (from brown algae, low viscosity, and medium viscosity), chitosan (from crab shells), genipin, $CaCl_2$, PBS, serum, cell growth media, fluorescein O-methacrylamide, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), rhodamine B isothiocyanate (RITC), and dextranase from *Chaetomium erraticum* were purchased from Sigma Aldrich. Tetramethylrhodamine cadaverine was purchased from Thermo Fisher. Methacryloxyethyl thiocarbamoyl rhodamine B was purchased from Polysciences. The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate was synthesized according to previously published procedures[1]. Dex-HEMA was synthesized and characterized as previously

described[2]. Dextran with M_r 15 kDa (from Leuc. Spp., Sigma Aldrich) was used as a substrate and was modified to a substitution degree of 20 and 25 percent methacrylate units per one sugar unit. The substitution degree was obtained from the ^{1}H NMR (CD3OD, 400 MHz) characterization of the Dex-HEMA. δ_H (400 MHz, D_2O) 6.02 (bs, 0.33H, =C*H*'H'' HEMA), 5.61 (bs, 0.34H, =CH'*H*'' HEMA), 4.85 (bs, 1H, O-C*H*-O Dex), 4.39 (bs, 0.70H, N-CO-O-CH_2 HEMA), 4.32 (bs, 0.70H, C-CO-O-CH_2 HEMA), 4.10-3.10 (m, 6H, Dex), 1.80 (bs, 0.97H, -CH_3 HEMA)

The number of HEMA groups per ribose unit of dextran (degree of substitution - *DS*) was calculated by comparing the integral intensity of the six dextran protons multiplet between 3.2-4.1 ppm (I_{DEX}) to integral intensity of methylene group of HEMA at 1.75 ppm (I_{HEMA}) as stated in equation (1).

$$DS = \frac{2 \cdot I_{HEMA}}{I_{DEX}} \cdot 100 \qquad (1)$$

An inverted microscope (Nikon Eclipse Ti-2) was used for microparticle production and observation. An X-Cite® 200DC mercury lamp was used as the light source, with a <420 nm dichroic mirror for photocrosslinking and with RITC and FITC cube filters (Semrock) for fluorescence imaging.

Alginate labeling. Sodium alginate (1 g) was dissolved in 20 mL water (pH adjusted to 6.5), and tetramethylrhodamine cadaverine solution (2 mg, 4 μmol dissolved in 500 μL DMSO) was added and mixed thoroughly. EDC (400 mg, 2 mmol) was added and the mixture was shaken for 30 min at RT. Then, another portion of EDC was added (400 mg, 2 mmol), followed by shaking for an additional 2 h. The crude product was purified by dialysis against water for three days

(water exchanged twice a day) and concentrated using a rotavap to a final concentration of 6% w/w (verified by gravimetry). Labeled alginate was used in a 1:4 vol:vol mixture with unlabeled alginate in all experiments.

Chitosan labeling. Chitosan (1 g) was dissolved in 20 mL of 1% acetic acid (w/w), and FITC (2 mg, 4 μmol dissolved in 500 μL DMSO) was added. The mixture was shaken overnight. The product was purified by dialysis against water for three days (water exchanged twice a day) and concentrated using a rotavap to a final concentration of 6% w/w (verified by gravimetry). Labeled chitosan was used in a 1:5 vol:vol mixture with unlabeled chitosan in all experiments.

Dextranase treatment. As an alternative to basic Dex-HEMA hydrolysis, the Dex-HEMA-alginate microgels were treated after $CaCl_2$ incubation with a solution of dextranase (1% w/v containing 2% w/v $CaCl_2$, 200 μL per well) and incubated for 48 hours at RT. After treatment, the microgels were washed 3 times with 2% $CaCl_2$.
The Dex-HEMA-chitosan microgels were treated after genipin crosslinking with a solution of dextranase (1% w/v, 200 μL per well) and incubated for 12 h at 37 °C. After treatment, the microgels were washed 3 times with water.

Oral Solid Dose formulation labelling. Alginate microgels were synthesized as described above, using 2x larger lithographic mask. A droplet (20 μL) with dispersed microgels was placed at the surface of OSD and allowed to dry at stream of warm air.

Mechanical properties.

Micro-compression test

TI 950 TriboIndenter (Bruker Corp.) nanomechanical test instrument was used to perform a compression testing of a single particle using a diamond flat end 100 μm 90° fluid cell conical probe[3]. Fully lubricated microgels (double network microgels and alginate microgels were immersed in 0.1% CaCl2 while microgels just after SFL were immersed in 0.12 % NaCl to keep the ionic strength of all solutions identical) were sedimented inside Petri dishes mounted on the vacuum stage. A position of the particle was determined using a light microscope which is mounted in the nanomechanical instrument. The transducer with the probe was removed 150 μm next to the particle to find the contact between the probe and bottom of the petri dish to find the Z-axis coordinates of the lower part of the particle. In this XY-axis position, the transducer was calibrated at the estimated Z-axis height of the particle to subtract the influence of the surrounding solution. The transducer was removed above the particle immediately and the compression test was activated. The maximum force P_{max} = 3000 μN with loading rate 300 μN/s which corresponded to a tip extension of about 1 μm/s was prescribed. The exact height of the particle was subtracted at the point of contact between the tip and the particle surface. The particle surface area was calculated from the diagonal length of the particle. The calculated high and area of the particle were used to determine the elastic modulus Es from engineering stress strains curves obtained from the compression testing. The elastic moduli were fitted in Matlab R2017a; R^2 > 0.95. Particles were deformed to maximum strain 15-30 %. We analyzed data up to the 5% strain after a non-linear toe region to eliminate viscoelastic influence on the mechanical properties of the particle[4,5]. 10 particles from each set were analyzed using the micro-compression test. All samples were measured within 31 hours.

Microindentation

TI 950 TriboIndenter (Bruker Corp.) nanomechanical test instrument was used to perform a micro-indentation of pure alginate beads (1.25% and 2.5%) using a diamond conospherical 90° fluid cell probe with the radius of the tip 100 μm. Samples Beads were prepared by placing a 100 μL droplet of sodium alginate (1.25 or 2.5% w/w) onto a glass slide, incubated in 2% $CaCl_2$ for 24 h and finally transferred to 0.1 % $CaCl_2$. The maximum force P_{max} = 3000 μN with loading rate 300 μN/s which corresponded to a tip extension of about 1 μm/s was prescribed. Indentation curves were fitted using Hertz model[6]. Poisson's ratio of the swollen alginate beads was assumed $\nu = 0.5$[7,8].

2. Optimal composition of initial polymer mixtures

The following parameters are crucial for the successful synthesis of the microgel particles:

1) The pregel mixture must be single-phase. Phase separation disables lithographic synthesis of microgels. The number of phases in the system is determined mainly by the concentrations of both polymers, but the phase diagram is also affected by other parameters, such as the molecular weights of the polymers and the presence of salts.

2) The crosslinking rate must be fast enough to allow the processing of the mixture with SFL. This is governed mainly by three parameters – the Dex-HEMA concentration, its degree of substitution, and the photoinitiator concentration.

3) The viscosity of the mixture must be low enough to enable purging through a microfluidic channel. The viscosity is determined by the concentrations and molecular weights of both polymers.

4) The leakage of entrapped biopolymer from freshly synthesized microgels must be slow enough to allow the washing of microgels and biopolymer crosslinking before it diffuses away from the microgels. This can be assured by the high crosslinking density of photocrosslinked polymer and the high molecular weight of the biopolymer.

We evaluated multiple parameters to meet these criteria; however, we have not fully explored the large number of parameters that may affect microgel synthesis. Here, we discuss the main features and trends observed in our system as we were developing it. This discussion focuses mainly on the alginate mixtures, as it was the first system developed. Additionally, Dex-HEMA and alginate have a greater propensity for phase separation compared to Dex-HEMA and chitosan.

Dex-HEMA molecular weight and substitution degree. We assessed three molecular weights—6, 15, and 40 kDa—and three degrees of substitution (DS, the relative number of HEMA groups per one glucose unit of dextran) —15, 20, and 25%. The 40 kDa polymer was not a suitable substrate, as it increased the viscosity of the mixture. Simultaneously, it tended to stick to the microfluidic channel walls during synthesis. DS greater than 25% cannot be used, as it promotes phase separation with alginate due to the increasing hydrophobicity of highly substituted Dex-HEMA (the limit for water solubility is around 35%). Both 20 and 25% DS worked similarly well, with the latter being more sensitive towards illumination; however, the alginate reaction mixtures were prone to phase separation, and thus 20% DS was chosen in this system. Both 6 and 15 kDa dextran worked similarly well, with the latter providing slightly better-resolved particles. We chose a 15% dextran concentration for our experiments, as higher concentrations

resulted in high viscosity in the mixture with a biopolymer, while lower concentrations dramatically decreased the sensitivity of the mixture to UV irradiation in the lithographic process.

Photoinitiator concentration. We evaluated concentrations of lithium phenyl-2,4,6-trimethyl-benzoylphosphinate (LAP), a highly efficient water-soluble photoinitiator, of up to ~8% w/w. However, in our binary polymer mixtures, high concentrations of LAP caused phase separation of the polymers. We attribute this to the ionic nature of LAP, resulting in increased ionic strength of the solution. A concentration of 0.25% w/w was the highest tolerated in both the Dex-HEMA-alginate and Dex-HEMA-chitosan systems. Despite the low concentrations, we were able to keep irradiation times for the SFL at ~200 ms.

Biopolymer concentration and molecular weight. We tested two molecular weight formulations of alginate as provided by the vendor – a 'low viscosity' fraction with Mr below 80 kDa and a 'medium viscosity' fraction with Mr 80-120 kDa. The low viscosity polymer diffused out of the hydrogel network of lithographic microgels too rapidly to allow ionic crosslinking (i.e., the microgels fully dissolved upon Dex-HEMA hydrolysis). The medium viscosity polymer leaked much more slowly and such microgels sustained the hydrolysis process. We compensated for the higher viscosity of the alginate and thus the entire mixture by choosing higher purging pressures for the SFL synthesis. We determined an optimal alginate concentration of 2.5% w/w. Higher concentrations resulted in mixtures that were too viscous, leading to a pressure increase, which resulted in channel destruction. On the other hand, a concentration of 2% did not provide finely resolved particles after template removal. Additional fine-tuning of the Dex-HEMA and alginate

concentrations may provide solutions with acceptably high viscosity and even slightly better performance than the one described in our study.

3. Construction and programming of the motorized stage coupled to the SFL

With SFL, microgels are created through radical polymerization. In the proximity of the top and bottom walls of the channel, the crosslinking process is scavenged by oxygen that penetrates through the PDMS wall from the air atmosphere. As a result of this oxygen inhibition layer, the synthesized microgels are separated from the channel walls by a thin (~1-2 μm) layer of the liquid substrate[9]. Therefore, the particles do not get stuck and can be flushed from the channel. We observed that the polymer mixtures used in our project affect the efficiency of this inhibition and cause freshly made particles to stick to the wall at the irradiation spot, which hampers the synthesis. To date, SFL has been almost exclusively used with polyethyleneglycol diacrylate as a substrate, and this problem has not been encountered[10-13]. We observed that sticking occurs after multiple irradiations at one spot and that longer delays between successive irradiations at the same spot suppresses the sticking. We hypothesize that the sticking originates from the depletion of the oxygen in the inhibition layer. To suppress the sticking, we coupled the SFL setup to a custom-built motorized stage and cycled the irradiation position inside the channel periodically between several positions (typically 6). This cycling prolonged the time interval between successive irradiation at the same spot and efficiently suppressed microgel sticking. Briefly, an Arduino Mega equipped with a CNC shield containing two A4944 stepper motor drivers was used to control the shutter, pump, and motion of the stage. The stage was connected

to two Nema 17 stepper motors with flexible shafts (SDP/SI, product nr. 7C12M4830533) for movement in the xy-plane and attached to a manual stage (ThorLabs product nr. XYT1/M), using connectors manufactured by 3D printing, similarly to previously published design[14]. The microfluidic chip was placed in a 3D-printed sample holder (see Figure S8). The Arduino Mega digital output directly controlled the SFL shutter (integrated to the X-Cite® 200DC illumination source) and a relay was used to operate a 3-way solenoid valve (Festo, cat. nr. MHA1-M5H-3/2G-0,6-HC, MHA1-AS-3-M3) switching between atmospheric pressure and ~0.5 bar overpressure from the nitrogen flask. We wrote a custom script that allows the irradiation and purge lengths and the motif of irradiation to be precisely controlled. A switch was attached to start and stop the SFL cycles.

4. Supplementary Figures

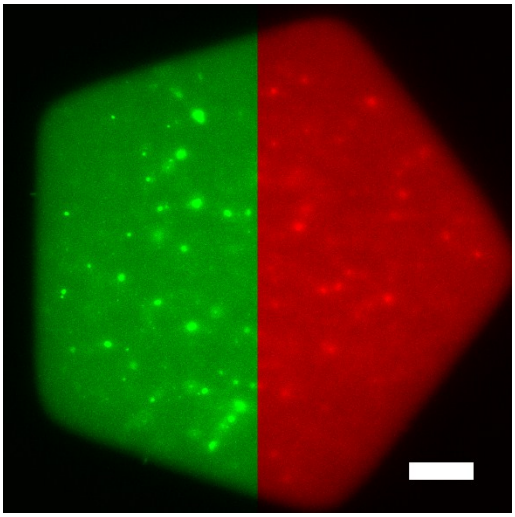

Figure S1. Scanning confocal image of fluorescently labelled dual network microgels, alginate (green)-Dex-HEMA (red). The distribution of both fluorescence intensities is uniform, showing no sign of microgel phase separation, that may occur during the semi-IPN – IPN transition, as has been previously described[15,16]. The bright spots belong to the agglomerates, observed in the alginate stock solutions. Their frequency can be reduced by filtration of the diluted alginate stock solution through a 0.2 µm filter, but they reappear over the course of long-term storage (within days). They are also weakly visible in the red channel through limited Förster resonance energy transfer between the fluorescein and rhodamine dyes. Scalebar corresponds to 10 µm.

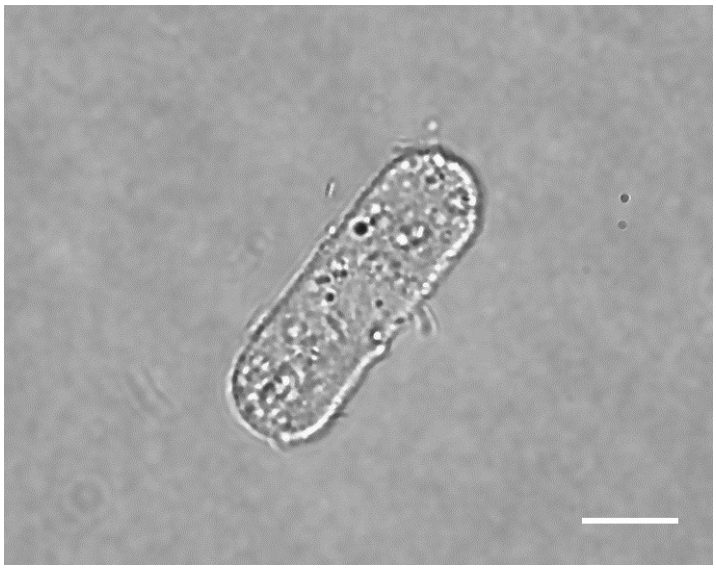

Figure S2. Micrograph of alginate anisotropic hydrogel obtained from hydrolysis taking ~10 min. Although the overall shape is preserved, the letters are not legible. The scale bar corresponds to 20 μm.

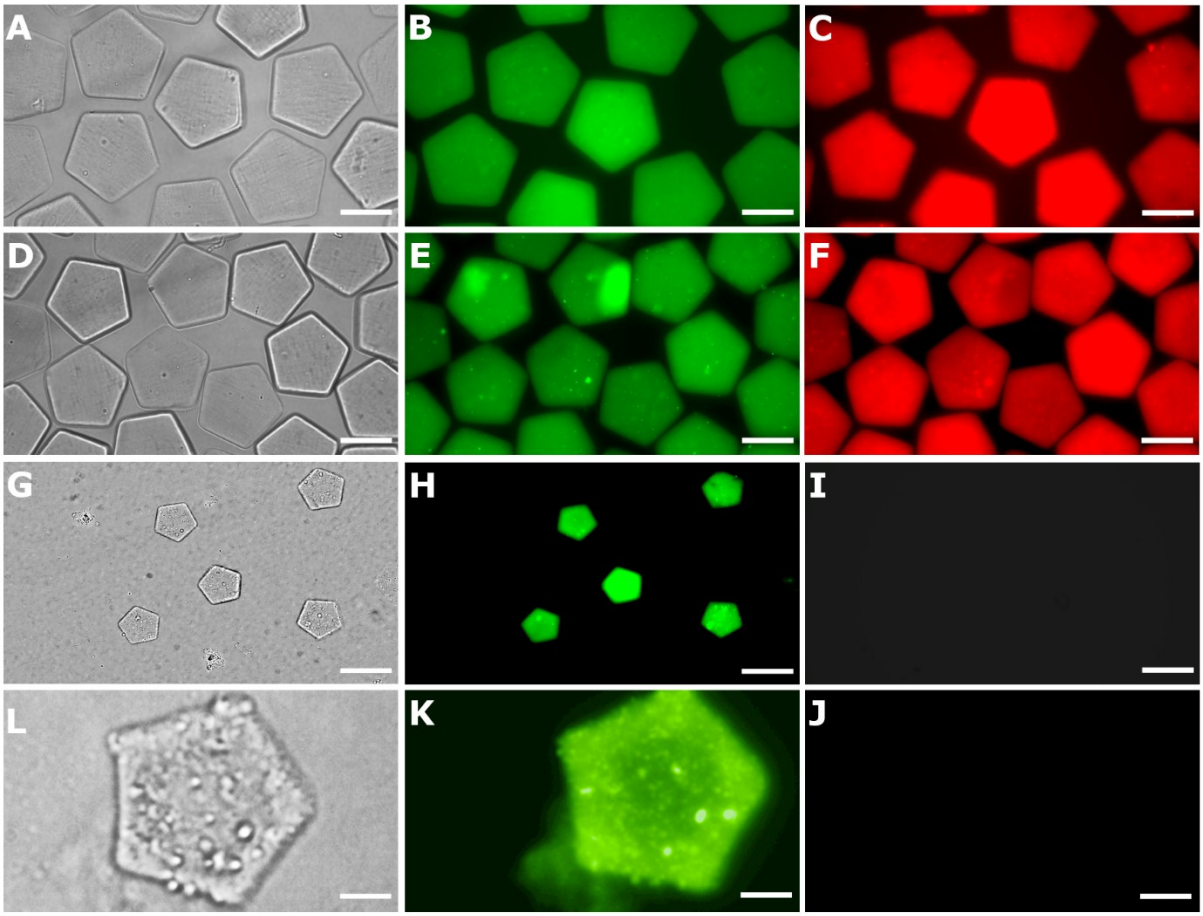

Figure S3. Micrographs of pentagon-shaped alginate hydrogels obtained from hydrolysis taking ~10 min. (A) Hydrogels obtained from lithographic synthesis with the alginate physically entrapped in the structure held together by photocrosslinked Dex-HEMA. (B and C) Fluorescent images of labeled alginate and labeled Dex-HEMA network, respectively. Particle size = 88 ± 2 μm. (D-F) The same sample after the addition of $CaCl_2$, (brightfield – D, alginate fluorescence – E, Dex-HEMA fluorescence – F). Particle size = 76 ± 2 μm. (G-L) Sample after addition of a base (brightfield – G, J; alginate fluorescence – H, K; Dex-HEMA fluorescence – I, L). Particle size = 39 ± 2 μm. Scale bars correspond to 50 μm in A-I and to 10 μm in J-L. All fluorescence images are in false colors.

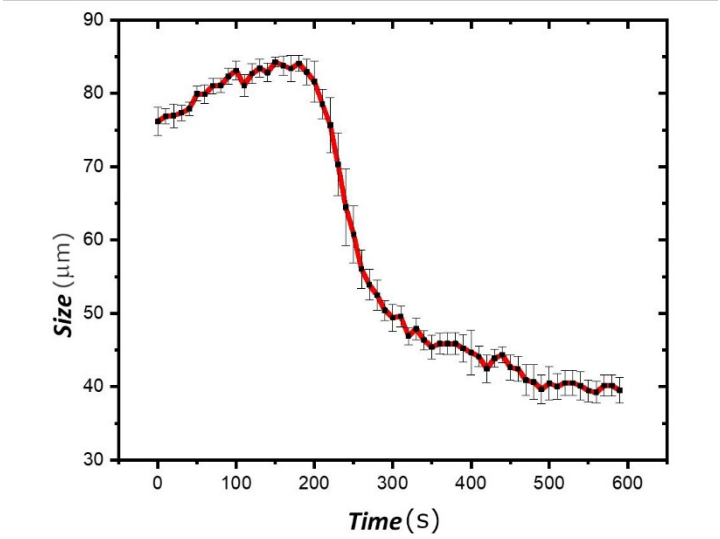

Figure S4. Plot of Dex-HEMA + Ca-alginate microgel size as a function of time during hydrolysis of the Dex-HEMA network. The hydrolysis process takes a total of 10 min. During the first 3 min, the microgel swells by ~10%. Then, the microgel rapidly shrinks to around 66% of its original size in ~100 s, followed by slow shrinkage to approximately 50% of its original size in 300 s. We attribute the initial swelling to the hydrolysis of Dex-HEMA and concomitant mesh-size increase. When the Dex-HEMA network is substantially hydrolyzed, the alginate chains gain enough conformational freedom to come into contact with each other and form new crosslinks responsible for the hydrogel shrinkage, thanks to the presence of Ca^{2+} ions in the solution.

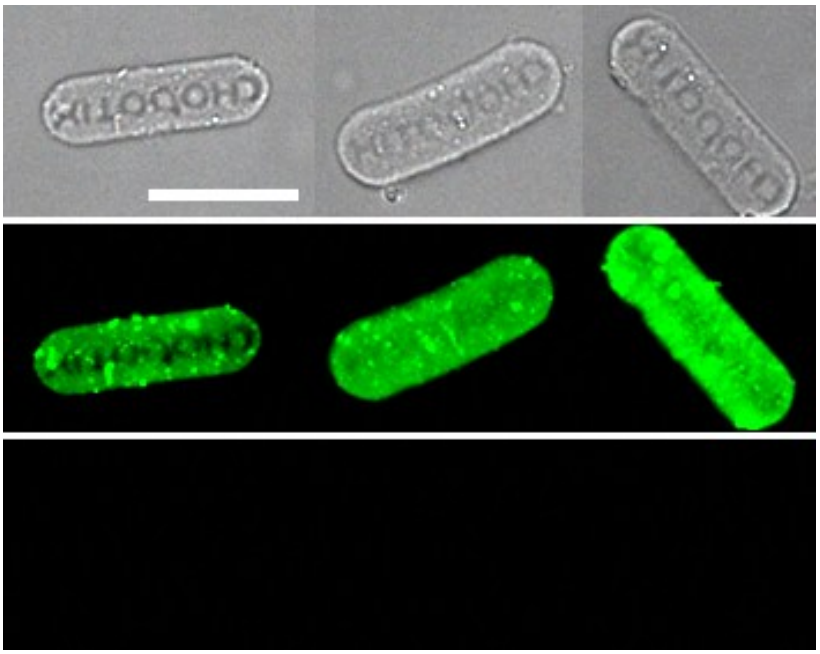

Figure S5. Alginate microgels obtained by simultaneous ionic crosslinking and basic cleavage (top to bottom line – brightfield image, fluorescently labeled alginate, fluorescently labeled Dex-HEMA). Scale bar correspods to 50 μm.

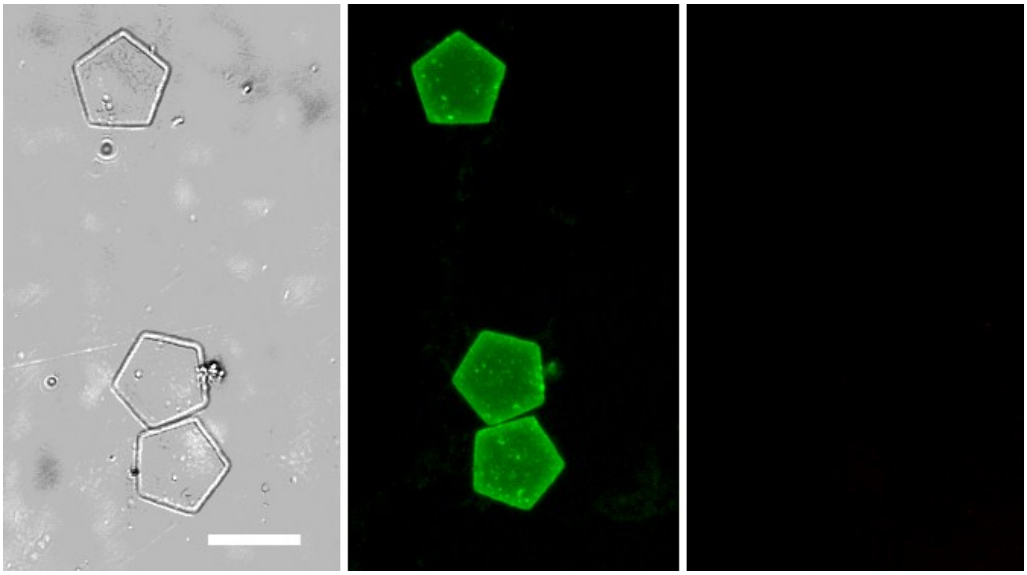

Figure S6. Alginate microgels obtained by enzymatic cleavage of Dex-HEMA. (A) Brightfield image, (B) fluorescently labeled alginate, (C) fluorescently labeled Dex-HEMA. Scale bars correspond to 50 µm. Fluorescence images are in false colors.

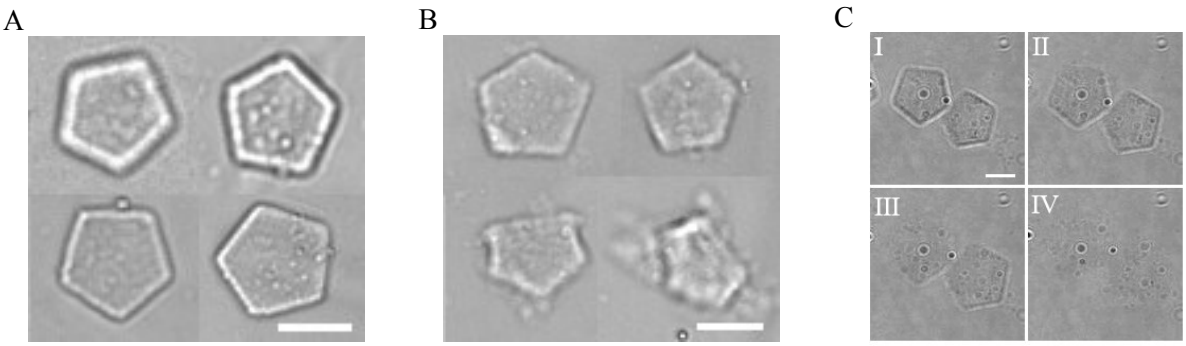

Figure S7. Stability of microgels in various solutions. A – in aqueous solutions contatining $CaCl_2$, the microgels show no signs of degradation for 3 months. B – after 6 month in pure water, some microgels maintain their shape, while some are deformed substantially. Scale bar corresponds to 50 μm. C – in PBS the microgels swell rapidly and completely dissolve within two hours (subsequent images I-IV). All scalebars correspond to 20 μm.

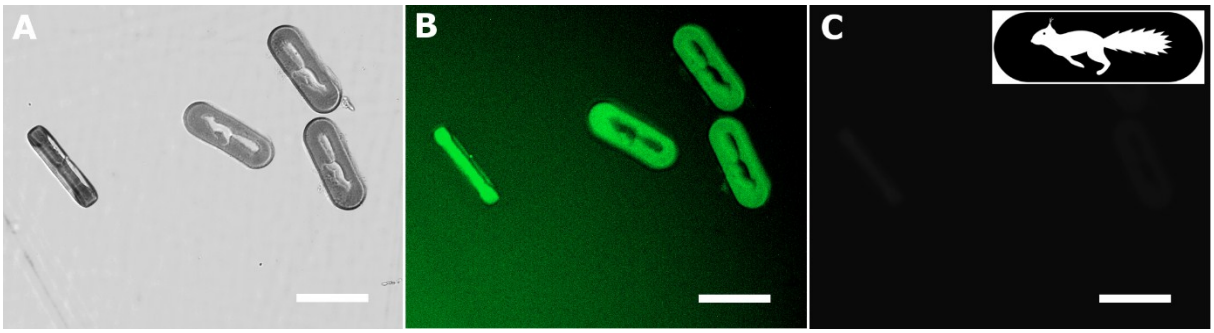

Figure S8. Genipin-crosslinked chitosan microgels obtained by enzymatic cleavage of Dex-HEMA. (A) Brightfield image, (B) fluorescently labeled chitosan, (C) fluorescently labeled Dex-HEMA. Scale bars correspond to 100 µm. The inset in the right image displays the design on the lithographic mask, with the black sections being transparent.

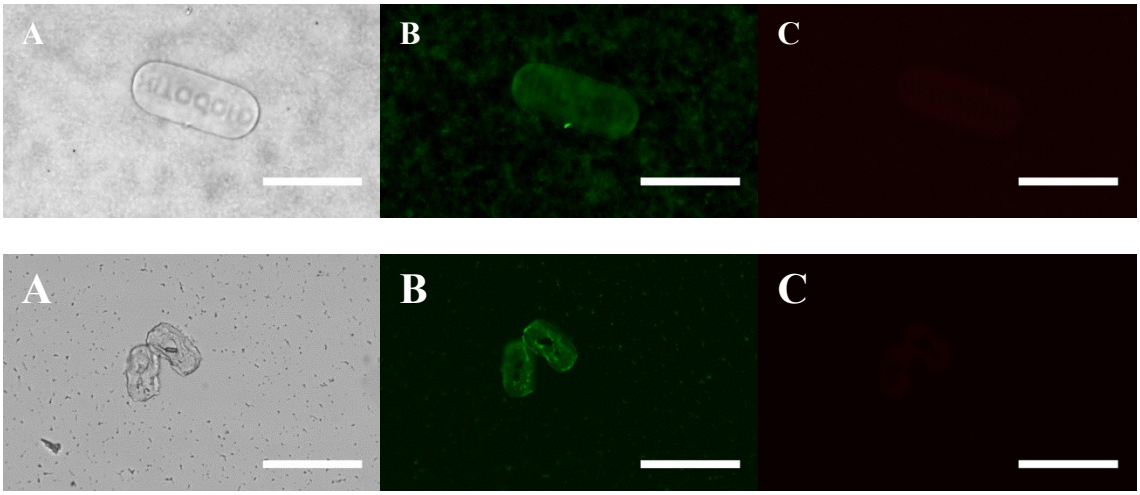

Figure S9. Phytic acid-crosslinked chitosan microgels obtained by enzymatic cleavage of Dex-HEMA. (A) Brightfield image, (B) fluorescently labeled chitosan, (C) fluorescently labeled Dex-HEMA. Scale bars correspond to 100 µm. This synthesis is not perfectly reproducible, and the bottom line shows one of the unsuccessful attempts.

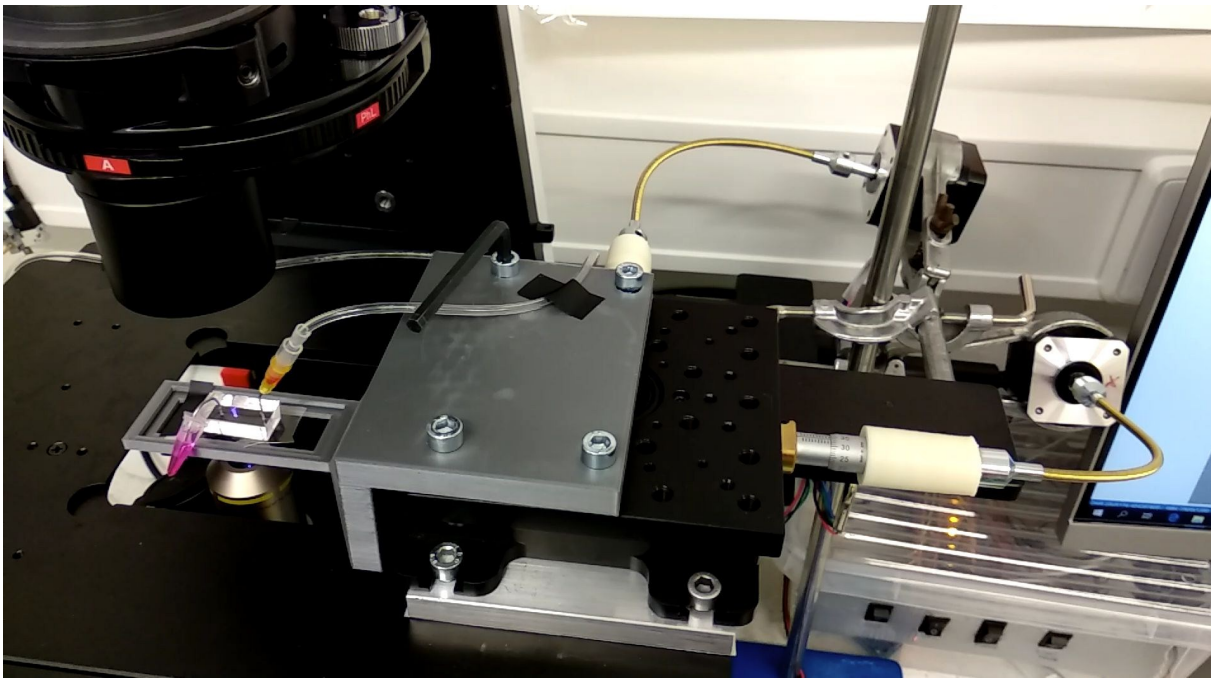

Figure S10. SFL setup coupled to motorized stage

5. References:

[1] B. D. Fairbanks, M. P. Schwartz, C. N. Bowman, K. S. Anseth, *Biomaterials* **2009**, *30*, 6702.

[2] W. N. E. van Dijk-Wolthuls, S. K. Y. Tsang, J. J. Kettenes-van den BoschW.E. Hennink, *Polymer* **1997**, *38*, 6235.

[3] J. Sepitka, P. Grznarova, T. Fuzik, J. Lukes, *Comput. Methods Biomech. Biomed. Engin.* **2014**, *17*, 40.

[4] A. J. Onyianta, M. Castellano, M. Dorris, R. L. Williams, S. Vicini, *Carbohydr. Polym.* **2018**, *198*, 320.

[5] R. M. Delaine-Smith, S. Burney, F. R. Balkwill, M. M. Knight, *J. Mech. Behav. Biomed. Mater.* **2016**, *60*, 401.

[6] C.-Y. Chui, A. Bonilla-Brunner, J. Seifert, S. Contera, H. Ye, *J. Mech. Behav. Biomed. Mater.* **2019**, *93*, 61.

[7] K. S. Anseth, Ch. N. Bowman, L. Brannon-Peppas, Biomaterials, **1996**, 17, 1647-1657.

[8] A. M. Padol, K. I. Draget, B. T. Stokke, *Carbohydrate Polymers*, **2016**, 147, 234-242

[9] D. Dendukuri, P. Panda, R. Haghgooie, J. M. Kim, T. A. Hatton, P. S. Doyle, *Macromolecules* **2008**, *41*, 8547.

[10] D. Dendukuri, S. S. Gu, D. C. Pregibon, T. A. Hatton, P. S. Doyle, *Lab. Chip* **2007**, *7*, 818.

[11] P. Panda, S. Ali, E. Lo, B. G. Chung, T. A. Hatton, A. Khademhosseini, P. S. Doyle, *Lab. Chip* **2008**, *8*, 1056.

[12] J. Lee, P. W. Bisso, R. L. Srinivas, J. J. Kim, A. J. Swiston, P. S. Doyle, *Nat. Mater.* **2014**, *13*, 524.

[13] D. C. Appleyard, S. C. Chapin, R. L. Srinivas, P. S. Doyle, *Nat. Protoc.* **2011**, *6*, 1761.

[14] R. A. A. Campbell, R. W. Eifert, G. C. Turner, *PLoS ONE* **2014**, *9*, DOI 10.1371/journal.pone.0088977.

[15] Sperling, L. H. & Hu, R. Interpenetrating Polymer Networks. in *Polymer Blends Handbook* (eds. Utracki, L. A. & Wilkie, C. A.) 677–724 (Springer Netherlands, 2014).

[16] Dragan, E. S. Design and applications of interpenetrating polymer network hydrogels. A review. *Chemical Engineering Journal* **243**, 572–590 (2014).