Alginate beads

Production of highly monodispersed alginate beads for mammalian cell encapsulation

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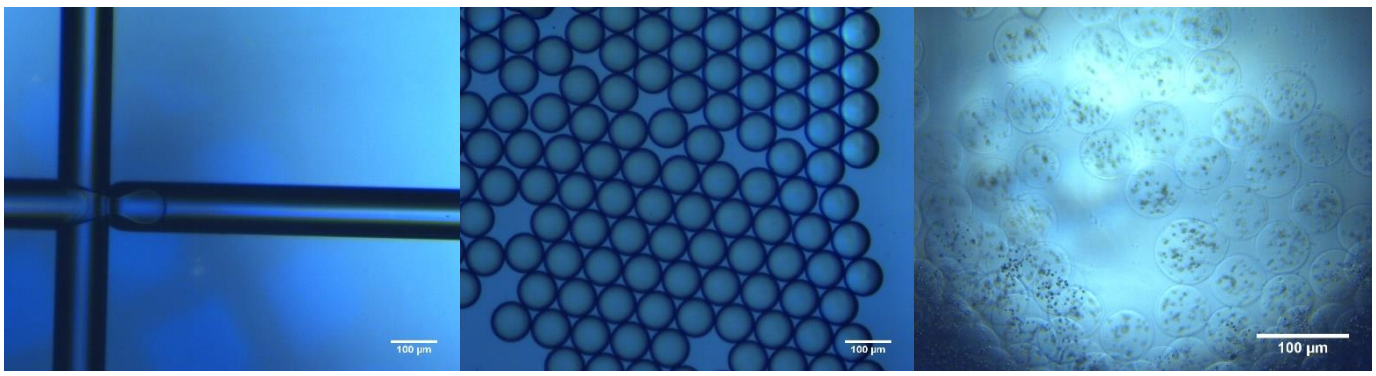

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1 Summary

This application note summarises the results for the encapsulation of mammalian cells in alginate beads using a single channel, 100 µm 3D pore flow focusing fluorophilic chip. 2 % (w/v) alginate was used to encapsulate mammalian cells. Cell viability was analysed using a nuclei dye (calcein) to visualise live cells. Different flow conditions were tested by altering the dispersed and continuous phase flow rates to achieve different droplet and bead size.

2 Introduction

Drug delivery, wound healing, cell and tissue culture all require inert biomaterials capable of catering to their different requirements. This makes naturally occurring anionic polysaccharide alginate a popular material for biomedical and pharmaceutical applications. Extracted from seaweed, alginate has low toxicity and is produced at relatively low costs.

Alginate has inherent structural similarities to extracellular matrices of living tissues making it a model system for mammalian cell culture and tissue engineering, i.e. organ and tissue replacement during organ or tissue failure. Another advantage of using alginate is its mild gelation requirements which can be performed using various methods. Mild gelation such as ionic crosslinking allows it to cater for its use with delicate actives such as proteins (Lee; & Mooney, 2013).

Alginate has been widely used in several industries for example: delivery of small chemical drugs, protein delivery since it minimises denaturation, wound dressings and cell culture. In each case a controlled reproducible product is crucial. A common method for producing alginate beads is extrusion, where a solution of alginate is extruded through a needle into a $CaCl_2$ bath to produce ionically crosslinked alginate beads. This however, produces large beads (several hundred micrometres) that have a wide size distribution and provide minimal control over droplet/bead size. Furthermore, resulting alginate beads are non-spherical with the tendency to fuse as droplets. The reproducibility and throughput of such methods is low, demanding the need for better techniques and control to produce alginate beads.

Dolomite Microfluidics offers such a platform to enable users to produce alginate beads with the desired size for their applications. Reusable glass chips enable the production of reproducible beads and allow real-time control to generate monodispersed droplets. Each droplet undergoes identical process conditions preventing fusion and product waste. Microfluidics approach for alginate bead production offers many advantages over conventional batch method synthesis.

The aim of the following work was to 1) produce monodispersed alginate beads 2) crosslink alginate droplets to produce monodispersed beads 3) identify parameters for high throughput 4) show biocompatibility by encapsulating live mammalian cells. The work carried out in this application note was adapted from Utech et al., 2015.

1. Lee;, K. Y., & Mooney, D. J. (2013). *Alginate : properties and biomedical applications*. *37*(1), 106–126. https://doi.org/10.1016/j.progpolymsci.2011.06.003.Alginate

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

3 Methods

3.1 Droplet system

Microfluidic droplet system (figure 1) uses pulseless Dolomite Microfluidics P-Pumps and can be driven from a PC via Dolomite Microfluidics Flow Control Centre (FCC) software. 100 µm 3D flow focusing fluorophillic microfluidic chip (Figure 3) connected via ID 0.25 mm, OD 1.6 mm FEP tubing was used throughout, manual valves were added in line for ease of operation. For the continuous phase (Q_c) a T-connector was used to deliver Fluosurf in HFE to the chip through the two lateral chip channels (Figure 3). The H interface (holds the chip in place) and the linear connector together seal the space between tubing and chip to prevent leakage and gas bubbles. All visualisation was possible through a Dolomite high speed digital microscope connected to a PC. The experimental setup is shown in figure 1 (for a list of all components see appendix).

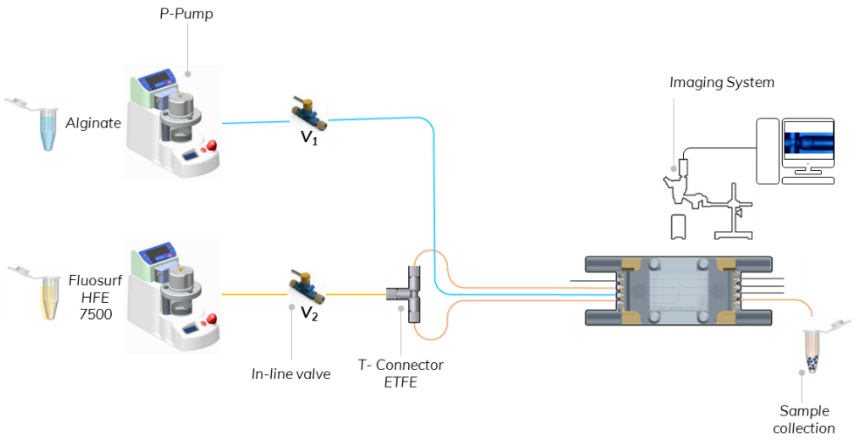


Figure 1: Schematic of the droplet system to produce 2 % (w/v) alginate droplets in 1 % (w/v) Fluosurf HFE 7500.

3.2 Materials

The dispersed phase (Q_d) 2 % (w/v) Alginate (Sigma Aldrich, U.K.), 0.1 M Calcium-EDTA (Sigma Aldrich, U.K.) were dissolved in deionised water (dH_2O). For the continuous phase (Q_c) 1 % (v/v) Fluosurf in HFE- 7500 (Dolomite Microfluidics, U.K.) was used. The collection phase comprised on 0.5 % (v/v) acetic acid (Sigma Aldrich U.K.) in the continuous phase. Fluoro-Stop (Sigma Aldrich U.K.) was used to break the emulsion, post crosslinking. Phosphate buffer saline (PBS) was used to suspended cells and beads.

A mixture of NIH/3T3 (ATCC® CRl-1658™) and HEK-293 (ATCC® CRl-1573™) at 10^6 cells mL^{-1} were stained using a calcein-AM assay (Thermo Scientific U.K.) for 5 minutes prior to encapsulation in alginate. Pelleted cells were resuspended in 2 % (w/v) alginate and checked for cell viability (Figure 3a).

3.3 Forming alginate droplets

All fluids used (Fluosurf, alginate, cell buffer) were pre-filtered using a 0.2 µm (Millipore U.K.) syringe filter prior to loading onto the system. The continuous phase pump was loaded with 1 % (v/v) Fluosurf in HFE- 7500 and the dispersed phase pump with 2 % (w/v) Alginate with 0.1 M Calcium-EDTA. The system was primed by running both fluids at 2 bar until liquid appeared at the linear connector (note: the tubing is not yet connected to the chip). Once this occurs (within seconds) close the valves and stop the pumps. The connector was then attached to the chip via the H-interface. The continuous phase (Q_c) was started first then the dispersed phase (Q_d) flow according to the flow rates listed in table 1.

The droplets were collected in a 200 µl solution of the continuous phase with 0.5 % (v/v) acetic acid. The experiment ran for 15 minutes or until enough material was collected for further analyses. Once the droplets were collected, 2 minutes of crosslinking time was allowed (mechanism of crosslinking is described in figure 2) before an emulsion breaker was added. Fluoro-Stop (Dolomite Microfluidics, U.K.) was added to the emulsion (2 to 3 times the volume

of the emulsion), the tube was gently inverted several times to create a good mix. The tube was left stationary to allow phase separation, sedimented oil was carefully pipetted out, leaving alginate beads. 100 µl of PBS solution was added to resuspend the beads, this was then gently centrifuged at 300 x g for 1 minute and the PBS pipetted off (note: washing step with PBS can be repeated several times to eliminate traces of oil). Finally, the beads were suspended in 100 µl of PBS and imaged using a microscope.

3.4 Cell encapsulation

Calcein dyed mammalian cells pelleted in PBS were resuspended in the dispersed phase to achieve a final working concentration of 10^6 cells mL^{-1}. The experiment was carried out as described above in Section *3.3*. After the final washing step and resuspending in PBS the cell viability was examined using a microscope with a fluorescent filter.

3.5 Cleaning the chip

Once the experiment had finished, the continuous phase was replaced with HFE only and the dispersed phase with water. The system was run until all traces of alginate in the system had been eliminated. Next, the system was rinsed with 100 % isopropanol at 2 bar and finally air. The chip was stored in an air tight container or zip lock bag away from dust and particles.

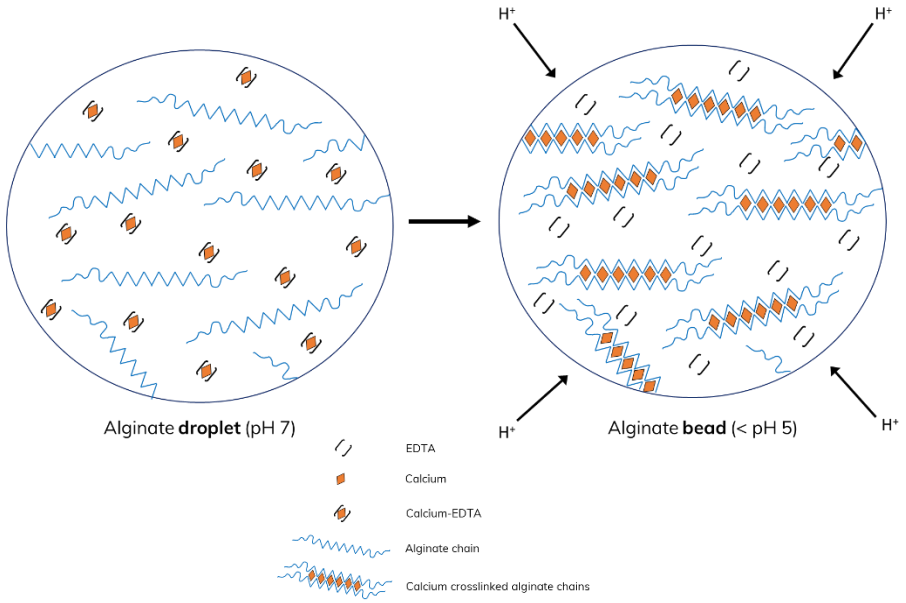


Figure 2: Mechanism of alginate crosslinking using 0.5 % (v/v) acetic acid in collection phase (1 % (v/v/) Fluosurf in HFE 7500). At low pH the Calcium-EDTA complex dissociates and releases Ca^{2+} ions to crosslink alginate chains.

4 Results

Firstly, a stable production of alginate droplets in the chip (Figure 3) was established by starting with low flow rates and slowly increasing to the desired drop size/ flow rate. For this experiment 2 % alginate was tested, this is an approximate upper limit of the alginate concentration which can successfully make beads without encountering issues with viscosity such as jetting. Lower alginate concentrations (0.5 % and 1 % were also successfully tested- data not shown). 2 % alginate is commonly used for biomedical applications.

The results in table 1 show the process parameters to achieve a certain droplet diameter and the respective particle diameter. The table also enlists the parameters for achieving the highest throughput for a given drop or bead size. Respective images of the drop and beads are shown in table 2. Post-crosslinking and washing the beads remain suspended in water- for better visualisation, a dye could be used. The beads produced post gelation are soft and preserve their shape. The beads retain their shape when compressed between two glass slides without external force. The highest production rate without jetting was achieved at Q_c 20 µl/min and Q_d 20 µl/min generating droplets of 113 µm and beads of 119 µm in diameter.

Calcein treated mammalian cells suspended in alginate prior to encapsulation (figure 4a) did not show loss of viability. Post encapsulation and washing, cells in alginate beads could be seen (figure 4b) and they remained viable (figure 4c).

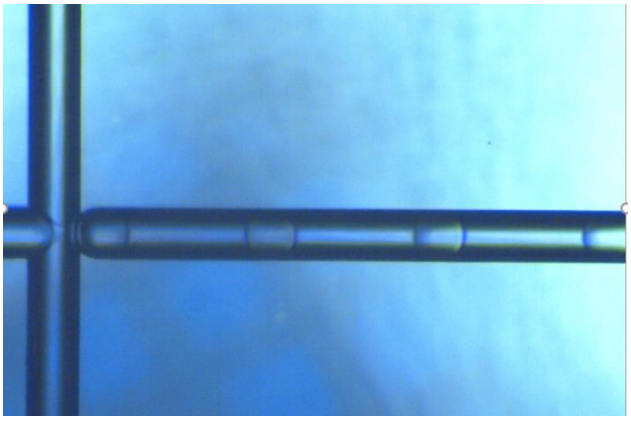
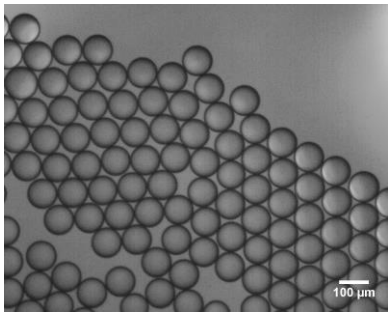
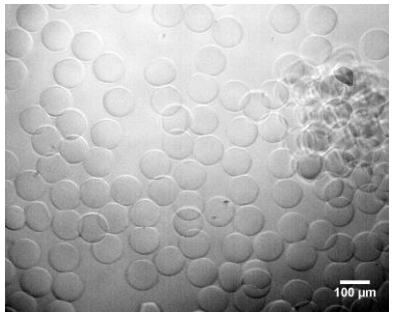
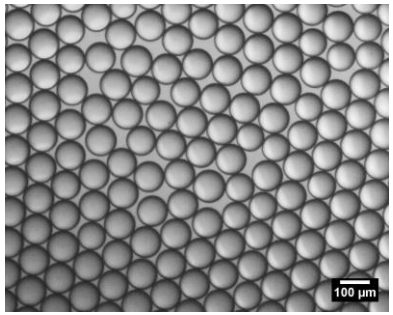


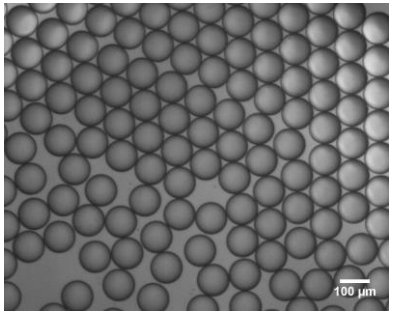
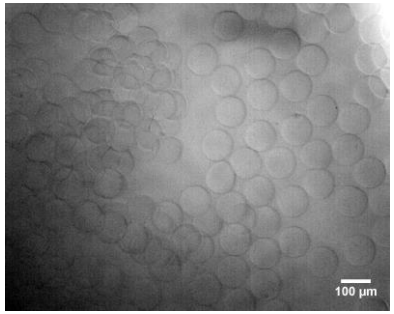
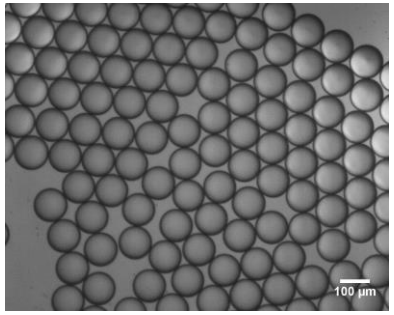
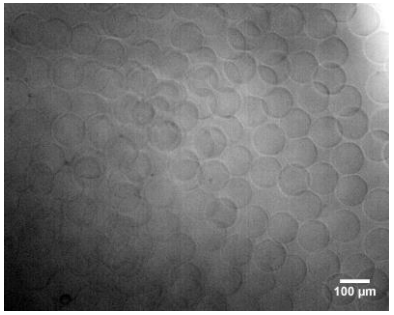
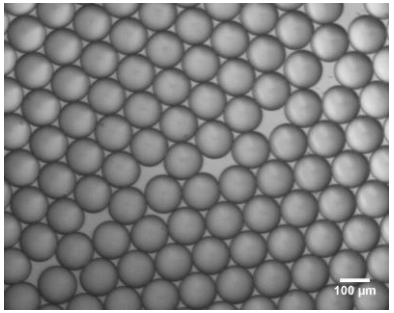
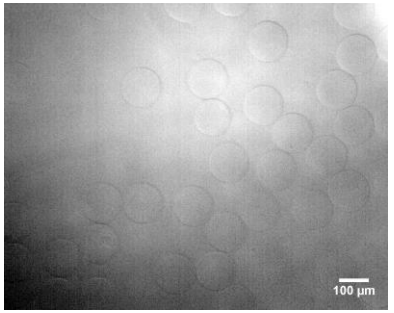
Figure 3: 100 µm 3D flow focusing fluorophilic single junction chip (3200515) showing formation of alginate droplets

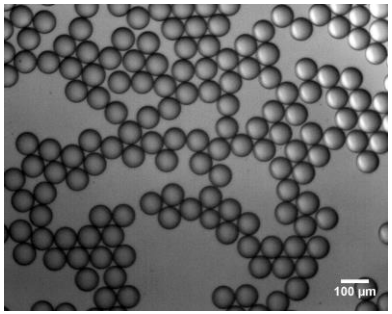
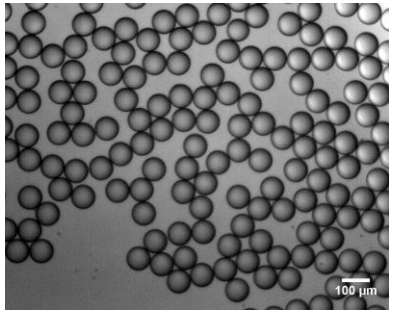
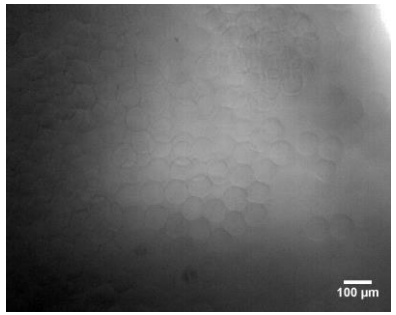
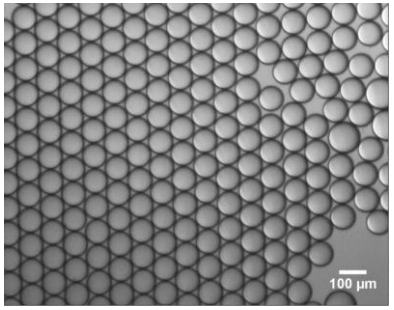
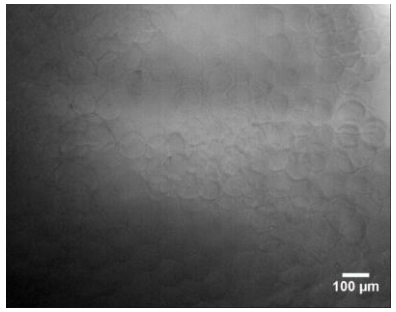
Table 1: Operating parameters to produce alginate droplets and particles. '- 'denotes data not available.

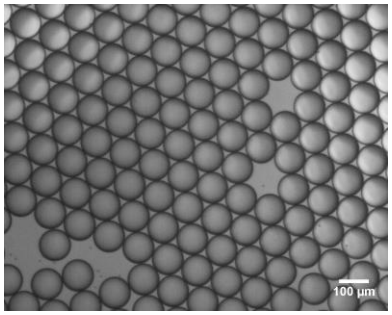
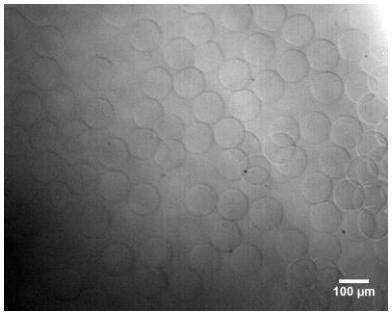
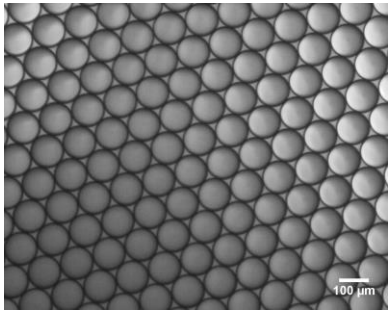
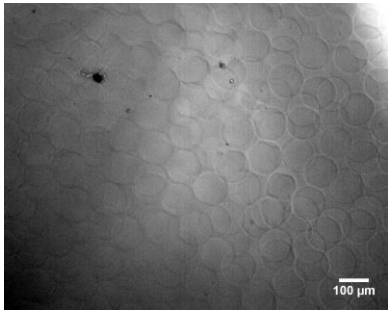
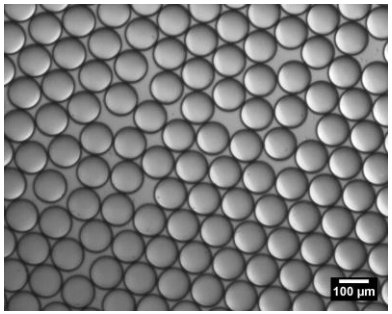
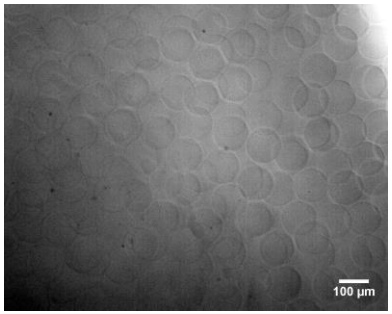
Q_d Flow rate (µl/min)	Pressure (mbar)	Q_c Flow rate (µl/min)	Pressure (mbar)	Frequency (Hz)	Drop volume (µl)	Droplet diameter (µm)	Coefficient of variance (CV %)	Particle diameter (µm)	Coefficient of variance (CV %)
2	117	10	110	74	0.00045	95	2	101	2
3	181	10	130	105	0.00048	97	3	-	-
4	243	10	144	120	0.00056	102	1	104	2
6	348	10	168	165	0.00061	105	1	107	1
10	559	10	217	180	0.00093	121	1	134	1
4	320	20	228	279	0.00024	77	2	-	-
6	386	20	243	373	0.00027	80	1	87	3
8	497	20	256	387	0.00034	87	1	97	3
12	533	20	280	360	0.00056	102	1	107	2
15	672	20	298	379	0.00066	108	0	111	2
18	930	20	335	442	0.00068	109	1	115	2
20	997	20	348	441	0.00076	113	1	119	2

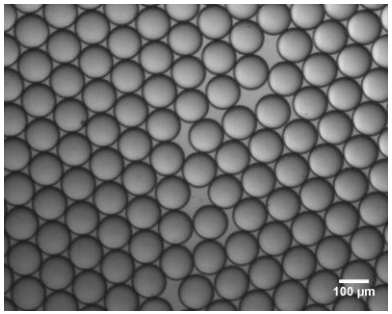
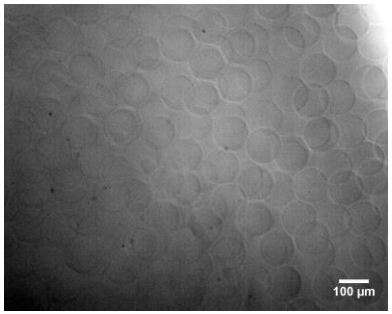
Table 2: Pictures of droplets and particles produced at varying flow rates and pressure

Q_d Flow rate (µl/min)	Q_c Flow rate (µl/min)	Droplet diameter (µm)	Particle diameter (µm)	Droplets	Particles
2	10	95	101		
3	10	97	-		-

4	10	102	104		
6	10	105	107		
10	10	121	134		

4	20	77	-		-
6	20	80	87		
8	20	87	97		

12	20	102	107		
15	20	108	111		
18	20	109	115		

20	20	113	119		

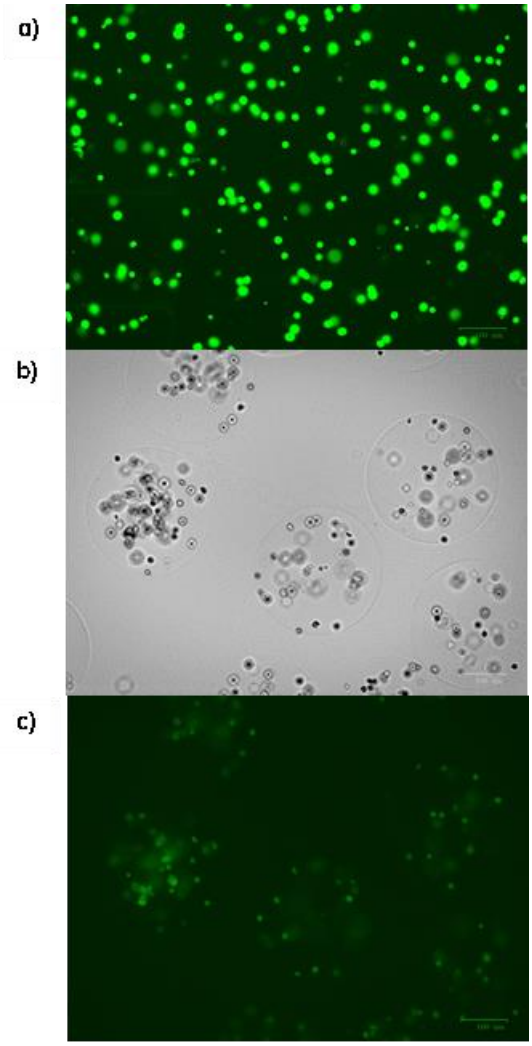


Figure 4: a) live mammalian cells before encapsulation; b) cells encapsulated in 2 % alginate beads; c) live mammalian cells encapsulated in 2 % alginate beads post washing.

5 Conclusions

2 % (w/v) alginate droplets were produce using a 100 µm 3D pore flurophilic chip. Upon collection the droplets were crosslinked and allowed to cure for a period of 2 minutes before breaking the emulsion and washing the beads with PBS. Cells were stained with calcein prior to encapsulation in the presence of alginate. This was to validate the live status and the impact of alginate on cell viability. Stained cells were encapsulated, and downstream processing carried out before they were analysed for loss of viability. Upon investigation it was concluded that alginate encapsulation and the downstream washing and crosslinking did not harm the mammalian cells. Live cells were visible in the alginate beads.

Due to the porous alginate structure, it is therefore possible to grow cells in beads. Nutrients and gas can readily be exchanged via the nanopores in the bead structure. Hence, this method can easily be adapted for creating microreactors and or other sensitive active agents requiring mild gelation and encapsulation.