Microfluid Nanofluid (2014) 16:773–777
DOI 10.1007/s10404-013-1264-z

SHORT COMMUNICATION

Microfluidic encapsulation of cells in alginate particles via an improved internal gelation approach

Samin Akbari · Tohid Pirbodaghi

Received: 30 May 2013 / Accepted: 10 September 2013 / Published online: 24 September 2013

Abstract An improved internal gelation approach is developed to encapsulate single mammalian cells in monodisperse alginate microbeads as small as 26 μm in diameter and at rates of up to 1 kHz with high cell viability. The cell damage resulting from contact with calcium carbonate nanoparticles as gelation reagents is eliminated by employing a co-flow microfluidic device, and the cell exposure to low pH is minimized by a chemically balanced off-chip gelation step. These modifications significantly improve the viability of cells encapsulated in gelled alginate particles. Two different mammalian cell types are encapsulated with viability of over 84 %. The cells are functional and continue to grow inside the microparticles.

Keywords Single cell encapsulation · Alginate · Droplet-based microfluidics

Samin Akbari and Tohid Pirbodaghi have contributed equally to this article.

S. Akbari (✉)
École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland
e-mail: samin.akbari@psi.ch

S. Akbari · T. Pirbodaghi
School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, USA

T. Pirbodaghi
Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland

1 Introduction

Cell encapsulation in hydrogel microbeads has promising applications in tissue engineering, regenerative medicine, and cell-based drug delivery (Drury and Mooney 2003; Pierigè et al. 2008; Slaughter et al. 2009; Schmidt et al. 2008; Nicodemus and Bryant 2008; Xu et al. 2009). Encapsulated cells can secrete therapeutic proteins in response to an external stimulus over an extended time period to treat various diseases including renal failure and diabetes (Sun et al. 1996; Prakash and Chang 1996). In cell transplantation therapy for ischemic heart diseases, direct injection of cells results in limited cell survival, while encapsulation in hydrogels improves cell growth and transplantation efficiency (Chachques et al. 2007; Yu et al. 2010). Of all hydrogels, alginate is one of the most suitable biomaterials for cell encapsulation due to its biocompatibility, biodegradability, similarities to the natural extracellular matrix, and ease of gelation (Lee and Mooney 2012). Alginate is a naturally derived polymer, which can physically cross-link with divalent ions to provide an ideal three-dimensional scaffold for cells that allows bidirectional diffusion of nutrients and waste products.

Alginate particles are typically produced by ejecting drops of alginate solution into a bath of divalent ions resulting in millimeter-sized polydisperse beads (Maguire et al. 2006; Hoesli et al. 2011; Mazzitelli et al. 2011). However, for use as carriers of drugs, proteins, or cells, it is desirable to precisely control particle size and monodispersity. Droplet-based microfluidics offers a powerful method to rapidly produce monodisperse alginate microdroplets with diameters of up to a few hundred micrometers (Tan and Takeuchi 2007; Capretto et al. 2008; Workman et al. 2008; Martinez et al. 2012; Teh et al. 2008; Choi et al. 2007). Smaller hydrogel particles (<50 μm in diameter) are

preferred for cell encapsulation because the small size: (1) enhances diffusion of oxygen and nutrients, improving cell viability over long periods of time (Drury and Mooney 2003; Sugiura et al. 2005); (2) decreases the sample volume and reaction times, enabling high-throughput single cell studies (Edd et al. 2008; Guo et al. 2012); (3) reduces the cellular reaction and the immune response to the particles (Sakai et al. 2006) and also facilitates particle injection for cell transplantation therapies (Yu et al. 2010); and (4) allows the particles to be sorted through the use of standard fluorescence-activated cell sorting (FACS) methods. However, producing small particles is challenging due to the high fluidic resistance of the viscous polymeric solutions in narrow microchannels increasing the inlet pressure for injection and therefore debonding the channels. Moreover, the in situ gelation of droplets in the microfluidic device quickly clogs the microchannels. Several gelation strategies, mainly based on an external or internal source of divalent ions, are used to polymerize the alginate drops. In the external gelation approach, divalent ions, generally Ca^{2+}, are added either by merging alginate drops to aqueous Ca^{2+} drops or by co-flowing alginate and calcium chloride ($CaCl_2$) solutions separated by a water stream through a microfluidic device (Choi et al. 2007; Shintaku et al. 2007). The use of aqueous drops to provide Ca^{2+}, or the use of a water stream to keep the alginate and $CaCl_2$ separate until drop formation, reduces the final concentration of alginate and therefore limits the stiffness of the microbeads. Additionally, the rapid gelation rate of these methods produces heterogeneous and mostly non-spherical particles (Martinez et al. 2012; Um et al. 2008). An alternative approach, internal gelation, allows better control over the particles' size and homogeneity (Tan and Takeuchi 2007; Workman et al. 2008). Tan and Takeuchi (2007) combined the internal gelation approach with droplet-based microfluidics in which a stream of alginate solution containing cells and $CaCO_3$ nanoparticles is used to form droplets, and then a stream of acidic oil is added to release the calcium ions and effect in situ gelation. They reported monodisperse particles as small as 94 μm diameter with 74 % cell viability. However, in their approach, the cell viability directly depends on the $CaCO_3$ concentration, making it difficult to tune the particle stiffness by varying the $CaCO_3$ concentration. Moreover, dispersion of the cells and $CaCO_3$ nanoparticles in the same alginate solution increases direct mechanical contact between them, which physically damages and breaks the cells prior to drop formation.

Here, we present an improved internal gelation technique to encapsulate mammalian cells in alginate microparticles as small as 26 μm in diameter with high viability independent of $CaCO_3$ concentration. By adding the stoichiometric equivalent of acetic acid with respect to the $CaCO_3$ concentration to the collected droplets and quickly transferring the gelled particles to fresh cell growth medium, the exposure of the encapsulated cells to low pH is minimized. The physical damage to the cells by $CaCO_3$ nanoparticles is also prevented by flowing the cells and the $CaCO_3$ nanoparticles in two separate streams of alginate solution prior to drop formation. Protecting the cells from chemical and mechanical harm during encapsulation increased the cell viability to 84 % after encapsulation.

2 Results and discussion

In our initial effort to duplicate the conventional internal gelation technique in which cells and calcium carbonate nanoparticles are suspended in the same alginate solution, we observed that direct mechanical contact between the cells and particles severely damaged the cells prior to drop formation. Cell breakage, obvious from the cell debris trapped in the inlet filter, is shown in Fig. 1, prevented an accurate measurement of cell viability.

To address this problem, we designed a microfluidic device consisting of three inlets with two flow-focusing junctions in series, as shown in Fig. 2. An aqueous alginate solution containing the cells is introduced into the rightmost inlet, labeled inlet 3 in Fig. 2a, and another solution containing the $CaCO_3$ nanoparticles is injected into the middle inlet. In the first junction, these fluids form a coaxial stream that extends into the second junction, where it is pinched off by oil into droplets, as shown in Fig. 2a, b. The nanoparticle-containing solution and the cell-containing solution have equal alginate concentrations; thus, mixing channels, which squeeze the drops and introduce further damage to the cells, are unnecessary.

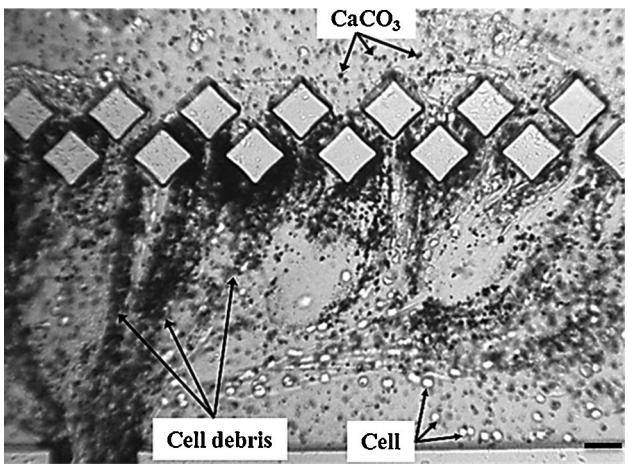

Fig. 1 Following the conventional internal gelation approach, a solution containing alginate, cells, and $CaCO_3$ nanoparticles was injected into a microfluidic device. Cells were damaged by contact with the $CaCO_3$ nanoparticles, resulting in the cell debris trapped in filter of the inlet channel of the microfluidic device. The *scale bar* is 50 μm

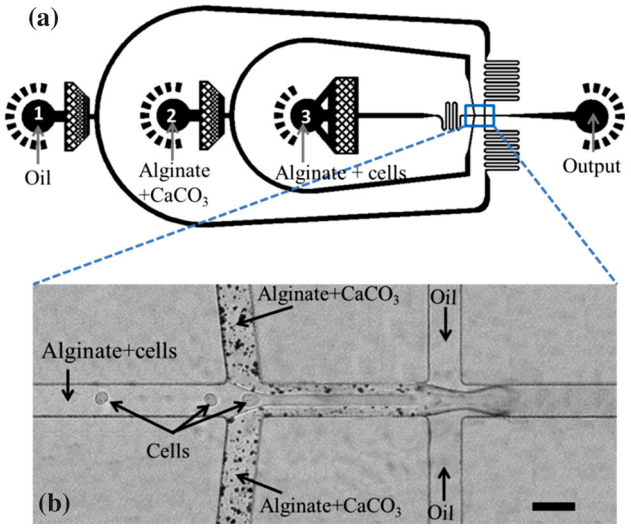

Fig. 2 **a** Schematic representation of the drop maker device with three inlets and two consecutive junctions. Aqueous alginate solutions containing $CaCO_3$ nanoparticles and cells are introduced into *inlets 2* and *3*, respectively, while the oil phase containing surfactant is injected into *inlet 1*. In the first junction, a coaxial stream consisting of the cell-containing inner fluid, surrounded by the nanoparticle-containing outer fluid, is formed. The coaxial stream extends into the second junction, where it is pinched off by the oil into droplets. **b** Bright-field image of the flow-focusing junctions. Prior to droplet formation, the cells are completely protected from direct mechanical contact with the $CaCO_3$ nanoparticles. The *scale bar* is 50 μm

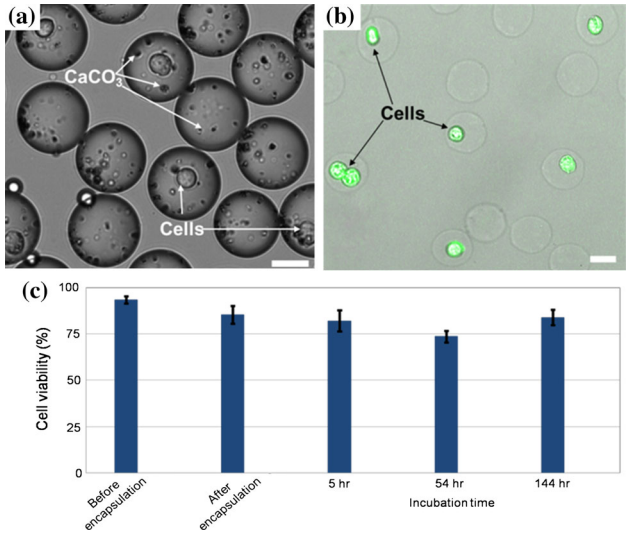

Fig. 3 **a** Bright-field image of 35-μm alginate droplets in oil, prior to gelation. Droplets contain cells and $CaCO_3$ nanoparticles. **b** Confocal microscopic image of the encapsulated cells inside the alginate microparticles after gelation and transfer to cell growth medium. *Green* fluorescence represents live cells stained by calcein AM. **c** Percentage viability of M6C cells determined from the fraction of live cells before and after encapsulation. The *scale bars* are 25 μm (color figure online)

To maintain the viability of the encapsulated cells, the gelation process must be precisely controlled. Previously reported techniques based on internal gelation utilize an acidic oil stream to release the Ca^{2+} ions of insoluble $CaCO_3$ particles and polymerize the alginate particles. This can result in prolonged exposure of the cells to low pH, reducing cell viability. To minimize the pH reduction in the particles during gelation, a stoichiometric equivalent of acetic acid with respect to the $CaCO_3$ is used, off-chip, after droplet collection. This amount is determined by: (1) calculating the molar quantity of $CaCO_3$ in the collected drops based on its initial concentration in alginate solution, the flow rates and the duration of drop collection; (2) determining the stoichiometric equivalent of acetic acid with respect to the moles of $CaCO_3$ using Eq. (1).

$$2CH_3COOH + CaCO_3 \rightarrow Ca(CH_3COO)_2 + H_2O + CO_2 \quad (1)$$

Finally, the acetic acid dissolved in oil (0.1 % v/v) is added to the drops causing gelation by diffusion into the droplets. After gelation, the surfactant is dissolved and the particles are washed three times with fresh cell growth medium to ensure all oil, and surfactant is removed and that optimal pH is restored. The gelation and washing steps are performed in 1–2 min.

To verify the effectiveness of this technique, we encapsulated antibody-secreting hybridoma cells (9E10 cell line) and mouse breast cancer cells (M6C cell line) within alginate microparticles. In Fig. 3a, droplets containing cells and $CaCO_3$ nanoparticles are shown in oil prior to gelation; in Fig. 3b, cell-containing microparticles are shown following their gelation and transfer to cell growth medium. To evaluate cell viability, the cells were stained with calcein AM (live stain, green) and ethidium homodimer (dead stain, red). A total of 84 % of 9E10 cells and 86 % of M6C cells were viable when assayed after the encapsulation and washing process. To test long-term viability, M6C cells were encapsulated into particles and incubated for 6 days, as shown in Fig. 3c. The viability decreases to 74 % at day 2 and increases to 84 % after 6 days due to cell division and growth.

Notably, MC6 cells continue to grow within these particles, forming multicellular colonies after 6 days of culture (Fig. 4). This feature is potentially very useful; for example, cancer cells might be grown into pseudo-tumors in these particles to provide a cost-effective tool to test new anti-cancer drugs.

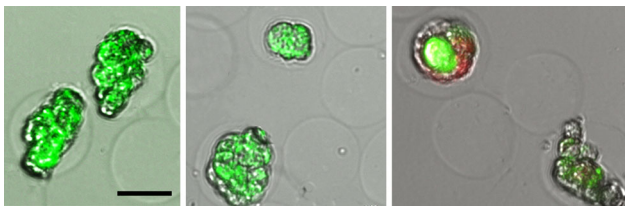

Fig. 4 Confocal microscopy images of growing M6C cells inside alginate microparticles after 6 days of incubation followed by staining with calcein AM (live stain, *green*) and ethidium homodimer (dead stain, *red*). It demonstrates that the encapsulated cells divide and grow inside the particles, eventually forming multicellular colonies that can stretch and deform their encapsulating microparticle. The *scale bars* are 25 μm (color figure online)

3 Conclusion

We introduced an effective microfluidic-based technique to encapsulate single cells in monodisperse alginate microparticles. Our method protects cells against mechanical and chemical harm during drop formation and gelation. The encapsulated cells have a high survival rate and are capable of proliferation inside the particles making the present method reliable and valuable for research applications such as cell transplantation, tissue engineering, and high-throughput cell analysis.

4 Materials and methods

4.1 Microfluidic device fabrication

The microfluidic device with channel dimensions of 20 μm in width and 25 μm in height was fabricated using soft lithography in polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning). To make the channels hydrophobic and prevent wetting, they were coated with Aquapel (PPG Industries, Pittsburgh, PA). Syringe pumps (Harvard Apparatus) were used to control the flow rates.

4.2 Preparation of fluids

Sterile alginate Pronova SLG100 (Novamatrix, Norway) was dissolved in 10 ml of cell growth medium containing 89 % v/v CO_2-independent DMEM (Gibco, Grand Island, NY), 10 % v/v FBS (Gibco), and 1 % v/v Penstrep (Gibco) to prepare 2.5 % w/w alginate solution. Based on the desired concentration of alginate microparticles, this solution was further diluted with cell growth medium and OptiPrep (Axis-shield, Norway). OptiPrep, typically used to prepare density gradients, is included at 16 % final concentration (v/v) to match the density of the alginate solution with that of the cells so that cells remain suspended during particle making. In the presented results, we have used two 1.5 % w/w alginate solutions: one containing the cells, and the other one with 70 mM calcium carbonate nanoparticles. We used the same flow rate for both of the solutions resulting in droplets of 35 mM $CaCO_3$ and 1.5 % w/w alginate. Fluorinated surfactant (Holtze et al. 2008) dissolved in Novec 7500 flurocarbon oil (1 % w/w) was used to stabilize droplets in oil phase and prevent drop coalescence.

4.3 Gelation and washing process

Prior to dropmaking, an acidic oil solution was prepared by dissolving 1 μl glacial acetic acid (Sigma, St. Louis, MO) in 1 ml oil (Novec 7500, 3M, St Paul, MN) resulting in 17.5 mM acidic oil solution. During dropmaking, droplets were collected every 20 min. The molar amount of calcium carbonate in the collected drops was calculated according to the flow rate of aqueous solution, and the stoichiometric equivalent of acetic acid dissolved in oil was added to the collected drops to initiate gelation. Then, the particles were gently pipetted up and down for 5 s to accelerate diffusion of the acid throughout the $CaCO_3$ nanoparticles, dissolving them and releasing Ca^{2+} ions. Next, the emulsion was centrifuged at 200 rpm for 3 s, and the oil phase was removed. Then, 1 ml of 20 % v/v of PFO (1H,1H,2H,2H-perfluoro-1-octanol 97 %, Sigma-Aldrich, USA) in Novec 7500 oil was added to release the surfactant from the particles. The mixture was centrifuged at 200 rpm for 3 s, and the oil/PFO phase was removed. The particles were then washed twice with fresh cell growth medium (centrifuging for 30 s at 300 rpm) to ensure that all acidic oil was removed. The particles resuspended in fresh growth medium and transferred to a 48-well plate and incubated at 37 °C and 5 % CO_2.

4.4 Viability measurement

We examined the cell viability using a Live–Dead assay kit, calcein (Invitrogen, Carlsbad, CA, live stain, green fluorescence) and ethidium homodimer-1 (Invitrogen, dead stain, red fluorescence). Four microliters of ethidium and 1 μl of 50 μM calcein are added to 1 ml of cell growth medium containing alginate particles encapsulating cells and incubated for 30 min in room temperature in dark.

Acknowledgments The authors thank Prof. D. A. Weitz, J. Heyman, A. Khavari, S. Utech, and R. Sperling for helpful discussions. S. Akbari, acknowledges the support of Prof. H. R. Shea and Swiss national foundation (Grant No. 200020-140394).

References

Capretto L, Mazzitelli S, Balestra C, Tosi A, Nastruzzi C (2008) Effect of the gelation process on the production of alginate microbeads by microfluidic chip technology. Lab Chip 8:617–621

Chachques JC, Trainini JC, Lago N, Masoli OH, Barisani JL, Cortes-Morichetti M, Schussler O, Carpentier A (2007) Myocardial assistance by grafting a new bioartificial upgraded myocardium (MAGNUM clinical trial): one year follow-up. Cell Transpl 16:927–934

Choi C-H, Jung J-H, Rhee Y, Kim D-P, Shim S-E, Lee C-S (2007) Generation of monodisperse alginate microbeads and in situ encapsulation of cell in microfluidic device. Biomed Microdevices 9:855–862

Drury JL, Mooney DJ (2003) Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials 24:4337–4351

Edd JF, Di Carlo D, Humphry KJ, Koster S, Irimia D, Weitz DA, Toner M (2008) Controlled encapsulation of single-cells into monodisperse picolitre drops. Lab Chip 8:1262–1264

Guo MT, Rotem A, Heyman JA, Weitz DA (2012) Droplet microfluidics for high-throughput biological assays. Lab Chip 12:2146–2155

Hoesli CA, Raghuram K, Kiang RLJ, Mocinecová D, Hu X, Johnson JD, Lacík I, Kieffer TJ, Piret JM (2011) Pancreatic cell immobilization in alginate beads produced by emulsion and internal gelation. Biotechnol Bioeng 108:424–434

Holtze C, Rowat AC, Agresti JJ, Hutchison JB, Angile FE, Schmitz CHJ, Koster S, Duan H, Humphry KJ, Scanga RA, Johnson JS, Pisignano D, Weitz DA (2008) Biocompatible surfactants for water-in-fluorocarbon emulsions. Lab Chip 8:1632–1639

Lee KY, Mooney DJ (2012) Alginate: properties and biomedical applications. Prog Polym Sci 37:106–126

Maguire T, Novik E, Schloss R, Yarmush M (2006) Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes. Biotechnol Bioeng 93:581–591

Martinez CJ, Kim JW, Ye C, Ortiz I, Rowat AC, Marquez M, Weitz D (2012) A microfluidic approach to encapsulate living cells in uniform alginate hydrogel microparticles. Macromol Biosci 12:946–951

Mazzitelli S, Borgatti M, Breveglieri G, Gambari R, Nastruzzi C (2011) Encapsulation of eukaryotic cells in alginate microparticles: cell signaling by TNF-alpha through capsular structure of cystic fibrosis cells. J Cell Commun Signal 5:157–165

Nicodemus GD, Bryant SJ (2008) Cell encapsulation in biodegradable hydrogels for tissue engineering applications. Tissue Eng Part B Rev 14:149–165

Pierigè F, Serafini S, Rossi L, Magnani M (2008) Cell-based drug delivery. Adv Drug Deliv Rev 60:286–295

Prakash S, Chang TMS (1996) Microencapsulated genetically engineered live *E. coli* DH5 cells administered orally to maintain normal plasma urea level in uremic rats. Nat Med 2:883–887

Sakai S, Mu C, Kawabata K, Hashimoto I, Kawakami K (2006) Biocompatibility of subsieve-size capsules versus conventional-size microcapsules. J Biomed Mater Res A 78A:394–398

Schmidt JJ, Rowley J, Kong HJ (2008) Hydrogels used for cell-based drug delivery. J Biomed Mater Res A 87A:1113–1122

Shintaku H, Kuwabara T, Kawano S, Suzuki T, Kanno I, Kotera H (2007) Micro cell encapsulation and its hydrogel-beads production using microfluidic device. Microsyst Technol 13:951–958

Slaughter BV, Khurshid SS, Fisher OZ, Khademhosseini A, Peppas NA (2009) Hydrogels in regenerative medicine. Adv Mater 21:3307–3329

Sugiura S, Oda T, Izumida Y, Aoyagi Y, Satake M, Ochiai A, Ohkohchi N, Nakajima M (2005) Size control of calcium alginate beads containing living cells using micro-nozzle array. Biomater 26:3327–3331

Sun Y, Ma X, Zhou D, Vacek I, Sun AM (1996) Normalization of diabetes in spontaneously diabetic cynomolgus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. J Clin Invest 98:1417–1422

Tan WH, Takeuchi S (2007) Monodisperse alginate hydrogel microbeads for cell encapsulation. Adv Mater 19:2696–2701

Teh S-Y, Lin R, Hung L-H, Lee AP (2008) Droplet microfluidics. Lab Chip 8:198–220

Um E, Lee D-S, Pyo H-B, Park J-K (2008) Continuous generation of hydrogel beads and encapsulation of biological materials using a microfluidic droplet-merging channel. Microfluid Nanofluid 5:541–549

Workman VL, Dunnett SB, Kille P, Palmer DD (2008) On-chip alginate microencapsulation of functional cells. Macromol Rapid Commun 29:165–170

Xu Q, Hashimoto M, Dang TT, Hoare T, Kohane DS, Whitesides GM, Langer R, Anderson DG (2009) Preparation of monodisperse biodegradable polymer microparticles using a microfluidic flow-focusing device for controlled drug delivery. Small 5:1575–1581

Yu J, Du KT, Fang Q, Gu Y, Mihardja SS, Sievers RE, Wu JC, Lee RJ (2010) The use of human mesenchymal stem cells encapsulated in RGD modified alginate microspheres in the repair of myocardial infarction in the rat. Biomaterials 31:7012–7020