Supplementary data for article:

Utech, S.; Prodanovic, R.; Mao, A. S.; Ostafe, R.; Mooney, D. J.; Weitz, D. A. Microfluidic Generation of Monodisperse, Structurally Homogeneous Alginate Microgels for Cell Encapsulation and 3D Cell Culture. *Advanced Healthcare Materials* **2015**, *4* (11), 1628–1633. https://doi.org/10.1002/adhm.201500021

ADVANCED HEALTHCARE MATERIALS

Supporting Information

for *Adv. Healthcare Mater.,* DOI: 10.1002/adhm.201500021

Microfluidic Generation of Monodisperse, Structurally Homogeneous Alginate Microgels for Cell Encapsulation and 3D Cell Culture

*Stefanie Utech, Radivoje Prodanovic, Angelo S. Mao, Raluca Ostafe, David J. Mooney, and David A. Weitz**

Supporting Information

Microfluidic generation of monodisperse, structurally homogeneous alginate microgels for cell encapsulation and 3D cell culture

Stefanie Utech[1], Radivoje Prodanovic[1,2], Angelo S. Mao[1], Raluca Ostafe[1,3], David J. Mooney[1,4], David A. Weitz[1,5]

[1] School of Engineering and Applied Sciences, Harvard University, Cambridge, Massachusetts, USA

[2] Faculty of Chemistry, University of Belgrade, Studentski trg 12, Belgrade, Serbia

[3] Molecular Biotechnology, Faculty of Biology, RWTH Aachen University, Aachen, Germany

[4] Wyss Institute for Biologically Inspired Engineering, Cambridge, Massachusetts, USA,

[5] Department of Physics, Harvard University, Cambridge, Massachusetts, USA

E-mail: weitz@seas.harvard.edu

Experimental part

Device fabrication

Microfluidic devices are fabricated by soft lithography.[1] Negative photo resist SU-8 (MicroChem, Newton, MA, USA) is spin coated onto a clean silicon wafer to a thickness of 10 µm, 25 µm or 50 µm and patterned by UV exposure through a transparency photomask (CAD/Art Service, Bandon, OR, USA). After developing the microstructure, a degassed 10:1 mixture of Sylgard 184 poly(dimethylsiloxane) (PDMS) (Dow Corning, Midland, MI, USA) and cross-linker (ratio 10 : 1) is poured onto the pattern, degassed and cured overnight at 65°C. The PDMS molds are peeled off the master and the channel inlets and outlets were made by using a 0.75 mm diameter biopsy punch (Harris Uni-Core, Ted Pella, Inc., Redding, CA). The PDMS replicas are bonded to a glass slide after oxygen-plasma activation of both surfaces and cured for one hour at 65 °C to enhance bonding. To avoid wetting of the channels by the inner phase, the devices are treated with Aquapel (PPG Industries, Pittsburgh, PA, USA) by flushing the channels with the solution as received, air drying and heating to 65 °C for one hour.

Alginate solution and calcium-EDTA complex formation

The alginate solutions are prepared by dissolving ultrapure sodium alginate (2 wt% Pronova UP MVG, Novamatrix FMC Biopolymer, Ayrshire, GB) in deionized water or cell culture medium

(Dulbecco's Modified Eagle Medium, Gibco Life Technologies, Grand Island, NY, USA), respectively. The calcium-EDTA solution is obtained by mixing 100 mM solutions of calcium chloride (J.T. Baker, Center Valley, PA, USA) and disodium-EDTA (Mallinckrodt, St. Louis, MO, USA) (ratio 1 : 1) and subsequent pH-adjustment using sodium hydroxide. The final pH-value of the solution is 7.2. To guarantee an even mixing of alginate and calcium-EDTA, the two solutions are mixed in a 1:1 ratio and vortexed previous to the drop formation.

Drop generation

To fabricate alginate beads, the alginate/calcium-EDTA mixture is loaded into polycarbonate syringes (BD Luer-Lok Disposable Syringes). A mixture of 1 wt% surfactant in perfluorinated carbon oil (3M Novec 7500 Engineered Fluid, 3M, St. Paul, MN, USA) is used as outer phase.[2] PE/2 tubing with an outer diameter of 1.09 mm and an inner diameter of 0.38 mm (Scientific Commodities, Lake Havasa City, AZ, USA) is used to connect the channel inlets with the syringes. Flow rates are controlled by syringe pumps (PHD 2000, Harvard Apparatus, Holliston, MA, USA).

Droplet generation is monitored with an inverted microscope equipped with a fast camera (Phantom V7, Vision Research, Wayne, NJ, USA). The flow rates are individually adjusted to obtain the aspired particle size (Table 1). Acetic acid (0.05 vol%) is added to trigger the release of calcium ions from the complex and hence induce gelation of the alginate drops. The pH range necessary to induce dissociation of the complex and release of calcium ions is determined in bulk systems and confirmed to be accurate for the micron-sized system by measuring the pH value after acid addition and breaking of the emulsion.

After gelation, the gels are transferred into aqueous medium by addition of a 20% (v/v) solution of perfluoro-1-octanol (PFO, SynQuest Laboratories, Alachua, FL, USA) in the perfluorinated carbon oil. To enhance the gel strength calcium chloride can be added during the transfer process. After centrifugation, the oil phase is discarded and the gels are re-suspended in water.

Table 1. Relationship between channel dimension (channel width/height at crossjunction), flow rates (Q_i: flow rate inner phase, Q_o: flow rate outer phase) and resulting particle diameters. An increase in the flow rate of the outer phase results in a decrease of the drop size and consequently the particle size for a given dropmaker.

10 µm dropmaker		**25 µm dropmaker**		**50 µm dropmaker**	
d(microgel)/µm	Q_i/Q_o	d(microgel)/µm	Q_i/Q_o	d(microgel)/µm	Q_i/Q_o
10	0.1	25	0.5	50	0.5
		22	0.1	40	0.1
		17	0.05	37	0.05

Cell culture

We grow D1 mesenchymal stem cells (MSCs) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 1% (v/v) Penicillin/Streptomycin. The cells are split every 5 days under sterile conditions and incubated at 37 °C and 5 % CO_2.

Cell encapsulation

Prior to the encapsulation, cells are trypsinized, centrifuged and resuspended in the calcium-EDTA/alginate mixture (2 w% alginate, 50 mM Ca-EDTA in DMEM) to gain the desired cell density.[3] Droplets are generated as described above. After gelation the microgels are transferred into aqueous medium by addition of 20 % PFO in perfluorinated carbon oil and subsequent centrifugation (1000 rpm, 1 min). The resulting oil phase is discarded and the gels are re-suspended in cell culture medium. Cell-laden gels are incubated at 37 °C under 5 % CO_2. Calcein-AM is used to determine the cell viability.

Imaging

For a better visualization of the homogeneity and structure of the alginate microgels, a FITC-labeled alginate is used.[4] Fluorescent imaging is performed using an inverted fluorescent microscope (Leica DM IRBE microscope, Leica Microsystems GmbH, Wetzlar, Germany) as well as a confocal laser scanning microscope (CLSM, Zeiss LSM510 META, Carl Zeiss North America, Thornwood, NY, USA).

Supplemental figures

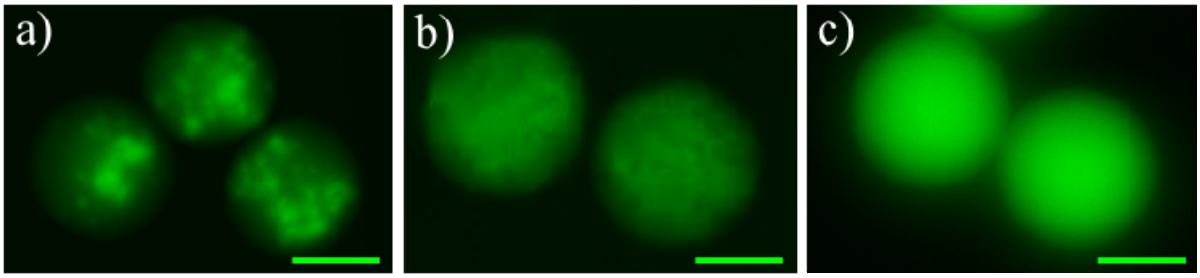

Figure S1. Comparison of different techniques for the microfluidic fabrication of alginate microgels. A fluorescently labeled alginate was used for the illustration of the particles structural integrity via fluorescent microscopy. a) Alginate microgels prepared by the incorporation of insoluble calcium carbonate ($CaCO_3$). The particles are mixed with the alginate solution prior to drop generation and are subsequently dissolved in presence of acetic acid. The freed calcium ions induce the gelation of the alginate droplets. The high magnification fluorescent images show that the particles are not homogenously dispersed within the droplets, which is reflected in the inhomogeneous distribution of the alginate chains. b) Particles prepared by mixing of alginate with a calcium solution. In a co-flow device, alginate and calcium chloride are simultaneously injected into the microchannel. Gelation occurs immediately upon contact between alginate and calcium chloride solution leading to clogging issue within several minutes. However, the particles obtained prior to clogging show an inhomogeneous internal structure due to the rapid gelation of alginate which does not allow for complete and homogeneous mixing of the two solutions. c) Alginate microgels prepared by acidic release of calcium ions from a water-soluble calcium complex. Under neutral pH, the calcium-EDTA solution can be mixed with alginate without gelation. After drop formation, gelation is induced by acid dissociation of the complex. Highly homogenous alginate microgels result.

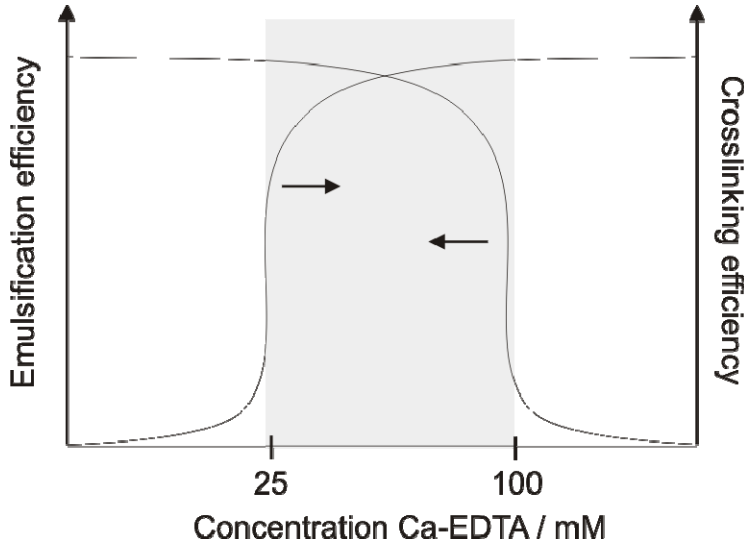

Figure S2. Schematic illustration of the relation between Ca-EDTA concentration, emulsification and crosslinking efficiency. Ideal concentrations for the internal gelation of alginate have been reported to be in the range of 25 to 100 mM. The crosslinking efficiency increases with increasing Ca-EDTA concentrations. We define the emulsification efficiency by the stability of the drop formation process. The emulsification efficiency decreases with increasing Ca-EDTA concentration due to the formation of insoluble precipitates and the increased viscosity when mixed with alginate solutions (2 wt%). This increase in viscosity is the result of premature crosslinking of alginate due to the presence of free calcium ions which are in equilibrium with the calcium-EDTA complex. The presence of insoluble precipitates and premature crosslinking hinder the drop formation process and thus the emulsification efficiency

The crosslinking efficiency is determined by the formation of stable alginate gels after purification and transfer to aqueous medium. If the concentration of Ca-EDTA is too low, the crosslinking process is incomplete, resulting in dissolution of the droplets after transfer into the aqueous phase.

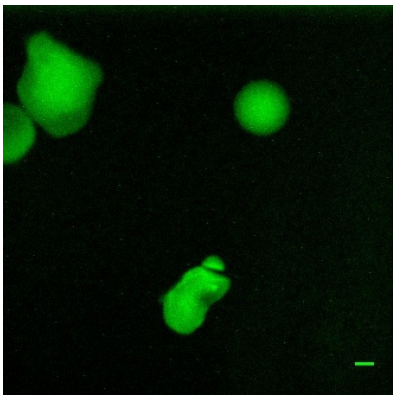

Figure S3. Alginate particles prepared from 2 wt% alginate, 100 mM Ca-EDTA and 0.001 % acetic acid. The low acid concentration is not sufficient to enable complete gelation of the alginate droplets. Mainly aggregated and disrupted particles result.

(1) Xia, Y. N.; Whitesides, G. M. *Annu. Rev. Mater. Sci.* **1998**, *28*, 153.

(2) Holtze, C.; Rowat, A. C.; Agresti, J. J.; Hutchison, J. B.; Angile, F. E.; Schmitz, C. H. J.; Koster, S.; Duan, H.; Humphry, K. J.; Scanga, R. A.; Johnson, J. S.; Pisignano, D.; Weitz, D. A. *Lab on a Chip* **2008**, *8*, 1632.

(3) Koster, S.; Angile, F. E.; Duan, H.; Agresti, J. J.; Wintner, A.; Schmitz, C.; Rowat, A. C.; Merten, C. A.; Pisignano, D.; Griffiths, A. D.; Weitz, D. A. *Lab on a Chip* **2008**, *8*, 1110.

(4) Rowley, J. A.; Madlambayan, G.; Mooney, D. J. *Biomaterials* **1999**, *20*, 45.