Microfluidic chip-based synthesis of alginate microspheres for encapsulation of immortalized human cells

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Cellular transplantation is a promising technology with great clinical potential in regenerative medicine and disease management. However, effective control over patient immunological response is essential. The encapsulation of cells within hydrogel microspheres is an increasingly prevalent method for the protection of cellular grafts from immune rejection. Microfluidic “chip” reactors present elegant solutions to several capsule generation issues, including the requirement for inter-capsule uniformity, high reproducibility, and sterile, good manufacturing practice compliance. This study presents a novel method for the *on-chip* production of stable, highly monodisperse alginate microspheres and demonstrates its utility in the encapsulation of an immortalized human-derived cell line. Four populations of immortalized human embryonic kidney cells (HEK293) were encapsulated *on chip* within monodisperse alginate capsules. Cell viability measurements were recorded for each of the four encapsulated populations for 90 days. © 2007 American Institute of Physics. [DOI: [10.1063/1.2431860](https://doi.org/10.1063/1.2431860)]

I. INTRODUCTION

The term “cell therapy” is now widely used to describe the process of transplanting nonautologous cells for therapeutic purposes. The scope of cellular therapy continues to broaden, and now extends into many areas of regenerative medicine, involving stem cells,¹ bone augmentation,² neuron stimulation,³ and artificial skin.⁴ The implantation of stable, allogeneic or xenogeneic cells, which are engineered to continually secrete functional molecules, is a highly promising approach to disease treatment. The simplest and most widely investigated approach to date has been the encapsulation of cells within cross-linked natural polysaccharide hydrogels such as alginate (a linear block copolymer of *D*-mannuronic acid and *L*-guluronic acid). Calcium-linked alginate hydrogels have been extensively tried and tested, and in many cases have given promising results in immuno-isolation in animals.^{5,6}

The prospect of long-term therapies for diseases such as Type I diabetes has attracted widespread interest. The World Health Organization (WHO) estimates that more than 180 million people worldwide suffer from diabetes, and this number is predicted to more than double by 2030.⁷ The National Diabetes Information Clearinghouse estimates that diabetes costs \$132 billion in the United States alone every year. The prospect of replacement of insulin-producing pancreatic

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islet beta cells has been described as “the ultimate treatment for Type I diabetes.”⁸ Clearly, the market for cell therapy is massive. Recently, the first clinical trials in nonimmunosuppressed human patients were undertaken.⁹ Preliminary data shows that two patients responded to Ca-alginate encapsulated islet grafts, although exogenous insulin was only decreased and not withdrawn completely.

There are many reasons why this considerable potential has not (yet) been fully realized, only some of which are discussed here. Cell-containing microspheres are often of a large diameter ($>500\text{ }\mu\text{m}$), with a broad size distribution. This means that nutrient diffusion to the center of the microspheres becomes an issue,¹⁰ and standardization of cell number per microsphere is difficult. There is also an inability to produce clinical-scale volumes of cell-containing microspheres. Good manufacturing practice (GMP) compliance becomes paramount when producing microspheres for use in clinical applications.

In response to this, the use of microfluidics is ideally suited to production of cell-containing microspheres. Linear emulsions can easily be produced in a sealed microenvironment, utilizing the phenomenon of segmented flow.¹¹ The diameter of the product microspheres can be accurately controlled, with size distributions of $<1\%$ coefficient of variance. Microfluidic devices containing flow-focusing junctions have previously been used as effective droplet generation systems. However, in most reports, alginate droplets thus formed are crosslinked off-chip, falling from the microfluidic chip into a setting bath of calcium ions.¹² Using such an approach, both standardization and sterility are difficult to achieve. Zhang *et al.*¹³ have succeeded in curing alginate droplets *in situ*, in a microfluidic device, using undecanol as a solvent for calcium ions. This methodology was used to demonstrate the encapsulation of polystyrene microbeads. However, cells were not encapsulated using this device. A method similar to that of Zhang *et al.* was previously employed in our laboratory, where a solution of calcium cyclohexanecarboxylate was dissolved in octanol. Solid alginate microspheres were successfully produced via this method (unpublished observations).

The primary objective of this work is to simplify and further develop the encapsulation and immunoisolation of live, therapeutically-active cells. It is therefore clear that the encapsulation process should not have any detrimental effect on the cellular payload. In our preliminary studies, aliphatic alcohols such as octanol were found to have an extremely cytotoxic effect on cultured human embryonic kidney cells (HEK293) and primary murine hepatocytes. This encapsulation method was therefore abandoned. In this article we will report the successful encapsulation of cells using an internal gelation method adapted for a microfluidic reactor.

II. MATERIALS AND METHODS

A. Microsphere formation

The microfluidic device consists of a 316 stainless steel manifold into which high performance liquid chromatography fluid connectors are introduced equatorially. Vertical through holes were sealed with nitrile rubber O rings, allowing fluid to flow to the top surface of a virgin polytetrafluoroethylene (PTFE) disc ($50\times 3\text{ mm}$) located on the manifold. A circular polyfluoroalkoxy polymer film gasket was placed in between the PTFE chip and a borosilicate glass disc ($50\times 5\text{ mm}$) cover. A 316 stainless steel clamping piece was bolted to the fluidic manifold, allowing the entire laminated assembly to be compression sealed. Microfluidic channels were machined into PTFE discs (Polyflon, Staffordshire, UK) using a Computer Numerical Controlled milling machine (Roland, Swansea, UK). A series of microfluidic “chips” was produced with varying channel dimensions. In one example, the fluidic input channels were of the dimension $500\text{ }\mu\text{m}^2$, opening out to $1000\text{ }\mu\text{m}^2$ in the sphere forming channel. This cross-sectional ratio (1:4) was conserved throughout the scalar series of fluidic chips.

Fluids were introduced into the microfluidic circuit using syringe drivers (KD Scientific—Linton Instrumentation, Norfolk, UK). In all cell encapsulation experiments, the stainless steel rig, glass, gasket, fittings, and PTFE chip were autoclaved and then air dried in an oven at $50\text{ }^{\circ}\text{C}$. Syringe drivers were cleaned with bactericidal wipes before being placed in a Class II hood to maintain sterility. All experiments were carried out in a Class II hood.

Nanocrystalline precipitated CaCO_3 , with an average particle size of 70 nm (donated by Speciality Minerals, Birmingham, UK) was suspended in either water or D-MEM/F12 medium (Invitrogen, Paisley, UK) at a concentration of 0.5% (w/v). Pronova UP MVM alginate (NovaMatrix™, Drammen, Norway) was added to the CaCO_3 suspension at a final concentration of 2% (w/v). The carrier phase contained sunflower oil (Statfold, Staffordshire, UK) and sunflower oil containing 0.5% acetic acid (v/v; Sigma, Dorset, UK). All cell encapsulation experiments used the human embryonic kidney cell line HEK 293 (American Type Culture Collection CRL-1573). These were resuspended in the alginate/ CaCO_3 mixture at a concentration of 1×10^6 cells/ml.

B. Cell culture

HEK293 cells and cell-containing microspheres were cultured in complete medium consisting of Dulbecco's modified Eagle medium (Invitrogen, Paisley, UK) with 10% fetal bovine serum (Autogen Bioclear, Wiltshire, UK) in an atmosphere of 5% CO_2 /95% air at 37 °C. Cells were dissociated using 0.02% (w/v) trypsin/0.05% (w/v) EDTA in PBS (Invitrogen, Paisley, UK).

C. Size measurement

Microsphere radii were measured using a light microscope (Motic, Suffolk, UK) with an attached camera. The coefficient of variance was calculated as the standard deviation of the derived diameters of microspheres as a percentage of mean diameter.

D. Viability measurement

Direct cell counts of individual microspheres were carried out using an adapted trypan blue exclusion method. Individual microspheres were placed on a haemocytometer and all excess medium removed. Alginate microspheres were dissolved by addition of 55 mM sodium citrate (Sigma, Dorset, UK). Cell viability (%) was subsequently determined by trypan blue exclusion.

E. Confocal microscopy

The viability of encapsulated cells was determined using live/dead viability/cytotoxicity kit for mammalian cells (Molecular Probes), according to the manufacturer's instructions. Briefly, the gels were incubated in 1 mL of Hank's buffered salt solution containing 2 μM calcein stain and 4 μM of ethidium homodimer-1 for 15 min at room temperature in the dark. Subsequently, the distribution of red (dead) and green (live) cells was visualized using a fluorescent microscope (Leica DM6000B upright microscope) at 20 $\times$.

III. RESULTS

A. Microfluidic method for microsphere formation

The heterogeneous process, first developed by Poncelet *et al.*,¹⁴ involving an insoluble calcium salt (CaCO_3), was considered to be the most applicable to a microfluidic flow reaction.

A mixture of nanocrystalline calcium carbonate and sodium alginate solution was used as the *functional phase* in the microfluidic reaction. Alginate droplets containing CaCO_3 were produced *via* segmentation with an immiscible hydrocarbon. Acetic acid in the reactive carrier phase reacted immediately with the immobile crystals of calcium carbonate (present at the interface between the droplets' surface and the organic phase), producing Ca^{2+} , H_2O , and CO_2 . The free Ca^{2+} ions were sequestered by the dissolved alginate chains and produced the desired ionotropic hydrogel.

The reaction between H^+ and calcium carbonate in the aqueous phase is fast, and resulted in rapid Ca^{2+} release and subsequent gelation. In the microfluidic device, the first point of contact for these two reagents is the junction. Consequently, as an aqueous fluid packet began to emerge from the junction, its interface immediately underwent reaction and crosslinking. As a result, a thin hydrogel skin formed across the junction (Fig. 1). The presence of this hydrogel solid caused the

FIG. 1. Schematic showing immediate interfacial hydrogel formation in a crossroads fluidic junction. The calcium carbonate in the functional fluid (shown in blue) reacts rapidly with the acidified oil. The Ca^{2+} ions thus released a form of solid hydrogel at the junction.

fluid flows to decelerate, and quickly produced further solid masses of alginate gel within the microfluidic channel. These eventually occluded the channel, thereby terminating the microsphere synthesis experiment.

B. Shielded segmented flow

To remedy the problem of immediate gelation, it was postulated that a two-component laminar flow could be used as the *carrier phase* stream. At the micron scale, turbulent fluid flow, which is responsible for fluid mixing, is constrained and indeed replaced by laminar flow. Under these conditions, fluids move in planes and with the exception of slow interfacial diffusion, do not undergo mixing. Therefore, it is possible to create a laminar flow of two *miscible* liquids, and maintain separation of the two for some considerable time.

The microfluidic device and junction was enhanced to include an additional fluid flow, into which pure sunflower oil was introduced. Hence, the carrier phase can be thought of as a reactive stream of acidified sunflower oil, containing a *stripe* of inert, unmodified sunflower oil; hereafter referred to as the *shielding flow* (Fig. 2). The two-phase laminar flows were created at microfluidic T-junctions, and approached the aqueous alginate mixture from either side, at an angle of 90° . The fluid flows were oriented such that the nonreactive shielding flows contacted the aqueous alginate flow at the junction. However, the acidified sunflower oil did not come into contact with the emerging aqueous droplets at the junction. This is pictorially demonstrated in Fig. 2(b).

FIG. 2. (a) Schematic showing microfluidic shielding junction with fluid inputs. Laminar streams of sunflower oil and acidified sunflower oil are shown. (b) A negative image of the shielding flow junction. The shielding flow is dyed in blue for ease of visualization. Beads are highlighted with arrows.

FIG. 3. Graphical representation of 5 alginate microsphere populations showing size distribution. A separate microfluidic chip and optimized flow rates were used to produce each sample.

This modification allowed the problem of immediate gelation at the junction to be overcome. The emerging droplets were insulated from the acidified fraction of the carrier phase by a layer of the shielding fluid [colored blue in Fig. 2(b)]. Spherical droplets of the alginate mixture were successfully generated at the junction, and these proceeded through the reactor channel. As the droplets were carried through the circuit, the shielding layer that surrounds them became gradually acidified, as protons diffused from the acidic surroundings. The eventual reaction between calcium carbonate and H^+ was seen to be slower and more controllable, since the relative flow rates (and

FIG. 4. Percentage cell viability measured using an adapted trypan blue method, as described in Sec. II D. Note that the increase in cell viability is concomitant with observing cell clusters. Error bars show standard error for four replicates.

consequently the thicknesses) of the two components of the laminar flow were entirely variable. Hence, by varying the relative proportion of the shielding fluid within the carrier phase, the rate of microsphere gelation could be adjusted.

The use of the shielding junction described above allowed monodisperse alginate microspheres to be produced in a microfluidic reactor. The diameter of the resulting microspheres could be accurately controlled, and this was found to be inversely proportional to carrier phase flow rate (in agreement with Ref. 13). The optimized microreactor ran unperturbed for several hours, until the feed syringes were exhausted. Using this method, approximately 1 ml of monodisperse microspheres was produced per hour. In order to investigate the flexibility of the chemical method and shielded segmentation junction, a series of microfluidic chips was manufactured, in which the channel dimensions were varied in a scalar fashion. It was observed that monodisperse alginate microspheres of any desired diameter in the range 80–400 μm could be produced by selecting the appropriate fluidic chip and corresponding fluid flow rates. Several samples of alginate microspheres were produced in this manner, their diameters and size distributions are shown in Fig. 3.

C. Microfluidic encapsulation of human cells

The microfluidic procedure for alginate microsphere synthesis described above was further adapted to enable the encapsulation of cellular material. The functional fluid formulation was modified to include the cell growth-medium D-MEM/F12. Four separate populations of HEK-293 cells were dissociated to form single-cell suspensions. Four samples of monodisperse alginate microspheres were obtained, containing approximately one hundred HEK293 cells, randomly distributed throughout each sphere. Approximately 500 μl of microspheres were produced for each population of cells. Using a modified trypan blue exclusion assay, the viability of each sample of encapsulated cells was quantified. Cell viability was measured at several time points, beginning immediately after the dissociation step (time point zero). The alginate capsules were seen to be stable in the cell growth medium and remained intact through numerous manipulations during medium changes. Weekly viability measurements were taken over the next 12 weeks. Over this time the viability of the encapsulated cells continued to increase. After 12 weeks no more cell capsules remained, and the viability measurement experiment was terminated. Live/dead cell ratios (for each sample at each time point) are reported in Fig. 4.

FIG. 5. (a) Light microscope image of cell-containing microspheres. (b) Confocal images of cell-containing microspheres stained with live/dead stains (24 h post encapsulation). (c) Confocal images of cell-containing microspheres stained with live/dead stains (14 days post encapsulation showing cell clusters. Live cells are stained green, and dead cells are stained red).

In addition to trypan blue viability readings, confocal microscopy using live/dead staining was carried out. Cells were seen to be randomly distributed through the microspheres 24 h after encapsulation [see Figs. 5(a) and 5(b)]. After 14 days of encapsulation, cells were observed in clusters. When observed using confocal microscopy, cell clusters were seen to be alive, while individual cells appeared dead [Fig. 5(c)]. Between one and four cell clusters of variable size were observed per microsphere.

IV. DISCUSSION

The interfacial reaction between CaCO_3 and H^+ is immediate, producing Ca^{2+} , CO_2 , and water. In a solution of sodium alginate, Ca^{2+} ions (liberated upon acidification) are immediately chelated by alginate molecules to form the widely accepted helical “eggbox” structures.¹⁵ This ionic crosslinking produces solid hydrogel, which has been widely used in cell encapsulation and immunoisolation experiments.^{5,6,16} The “internal” gelation method was adapted to suit a microfluidic flow reactor, as described above. However, it became immediately clear that the rapid, uncontrolled cross-linking (and therefore gel-forming) reaction at the emerging interface of a droplet caused problems at the fluidic junction. The presence of solid hydrogel at the junction greatly affects the surface properties of the PTFE microfluidic chip, which is intrinsically hydrophobic. The hydrophilic nature of the hydrogel solid causes the aqueous flow stream to be attracted to the surface, thereby disrupting the segmented flow conditions and precluding the formation of droplets.

It was postulated that the gelation reaction could in effect be “postponed” if the aqueous droplets were formed in the absence of H^+ , and exposed a moment later to the acidified immiscible phase. Previously, microfluidic laminar flows have been used to create such chemical gradients and to perform liquid-liquid extractions.^{17–19} These reported experiments involve combinations of miscible fluids or stratified flows containing an immiscible “membrane” flow. By introducing an unreactive laminar stream into the carrier fluid a diffusion barrier was created, which served to delay the interfacial reaction.

The “shielded” segmented flow described above was designed, and proved to be effective in this application. As the aqueous droplet emerges from the fluidic junction, it meets only the nonreactive portion of the carrier phase. Hence, the droplet elution is not hindered by the formation of thin hydrogel film as before. Diffusion of H^+ within the laminar oil flow begins to occur post segmentation, and thus the reaction of calcium carbonate with H^+ is more gradual. This allows the aqueous droplets to crosslink more slowly as they travel through the microchannel.

As described above, it is imperative that the capsule-forming reaction is compatible with the survival and growth conditions for a cellular payload. In order to limit the possible deleterious effect of H^+ and Ca^{2+} on cells, the minimum concentrations of glacial acetic acid and calcium carbonate required to induce the cross-linking reaction were identified. Using these conditions, the microfluidic flow reaction produced stable, extremely monodisperse alginate microspheres.

HEK293 was used as a model cell line in these experiments due to its high growth rate, hardiness, and ease of transformation. In the future, this novel method could allow the encapsulation of a wide variety of cells and cell lines, designed for different therapeutic applications, under reproducible and sterile conditions. The immortalized cell-line HEK293 was successfully encapsulated using the described method. Initially, viability of cells was seen to decrease sharply after encapsulation. Upon investigation (using confocal microscopy), dead cells were found to be randomly distributed throughout the microspheres [Fig. 5(a)]. It is unclear whether the encapsulation method itself or a lack of cell-cell interactions is the cause of this decrease. Here we present only preliminary results, and further work into investigating the cause of the initial drop in cell viability has yet to be carried out. After approximately 14 days, cell clusters were observed inside the microspheres [Fig. 5(b)], with a corresponding increase in viability readings. The increase in viability was in direct relation to the appearance of cell clusters. It is proposed that cells in close contact at encapsulation are able to proliferate to form the cell clusters observed. Viability (live/dead cell ratio) stabilized at 70% after a month and this was seen to continue, until no microspheres remained (at three months post encapsulation).

Similar results were observed for three of the four replicate encapsulated populations. By contrast, the fourth population exhibited markedly reduced viability 24 h post encapsulation. The cell viability continued to decrease, relative to the other three populations, until at 15 days after encapsulation, all encapsulated cells were dead. Cells encapsulated in this population were not observed to form cell clusters. We have not established a satisfactory explanation for the failure of this one batch, but it is noteworthy that the fate of each batch could be determined within 24 h of production, which will be an important factor in quality control for any therapeutic application.

In the context of cell encapsulation, the ingress of nutrients and oxygen toward the center of a sphere is extremely important. Hence, porosity and sphere size are factors in determining optimum conditions for cells contained within a hydrogel matrix. Larger alginate microspheres (500–2000 μm) can easily be generated via standard droplet generation techniques such as air-jet break-up.^{20,21} However, in our experience it is significantly more difficult to produce spheres with a diameter of $<400 \mu\text{m}$ using these conventional methods. By virtue of having decreased diffusion paths, beads with smaller diameter are arguably more effective in averting cell hypoxia.¹⁰ Using microfluidic devices with the shielded segmented flow junction described, we can now reliably synthesize monodisperse samples of alginate microspheres in the range 80–400 μm .

An important issue for the development of cell therapies toward clinical trial application, will be the need to develop GMP-compliant methods for the production, manipulation, and validation of encapsulated cells.⁷ Few existing droplet-generation protocols are sufficiently well developed to enable the reproducible production of cell capsules under GMP conditions. Microfluidic devices are intrinsically “sealed environments”; and this will likely prove to be a significant advantage in the attainment of GMP standards. Furthermore, the microfluidic device described here is entirely constructed from “generally regarded as safe” materials.

In the context of microsphere production, the output from a single-circuit microfluidic reactor is typically in the range of 1–20 ml/h (depending *inter alia* on reactive-fluid flow-rates and microsphere diameter). Pharmaceutical and life-science applications are likely to require tens or hundreds of liters of microsphere products for effective clinical trials. Clearly, it would be an extremely laborious task to produce these quantities of microspheres from such a single-circuit microreactor. Indeed, a single-circuit microfluidic device as described here is suitable primarily for the production of much smaller sample volumes for analysis and proof-of-principle studies. If microfluidic devices are to advance and add further value to the encapsulation of live cells, further work toward scaling-up and increasing production volumes must be performed. It is highly likely that this could be achieved via the development of GMP-compliant multicircuit-array devices.

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¹ X. Wang, W. Wang, J. Ma, X. Guo, X. Yu, and X. Ma, *Biotechnol. Prog.* **22**, 791 (2006).

² J. C. Pound, D. W. Green, J. B. Chaudhuri, S. Mann, H. I. Roach, and R. O. Oreffo, *Tissue Eng.* **12**, 2789 (2006).

³ K. Moriyasu, H. Yamazoe, and H. Iwata, *J. Biomed. Mater. Res.* **77**, 136 (2006).

⁴ Z. H. Hu, S. Z. Chen, Y. Jin, Y. Xiong, W. Wang, X. J. Ma, and M. Song, *Singapore Med. J.* **47**, 504 (2006).

⁵ R. Calafiore, G. Basta, G. Luca, M. Calvitti, G. Calabrese, L. Racanicchi, G. Macchiarulo, F. Mancuso, L. Guido, and P. Brunetti, *Biotechnol. Appl. Biochem.* **39**, 159 (2004).

⁶ A. Omer, V. Duvivier-Kali, J. Fernandes, V. Tchpashvili, C. K. Colton, and G. C. Weir, *Transplantation* **79**, 52 (2005).

⁷ The prevention of diabetes and its complications, WHO Review 2006.

⁸ C. M. Shi and T. M. Cheng, *World J. Gastroenterol.* **10**, 2550 (2004).

⁹ R. Calafiore, G. Basta, G. Luca, A. Lemmi, M. P. Montanucci, G. Calabrese, L. Racanicchi, F. Mancuso, and P. Brunetti, *Diabetes Care* **29**, 137 (2006).

¹⁰ J. C. Ogbonna, M. Matsumura, and H. Kataoka, *Process Biochem.* (Oxford, U.K.) **26**, 109 (1991).

- ¹¹ M. Joanicot and A. Ajdari, *Science* **309**, 887 (2005).
- ¹² K. S. Huang, T. H. Lai, and Y. C. Lin, *Lab Chip* **6**, 954 (2006).
- ¹³ H. Zhang, E. Tumarkin, R. Peerani, Z. Nie, R. M. Sullan, G. C. Walker, and E. Kumacheva, *J. Am. Chem. Soc.* **128**, 12205 (2006).
- ¹⁴ D. Poncelet, R. Lencki, C. Beaulieu, J. P. Halle, and R. J. Neufeld, *Appl. Microbiol. Biotechnol.* **38**, 39 (1992).
- ¹⁵ I. Braccini and S. Perez, *Biomacromolecules* **2**, 1089 (2001).
- ¹⁶ Y. Gao, H. C. Jiang, J. Xu, S. H. Pan, and Y. D. Li, *Transplant. Proc.* **37**, 4589 (2005).
- ¹⁷ M. Surmeian, M. N. Slyadnev, H. Hisamoto, A. Hibara, K. Uchiyama, and T. Kitamori, *Anal. Chem.* **74**, 2014 (2002).
- ¹⁸ T. Maruyama, H. Matsushita, J. Uchida, F. Kubota, N. Kamiya, and M. Goto, *Anal. Chem.* **76**, 4495 (2004).
- ¹⁹ J. Atencia and D. J. Beebe, *Nature (London)* **437**, 648 (2005).
- ²⁰ G. L. Fiszman, A. L. Karara, L. M. E. Finocchiaro, and G. C. Glikin, *Electronic Journal of Biotechnology* (2002), available at: <http://www.ejbiotechnology.info/content/vol5/issue3/full/5/>
- ²¹ S. Koch, C. Schwinger, J. Kressler, C. Heinzen, and N. G. Rainov, *J. Microencapsul.* **20**, 303 (2003).