The use of microfluidics to encapsulate therapeutic cells

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Introduction

Microfluidics is the science of handling small volumes of fluid (liquid or gas). In a microfluidic device, immiscible fluids form segmented flows, which are often described as linear-emulsions. Such emulsions (formed in microfluidic channels) are highly monodisperse (Anna et al. 2003), unlike those formed *via* traditional emulsification methods, which typically have a large droplet size distribution (Poncelet et al. 1992). Fluid droplets containing polymers such as alginate can undergo chemical reaction within the microfluidic device to produce equally monodisperse, microspherical beads. As it is easier to manipulate and precisely regulate individual droplets properties using microfluidic circuits, reaction times and cell-reagent exposure times can be strictly controlled. In addition, beads, and thus biological molecules/cells within them, are not exposed to toxic substances or high shear environments.

Microfluidics has previously been used to produce beads from the naturally-occurring polysaccharide alginate, with varying success. Most successful methods use microfluidic circuits merely as an alternative emulsion production technique, allowing droplets produced to fall into setting baths as in traditional bead generation methods (Huang et al. 2006, 2007). Two groups have recently reported the synthesis of alginate microspheres *via* similar microfluidics-based droplet generation methods containing flow-focusing fluidic junctions. Zhang et al. (2007) have developed a microfluidic method for cross-linking alginate droplets on-chip by suspending calcium acetate in the continuous phase. Calcium acetate dissolves upon contact with the surface of the aqueous alginate-containing dispersed phase, inducing ionotropic cross-linking to produce externally gelled microspheres. Zhang and co-workers attempted to adapt Poncelet's (1992) method for internal gelation emulsification, involving an aqueous suspension of calcium carbonate and an acid-containing continuous phase, to their microfluidic platform. However, they reported some difficulties in achieving solid beads.

Huang et al. (2007) report a stable segmented flow system comprising an alginate-calcium carbonate dispersed phase and an inert continuous phase. This microfluidic device enabled the generation of droplets, which were subsequently cross-linked off chip in an acid-containing setting bath. Neither group has reported the successful use of a microfluidic device for the encapsulation of living cells within the resultant microspheres. Here we present a successful microfluidic method for encapsulating a therapeutically active cell line.

Material and methods

Pronova UltraPure, medium viscosity, high mannuronate (UP MVM) alginate was purchased from NovaMatrix™ (Drammen, Norway). Microcrystalline precipitated calcium carbonate, with an average particle size of $0.07\mu m$ was a gift from Specialty Minerals (Birmingham, UK). High oleic sunflower oil was purchased from Statfold (Stafordshire, UK). All cell culture materials were purchased from Invitrogen (Paisley, UK). Dopamine ELISA was purchased from Oxford Biosystems (Oxford, UK). PC12 cells were a kind gift from Dr Jack Ham (Cardiff University, UK).

Cell encapsulation experiments were carried out as previously described (Workman et al. 2007, 2008) briefly, all microfluidic apparatus was autoclaved and then air dried in an oven set to 50˚C. Alginate (2% w/v) and calcium carbonate (0.5% w/v) were suspended in D-MEM/F12 medium without serum at 37ºC. Cells were scraped from flasks, washed, counted and then resuspended in alginate-calcium carbonate mixture at varying concentrations. The alginate was segmented using sunflower oil as a shielding flow and sunflower oil containing acetic acid (0.5% v/v) as the reactive fluid. Upon emergence from the encapsulation apparatus the beads were collected in D-MEM/F12 medium and then maintained in cell culture flasks containing D-MEM/F12 supplemented with 5% fetal calf serum, 10% heat-inactivated horse serum and penicillin-streptomycin.

Viability was assessed by placing single beads on each half of a haemocytometer. After medium was removed sodium citrate solution (55mM) was added to each bead followed by trypan blue. A coverslip was placed over the beads and gentle pressure applied to squash the beads. Cell and viability counts were carried out according to literature precedent (Strober, 2001).

PC12 containing beads (50) maintained for 14 days were incubated with 600µl HBSS (basal) or 600µl HBSS with 100mM KCl (induced) for 45mins. Medium was subsequently collected and frozen. Dopamine was extracted and detected as per manufacturers' instructions.

Results and Discussion

A novel method was developed to allow cell encapsulation *via* a microfluidic platform (Workman et al. 2007, 2008). This involved adapting an emulsion-based, internal gelation procedure (Poncelet et al. 1992). Aqueous sodium alginate mixed with $CaCO_3$ and cells is introduced into the central channel (Figure 1A). Sunflower oil mixed with acetic acid is supplied to the outermost channels (Figure 1C). Sunflower oil is supplied to the intermediate channels (Figure 1B) to act as a shield preventing the alginate solution from coming into contact with the acidified oil flow. If this were not present immediate crosslinking of alginate would occur at the junction, thus preventing subsequent flow. Between channels B and A the two oils flow in a laminar fashion, with minimal diffusion of H^+ into the protective sunflower oil. After droplet formation at the junction, H^+ diffuses into the alginate droplet, thus liberating Ca^{2+} from $CaCO_3$, which causes gelation of the alginate.
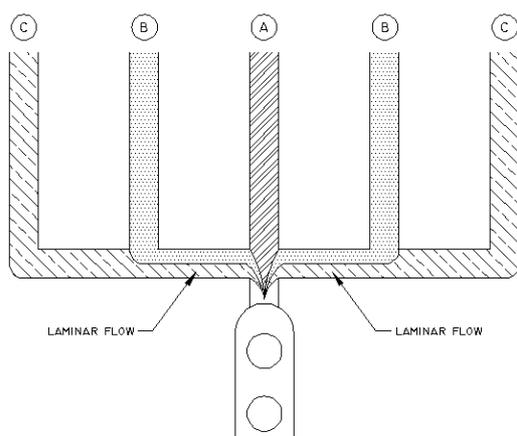

Figure 1: A representation of the shielded junction employed to generate alginate microspheres.
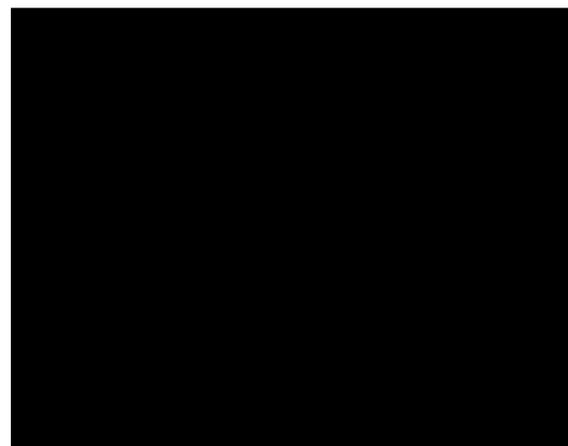

Figure 2: Light microscope image of encapsulated PC12 cells, showing small cell clumps present after encapsulation. Scale bar = 500µm

PC12 cells were successfully encapsulated using this method (Figure 2). In initial experiments a cell concentration of 1×10^7 cells per ml was employed. Beads with a diameter of 500μm were produced, containing ~200 cells per bead. No decrease of cell viability was observed after encapsulation and a viability of 80% was maintained for 50 days in culture (data not shown). Although viability was high, cells did not proliferate to greatly increase in number. To investigate the effect of initial cell concentration on cell proliferation, initial cell concentrations of 1×10^7 (low), 2×10^7 (medium) and 4×10^7 (high) cells per ml were encapsulated. Again viability remained high and consistent over time (Figure 3). Although all three types of beads showed cell proliferation (Figure 4), higher initial concentrations of cells allowed increased cell expansion.
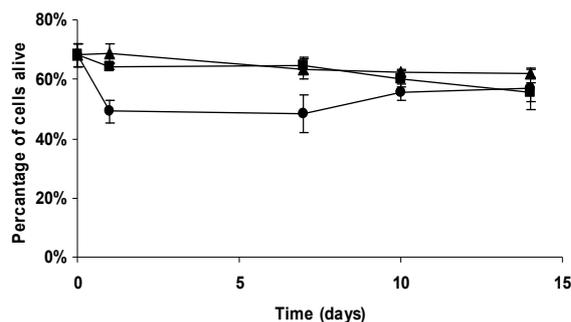

Figure 3: Percentage of live cells after encapsulation. Initial cell concentrations of 1×10^7(●), 2×10^7(■) and 4×10^7(▲) cells per ml were encapsulated.
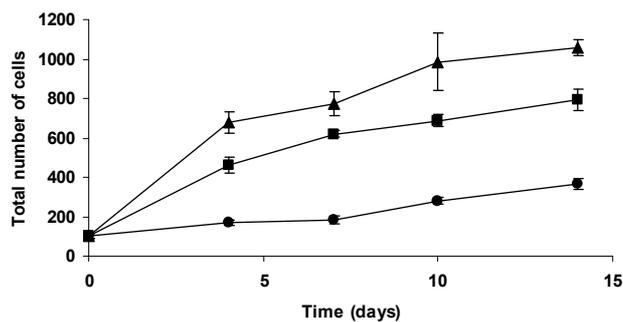

Figure 4: Total number of cells per bead after encapsulation. Initial cell concentrations of 1×10^7(●), 2×10^7(■) and 4×10^7(▲) cells per ml were encapsulated.

After 14 days in culture, dopamine expression from these encapsulated PC12 cells was investigated. Beads from low and medium initial cell concentrations had basal dopamine expression of 1.5 pg/bead (Figure 5). Beads from high initial cell concentration had basal dopamine expression of 2.4 pg/bead. After potassium induction all bead types showed an increase in dopamine expression with increasing amounts of dopamine being expressed with more PC12 cells being present.
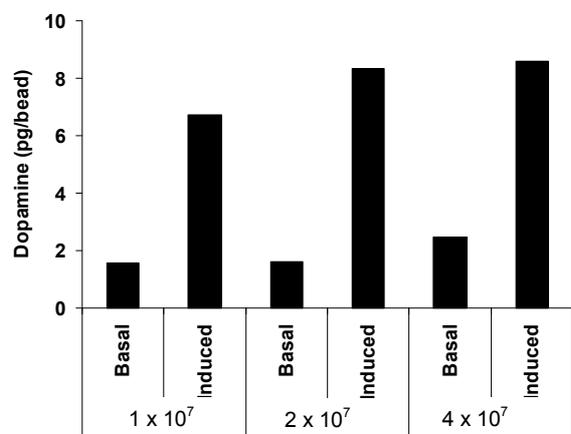
Figure 5: Amount of dopamine expressed per bead for three different initial encapsulation concentrations.
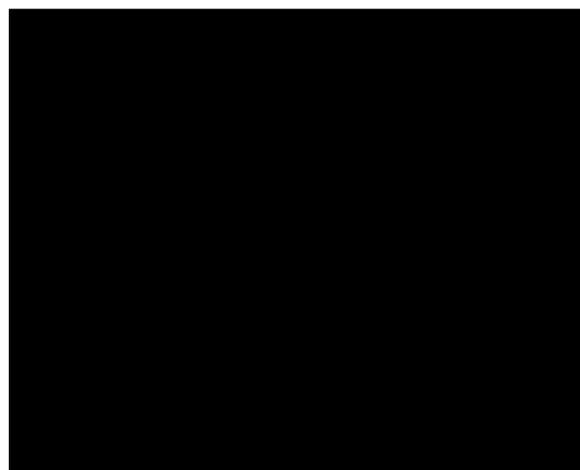
Figure 6: Cresyl violet stained histological section showing alginate beads present 15 days after implantation. Scale bar=100μm

Alginate beads produced using the presented microfluidic method were stabilized using 5mM BaCl$_2$. These beads (3μl) were injected into rat striatum. At various time points histology of rat brain was carried out. Barium stabilized beads could be observed up to 15 days after implantation (Figure 6).

Conclusions

The work presented here illustrates the use of a microfluidic method to produce alginate beads. Therapeutically active cells have been encapsulated using this method. Not only are cells shown to be viable and to proliferate within the produced beads, but they have been shown to express dopamine, both basally and upon potassium induction. Although preliminary implantation experiments show that microspheres are only stable for 15 days, further work in this area may produce microspheres which persist in the brain for longer time periods.

The ultimate goal of the current study is to introduce microspheres containing PC12 cells, expressing dopamine, into a murine model of Parkinson's disease. The hypothesis being that the dopamine produced would rescue the Parkinson's phenotype and allow behaviour to return to near normal.

Acknowledgements

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