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Microfluidic Encapsulation of Cells in Alginate Capsules for High Throughput Screening

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Abstract—**Microdroplet systems can drastically reduce costs and increase throughput in high throughput screening (HTS) assays. While droplets are well suited for biomolecular screening, cell-based screens are more problematic because eukaryotes typically require attachment to solid supports to maintain viability and function. This paper describes an economical, off-the-shelf microfluidic system which encapsulates eukaryotic cells in gelatinous alginate capsules for the purpose of HTS. The flow-through system consists of i) a cross junction, which forms monodisperse droplets of alginate and cell suspension in an immiscible carrier fluid, followed by ii) a T junction which delivers $BaCl_2$ to crosslink and solidify each droplet. With an appropriate carrier fluid, the system is self-synchronized and can produce cell-alginate-$BaCl_2$ capsules with virtually 100% reliability. Droplet volumes and frequency are determined by flow rates and the diameter of the cross junction. The present implementation, which utilizes 1.5 mm Teflon tubing and plastic junctions, can generate 0.4-1.4 µL droplets at frequencies >10 droplets/sec. Cell viability is >80% at 4 hours post-encapsulation. With low recurring cost (<$2) and no need for automation robots, this can be an initial step towards economical cell-based HTS.**

Keywords: droplet, high throughput screening, alginate

I. Introduction

High throughput cell-based assays provide rich information needed in the fields of drug discovery, toxicity testing, genomics, proteomics, and cell biology. Examples include second messenger assays for monitoring signal transduction, reporter assays for monitoring gene expression, and cell viability assays to test for toxicity to external stimuli [1-2]. Current approaches to high throughput screening (HTS) employ microplates with densities ranging from 96-1536 wells/plate, and reaction volumes ranging from 1 mL to 1 µL. The primary concerns with existing technology are the reagent costs and limited throughput. Typical HTS facilities screen 10,000 to 100,000 compounds/day [1] at a cost of $1/assay, determined primarily by the cost of reagents and consumables. Added to this is the initial capital cost of liquid handling robots. Assay throughput is typically < 1 assay/second, limited by reaction rates and the ability of the automation robots to transfer liquids from one container to another.

Microdroplet-based HTS has the potential to reduce assay volumes, increase throughput, and eliminate the need for liquid handling robots, all of which can result in significant cost savings [3]. In microdroplet-based screening, a stream of droplets containing reagents or cells is generated in a microfluidic channel using simple geometries [3-4]. The droplets are separated from each other by an immiscible carrier fluid, allowing each droplet to serve as an isolated reaction container. Droplets can be merged with each other to perform biochemical assays, and inline detectors can provide real time readout of each droplet as they flow by the detector. Microdroplet technology can potentially provide 1) 10-1000x reduction in assay volumes, and 2) throughputs up to 1000 droplets/sec [5-7].

To date, microdroplet systems have been applied primarily towards screening biomolecules. For example, Ismagilov and colleagues used droplet-based screens to optimize conditions for protein crystallization [3,8]. However, there is growing need to perform cell based assays, for the reasons mentioned above.

Weitz, Toner, and colleagues demonstrated the encapsulation of single cells into aqueous droplets for the purposes of performing cell-based HTS [5]. Encapsulation directly into an aqueous droplet is a suitable approach for many prokaryotic cells; however, eukaryotes typically require a solid support for cell attachment in order to remain viable and maintain normal function. This requirement is one of the factors limiting the adoption of microdroplet systems for high throughput cell screens.

To address this issue, this paper presents a microfluidic system for encapsulating cells into hydrogel capsules which can support cell growth and proliferation. The capsule is made by combining solutions of alginate and Ba^{2+}, a multivalent ion which acts as a crosslinking agent. VandeVord and colleagues have previously demonstrated that this type of capsule maintains cell viability >80% and also supports induced secretions in Schwann cells [9]. The capsules can be functionalized with a cell-matrix emulator (Matrigel), and can be further optimized to support cells with additional reagents or proteins. The microfluidic system generates alginate droplets containing cells and then solidifies them inline by adding a fixed amount of the crosslinking agent to each droplet. The resulting capsules are monodisperse, and have a fixed distance between them. The capsules are stored inside a flexible, gas-permeable tube which allows for *in situ* culture and economical transport between screening facilities. Section II describes the system concept, section III outlines the experimental setup, section IV discusses results, and section V concludes.

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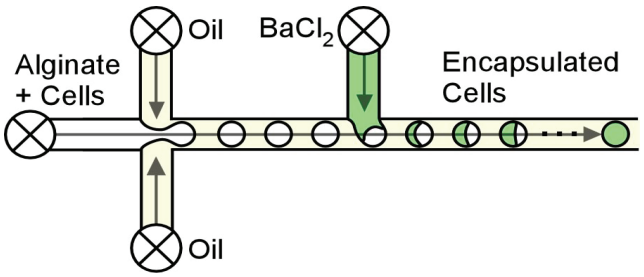

Figure 1: Microfluidic method used to encapsulate cells in monodisperse, gelatinous alginate capsules.

II. Concept

The system consists of two components, a microfluidic droplet generator and a merging junction (Fig. 1). The droplet generator is a cross junction which produces droplets in a manner similar to flow focusing [4]. A single stream containing the solution of alginate and cells is merged with two orthogonal streams of an immiscible carrier fluid (typically oil or organic solvent [3-6]). Shear forces induced by the opposing oil streams cause the droplet to break off in a reproducible manner, resulting in monodisperse droplet formation. The size and frequency of droplets are determined by the diameter of the cross junction and the relative flow rates of the aqueous and oil streams. They are related by the equation $Q = F_d * v_d$, where Q is the flow rate of the aqueous phase, F_d is the droplet frequency, and v_d is the droplet volume.

The stream of alginate/cell droplets enters a merging junction (a T connector) which adds the crosslinking agent $BaCl_2$ to each droplet. Assuming there is sufficiently high interfacial tension between the aqueous and carrier phases, a portion of the incoming $BaCl_2$ stream is spontaneously wicked into each droplet as it flows by the junction. Furthermore, if the flow rates of alginate and $BaCl_2$ solutions are equal, then a fixed amount of $BaCl_2$ is added to each droplet, and the process of droplet merging becomes self-synchronized. For repeatable system performance, it is important to maintain constant flow rate. Pressure spikes due to sudden changes in flow rates or movements in the capillary will destabilize the system and result in sporadic droplet merging. In general, however, the approach of merging a set of droplets with a stream is more reliable than other systems where two sets of droplets are then independently generated and merged [10].

The addition of $BaCl_2$ crosslinks the alginate solution, forming a gelatinous structure which 1) prevents the cells from migrating outside the droplet, and 2) provides a solid support in which eukaryotic cells can proliferate [9].

III. Experimental Setup

A. Microfluidic Setup

The fluidic apparatus for cell encapsulation is assembled with low cost, off-the-shelf components and requires no microfabrication. Teflon capillaries with 1.5875 mm inner diameter (Small Parts, Miramar FL) are chosen because Teflon is chemically inert, hydrophobic, and has high gas permeability. Chemical inertness and hydrophobicity ensures that the aqueous droplets do not wet the channel walls, thus preventing cross-contamination. Gas permeability facilitates the exchange of gasses which makes it possible to culture cells directly in the tubing. The cross connector and T connector (Value Plastics, Fort Collins CO) have barb fittings which do not require any glue for the low pressures used in this experiment.

B. Alginate Capsule Formation

Alginic acid sodium salt from brown algae powder with high M content (#71238, Sigma-Aldrich, St. Louis MO) was initially dissolved in buffer (13 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-and 0.84% NaCl, pH 7.4, Sigma-Aldrich) to make a 1% working solution and then filter sterilized using a vacuum pump. Barium (Ba^{2+}) ions (Sigma-Aldrich) were chosen for alginate gelation since they have been shown to synthesize mechanically stable microcapsules with negligible swelling [11]. Ba^{2+} has a higher affinity to alginate cross-linking binding sites compared to commonly employed divalent cations Ca^{2+} and Sr^{2+}, suggesting better gel strength and minimal degradation. Using high M alginate mixed with Ba^{2+} produces elastic and flexible gels [9].

The carrier fluid used was 10 centistoke silicone oil (Corning 200, Dow Corning, Midland MI). The immiscibility of the carrier fluid with the aqueous phase provides an isolated micro-environment within the droplet and eradicates the problem of cross-contamination by diffusion or surface interaction. After testing carrier fluids of various viscosities it was experimentally concluded that oils of higher viscosity reduces the merging of capsules after with one another they have been formed [12].

C. Cell Preparation

C6 astrocytoma cells (ATCC, Manassas VA) were grown in tissue culture flasks using F-12K medium supplemented with 10% horse serum, 2.5% FBS, and antibiotics. For testing, cells were trypsinized, counted using a hemocytometer, and resuspended the 1% alginate solution. C6 cells were chosen for their phenotype resemblance of native astrocytes. Other cells that have successfully been encapsulated in alginate droplets include Schwann cells, pancreatic cells, liver cells, fibroblasts, and myoblasts [13].

D. Experimental Procedure

The 1% alginate solution was combined with the cells at a concentration of 200,000 cells/mL. The cell-alginate solution and 50 mM $BaCl_2$ solution were filled in separate 3 mL syringes. The 10 cst silicone oil was filled in two 10 mL syringes. All syringes were mounted on a single syringe pump (model KDS230, Holliston MA); thus, the relative flow rates of each fluid were determined by the diameters of the syringes. Typical flow rates were 60-300 µL/min on the oil syringe and 20-100 µL/min on the alginate and $BaCl_2$ syringes. In order to synchronize the coalescence of alginate and $BaCl_2$, identical flow-rates were used on both reagents. After the capsules were generated, they were collected in a beaker for subsequent viability analysis.

E. Viability Analysis

The Live/Dead® viability/cytotoxicity assay (Invitrogen, Carlsbad, CA) was employed to determine cell viability post encapsulation. The capsules containing the cells were rinsed three times using phosphate buffer saline solution (PBS) and then stained. The stain consisted of 200 µL of solution prepared from 5 mL Dulbecco's Modified Eagle Medium (DMEM) phenol-free culture media, 10 µL ethidium homodimer-1 (Etdh-1: detects dead cells) and 2.5 µL calcein AM (to detect live cells). Capsules were then incubated at 37 °C for 30 minutes. Fluorescence images were obtained using green (495-515 nm) and red (560-595 nm) emission filters. Images of different focal planes within the capsule were taken and then analyzed using an image processing software (ImageJ, National Institutes of Health). Percent surviving cells were determined by measuring the area of green signals divided by the area covered by the total number of cells (green + red fluorescence) at a given focal plane.

IV. Results and Discussion

The size and frequency of droplets generated at the cross junction are determined by the relative flow rates of the alginate and the two oil streams. In general, the droplet volume increases monotonically with increasing water flow rate, and with increasing ratio of water to oil flow rate (Fig. 2a). The geometry of the tubing also plays an important role in determining droplet size [4]. With the 1.5875 mm ID tubing used here, the droplet volume can be varied from 0.4 to 1.4 µL at the given flow rates. The frequency of droplet formation increases linearly with flow rate, reaching up to 2 droplets/sec at 60 µL/min (Fig. 2b).

The cell-alginate droplets formed at the cross junction merge with the 50 mM $BaCl_2$ solution at the T connector (Fig. 3). Due to the low volume and high surface area of the droplets formed (typical volumes are on the order of microliters), the mixing between the $BaCl_2$ and alginate solution occurs in about 3-5 seconds. A food coloring dye was added to the $BaCl_2$ solution to visualize the mixing of the two compounds.

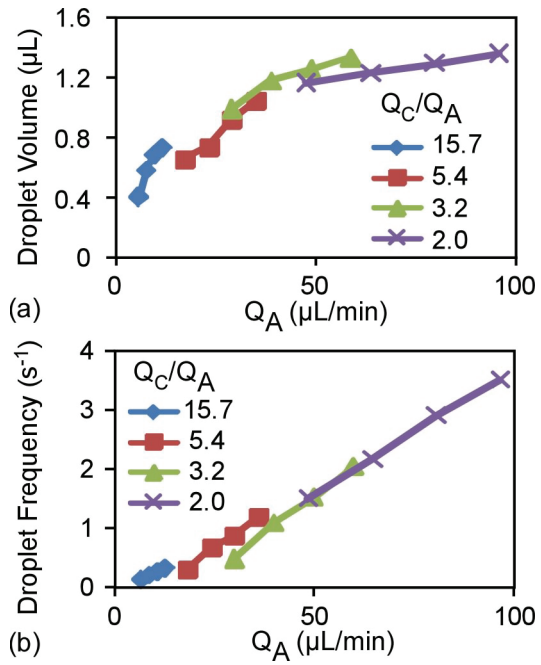

Figure 2: (a) Droplet volume versus the flow rate of alginate solution (Q_A) and the carrier fluid (Q_C). (b) Droplet frequency vs. Q_A and Q_C.

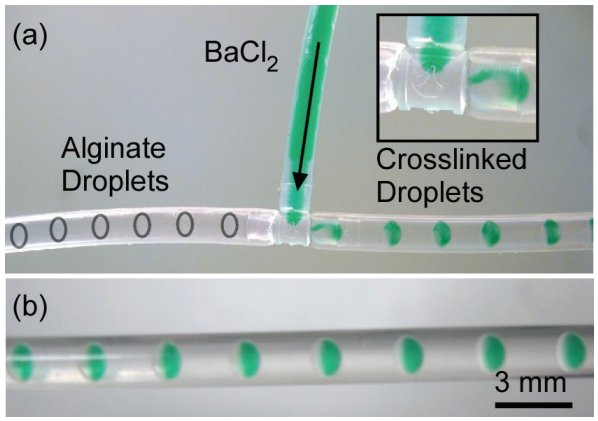

Figure 3: a) Experimental results of the merging of 1% alginate droplets and 50 mM $BaCl_2$ solution in 10 cST silicone oil. Inset illustrates the manner in which the $BaCl_2$ stream wicks into each droplet. (b) Droplets 2 cm downstream the T junction, showing the progression of mixing.

For reliable merging to occur at the tee junction, the carrier fluid should be chosen appropriately. The interfacial tension, viscosity, and surfactant concentration in the carrier fluid determine whether spontaneous merging occurs [3]. Empirically, it was determined that when 10 cst silicone oil is used as the carrier fluid, the merging takes place instantaneously at the T junction due to the wicking effect described earlier (Fig. 3). Other carrier fluids with lower viscosity showed sporadic patterns in merging (Fig. 4). The robust performance of the silicone oil as a carrier fluid allows the droplet generator to be operated a flow rates of up to 300 µL/min, resulting in throughputs of >10 droplets/sec.

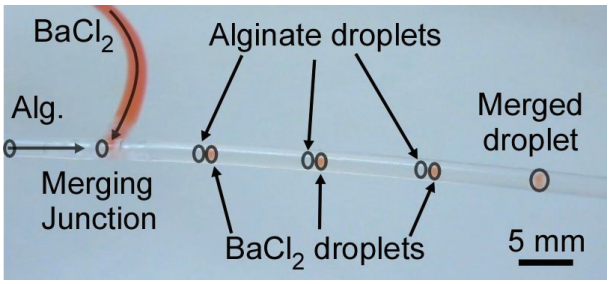

Figure 4: Effect of carrier fluids with low interfacial tension (e.g. soybean oil). Instantaneous merging does not occur; instead two interleaved droplets are formed at the tee junction. Merging occurs downstream.

An alternate scheme for encapsulating cells investigated in this effort was found to be unreliable, and is included here for completeness. In this scheme, the alginate/cell solution and the 50 mM $BaCl_2$ solution were added into a Y-junction.

This method was found to be unreliable because the solutions became crosslinked in the mixing region, causing gelation before the droplets could be formed. As a result, the beads formed a long gelatinous tail.

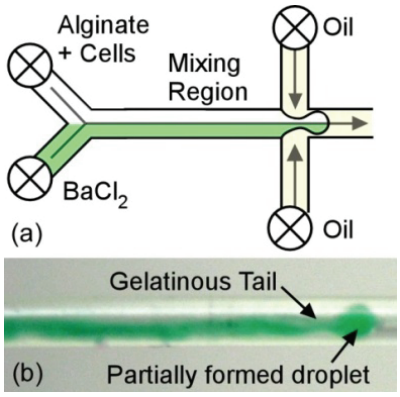

Figure 5: (a) Unsuccessful design Y-junction for mixing the two compounds, followed by a droplet generator. (b) Results showing a partially formed droplet and gelatinous tail.

After forming micro-capsules using the optimal system (Figs. 1 and 4), the Live/Dead® assay was performed to determine cell viability (Fig. 6). The poly-anionic dye calcein AM is well retained within live cells, producing an intense uniform green fluorescence in live cells at a wavelength of 495-515 nm. Etdh-1 enters cells with damaged membranes and produces a bright red fluorescence upon binding with nucleic acids at a wavelength of 560-595 nm. Cell viability, calculated using the procedure described above, was found to be 80% at 4 hours post-encapsulation and 60% after 9 hours.

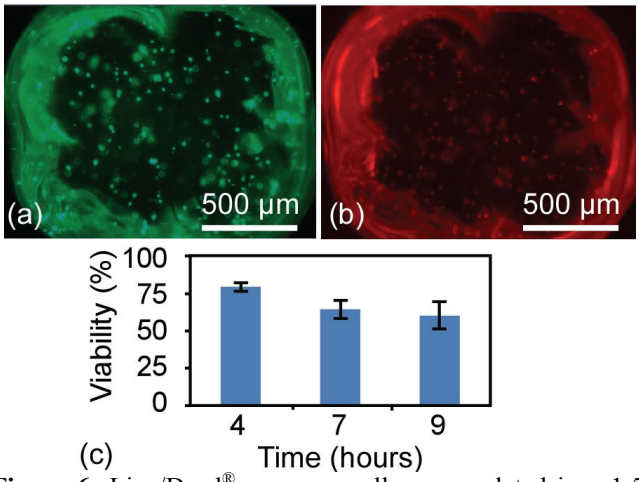

Figure 6: Live/Dead® assay on cells encapsulated in a 1.5 mm diameter alginate capsule. (a) Live stain (495-515 nm), representing cells with intact membranes. (b) Dead stain (560-595 nm) on the same capsule and focal plane, showing cells with damaged membranes. (c) Cell viability at 4, 7 and 9 hours post-encapsulation.

V. Conclusion

This paper describes a simple and economical system for generating streams of alginate beads containing viable eukaryotic cells. The order and monodispersity of the capsule stream, along with the ability to add additional reagents to the droplets, makes this system useful for high throughput cell-based assays. Applications include cell-based screening of drugs, organic compounds, and proteins. The system can be integrated with inline optical detectors for assay readout without laboratory automation robots.

Acknowledgment

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