ALGINATE HYDROGEL BASED 3-DIMENSIONAL CELL CULTURE AND CHEMICAL SCREENING PLATFORM USING DIGITAL MICROFLUIDICS

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ABSTRACT

We report the development of a microfluidic device that is the first of its kind to generate uniform arrays of individually addressable cell seeded calcium alginate gels for 3D cell culture using electrowetting on dielectric (EWOD) digital microfluidics (DMF). This is combined with EWOD DMF's multiplexing abilities to demonstrate how a single device is capable of forming cell seeded alginate hydrogels, generating different concentrations of chemicals and delivering these generated chemical concentrations to different alginate gels to observe the effect of chemicals on 3D cultured cells. This lays the foundation for a more efficient and versatile 3D cell culture and chemical screening platform.

INTRODUCTION

The application of alginate hydrogels for 3D cell culture has already been well documented [1]. Incorporating alginate hydrogels into continuous microfluidic devices for cell culture and screening purposes, however, has been found to be problematic due to the difficulty in forming well controlled gel shapes. As such, methods have been limited to continuous microfluidic devices that formed a single large hydrogel exposed to a chemical gradient [2]. Robotic spotters have been used to form multiple alginate spots on a single device, but could not isolate alginate gels and exposed them all to the same chemical concentrations [3]. Current approaches mostly rely on droplet microfluidics since other methods fail to generate alginate hydrogels of regular and uniform shapes. However, droplet microfluidic methods fail to allow for integrated operations and require separate devices to carry out different steps [4].

In order to allow for 3D cell culture and chemical screening using alginate hydrogels, an electrowetting on dielectric (EWOD) digital microfluidics (DMF) approach is proposed. EWOD DMF allows for discrete nanoscale volumes of drops to be generated and transported on chip [5]. This can be utilized to accurately direct the motion of sodium alginate and calcium chloride droplets and control their method of merging to yield uniformly shaped alginate gels. This can then be used for cell culture and chemical screening applications.

DESIGN

The formation of alginate gels on EWOD DMF has never been studied in detail before. Conventionally, drops of sodium alginate are immersed in calcium chloride solution to form 3D alginate gel environment. Therefore, the most obvious immediate method to form an alginate gel on EWOD DMF would be to merge drops of sodium alginate and calcium chloride. The schematic of operations required to carry out such a mode of gel formation on EWOD DMF is shown in Figure 1. However, this approach was found to yield very irregular shaped gels with alginate hydrogels.

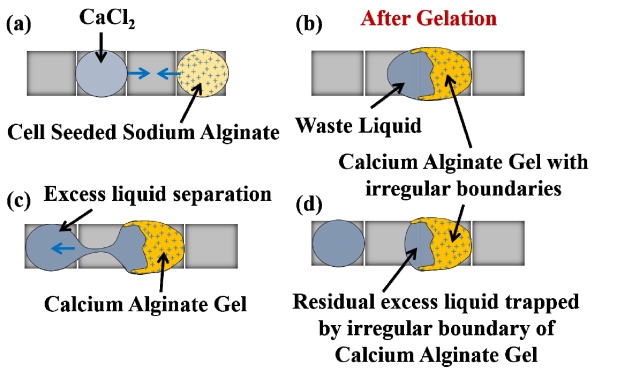

Figure 1: Schematic showing how direct merging of sodium alginate with calcium chloride (a) would result in the formation of irregularly shaped gels (b) which would trap liquid during removal of excess liquid (c)-(d).

Irregular shaped gels were found to retain pockets of liquid that hamper complete removal of excess liquid. These pockets of liquid would dilute any incoming liquid, making it impossible to accurately deliver specific concentrations of chemicals to the cells in the gel for screening purposes and studies. Without the ability to form gels with regular shapes and size, a reliable 3D cell culture and chemical screening would be impossible.

Gels with irregular shape also provided additional resistance to the separation of excess liquid. Applying greater force to remove excess liquid would result in the gel being displaced from its gel formation site and being carried away with the excess liquid. Such events would lead to the failure of the DMF system.

Thus in order to ensure uniform gel formation, a new encapsulation design was developed. This method relies on delivering sodium alginate to a circular gel post followed by encapsulation with calcium chloride. Once the sodium alginate is completely encapsulated by calcium chloride, the two liquids are allowed to merge to initiate gel formation. This method would immediately define the outside boundary of the alginate gel resulting in a uniformly circular gel.

The ability to separate the encapsulation step from the merging step relies on the adoption of a separator ring as seen in Figure 2. This separator ring allows the sodium alginate to be surrounded by the calcium chloride without accidental merging occurring. Merging is then carried out on demand by actuation of the separator ring electrode.

In order to ensure that premature merging of sodium alginate and calcium chloride does not occur, precision dispensing of cell seeded sodium alginate is carried out at the gel formation sites. Thus merging of the two drops only occurs by deliberate actuation of the separator ring electrode only after complete encapsulation.

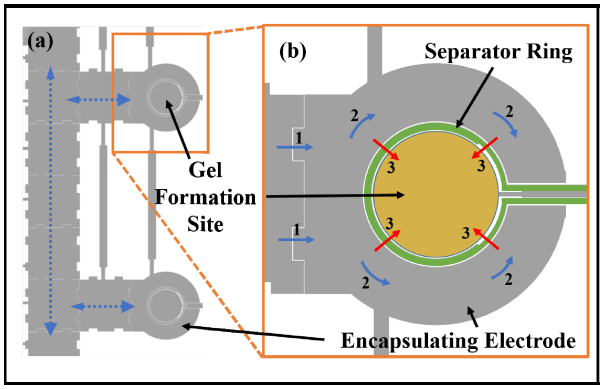

Figure 2: (a) shows a magnified mask design around the gel formation sites of a EWOD platform. Blue dotted arrows show the pathways for fluid. Inset magnified image (b) shows the separator ring electrode (green) that allows for 'on-demand' merging of the calcium chloride with sodium alginate. Blue arrows with numbers show how calcium chloride enters the encapsulating electrode (1) and then encapsulate the gel formation site (2). Actuation of the separator ring electrode causes calcium chloride to flow into and merge with sodium alginate at the gel formation site as seen by red arrows (3).

METHODS AND MATERIALS

Cell Culture and Alginate Hydrogel Preparation

Human breast cancer cells (MCF-7) obtained from American Type Culture Collection (ATCC) were maintained in culture medium (D-MEM/F12, Life Technologies, Carlsbad, CA), and supplemented with 4% fetal bovine serum, 2 mM l-glutamine, 100 µg/mL penicillin/streptomycin, and 0.01 mg/mL insulin. Cells were incubated in 25 cm^2 T-flasks at 37°C and 5% CO2 while being sub-cultured every 3-4 days at ~ 80% confluency.

In order to prepare 0.5% wt/vol sodium alginate solutions, first stock solutions of sodium alginate were formed by dissolving low viscosity sodium alginate powder (Sigma Aldrich, St. Louis, MO – CAS 9005-38-3) in deionized water to obtain a concentrated 4% wt/volume sodium alginate solution. This concentrated alginate solution was diluted in a 1:4 ratio with MCF-7 cells suspended in DMEM/F12 to obtain a 0.5% wt/vol of cell seeded sodium alginate solution with a concentration of 0.5-1 x 10^6 cells/mL. 100 mM calcium chloride solutions were prepared by dissolving calcium chloride crystals (Sigma Aldrich) in DMEM/F12 culture media. In order to prevent biofouling [6], pluronic F-68 was added to all culture media containing reagents at 0.04% wt/vol concentration.

EWOD Device Fabrication and Setup

EWOD devices were fabricated at the University of Texas at Arlington's Nanofabrication facility in a manner similar to that used by Wijethunga et al [7]. Briefly, a bottom EWOD chip consists of patterned ITO electrodes on a glass substrate, 5µm-thick dielectric layer of Su8-2005, and 150 nm-thick topmost hydrophobic layer of Teflon whereas a top EWOD chip is formed with an ITO and a Teflon layers only. EWOD bottom and top chips were assembled by means of 100 µm thick double sided kapton tape and mounted into a custom holder that interfaced with a DAQ through which AC voltages could be transmitted from an AC signal generator and amplifier to the EWOD electrodes. Signals transmitted to the EWOD were programmed through scripts that were fed into a LABView program that controlled the DAQ.

Fluorescent Dye Preparation

Fluorescent dyes Hoechst 33342 (Life Technologies) was used to stain all the cells present in the hydrogels while Propidium Iodide (PI) (Life Technologies) was used to stain all the dead cells. H-33342 and PI were dissolved in sterile culture grade water to obtain final dye concentrations of 5ug/ml of H-33342 and 8ug/mL of PI.

DMSO Preparation and Dilution Protocol

A stock solution of DMSO was prepared by mixing sterile culture grade DMSO (Sigma Aldrich), deionized water, Hoechst-33342 and Propidium Iodide to obtain a 50% v/v DMSO solution with the same final dye concentration of H-33342 and PI as used to prepare the fluorescent dye solution described earlier. This solution was loaded into the reservoir and on chip dilution was carried out using a serial dilution protocol similar to that used by Park et al [8]. In this manner, concentrations of 50%, 25% and 12.5% DMSO were obtained while the 0% concentration was a control drop of fluorescent dye.

Targeted Chemical Delivery Experiment Protocol

In order to carry out the targeted drug delivery experiments, MCF-7 cell seeded alginate hydrogels were created at designated tissue post sites. This was done by dispensing cell seeded sodium alginate drops and delivered to designated tissue post sites. Calcium chloride drops was similarly dispensed from its parent reservoir and brought to the tissue post sites where they were merged with sodium alginate drops and gelation was allowed to occur. During this gelation process, simultaneous preparation of different concentrations of DMSO (0%, 12.5%, 25%, and 50%) was carried out in another area on the same chip using the DMSO dilution protocol as described earlier. After allowing sufficient time for gelation (~7 minutes), excess liquid from the tissue posts was extracted and dispensed to waste. The 4 different DMSO solutions were then delivered to the target tissue posts and the whole chip was incubated for 30 minutes in a 37°C, 5% CO2 humidified incubator following which fluorescence images were then taken using the protocol described below.

Fluorescence Microscopy and Cell Counting

Fluorescence images were taken using fluorescence microscope (Olympus BX-51) to visualize the cells in the gel posts stained by the fluorescent dyes. H-33342 dye stained all the cells present blue when viewed under DAPI filters while dead cells were labelled red by PI dye when viewed under TRITC filters.

Fluorescent images thus obtained were combined using ImageJ (NIH) so that the dead cells labelled red were overlaid with all the cells present in the sample that were stained blue. The resulting image clearly allowed viable cells to be distinguished from dead cells.

For experiments where viability was measured, cell counting was carried out manually using combined fluorescent images and the cell counter plugin in ImageJ software. This yielded the total number of live and dead cells, which were then used to calculate the viability percentage. The targeted chemical delivery experiment was repeated 3 times and results were presented as viability percentage ±1 standard deviation.

RESULTS

The proposed design for uniform gel formation was tested by delivering extra-large drops of sodium alginate to the gel formation site. Precision dispensing of sodium alginate was carried out directly at the gel formation site as seen in Fig 3 (a)-(c) resulting in sodium alginate drops that stayed within the boundary of the gel formation site.

Calcium chloride was then delivered to the encapsulating electrode and allowed to surround the sodium alginate as seen in Fig 3 (d)-(e). The separator ring was found to be successful in preventing merging of the two liquids as clearly seen in Fig 3 (e) where encapsulation has taken place but the drops are still separated. In order to initiate gel formation, the separator ring electrode is then actuated as seen in Fig 3 (f), which causes merging of the sodium alginate and calcium chloride to occur. After allowing for 7 minutes of gelation time, excess liquid removal was successfully carried out as seen in Fig 3 (g)-(i). This method resulted in uniformly circular gels that were of consistent size and shape and could be repeatedly formed.

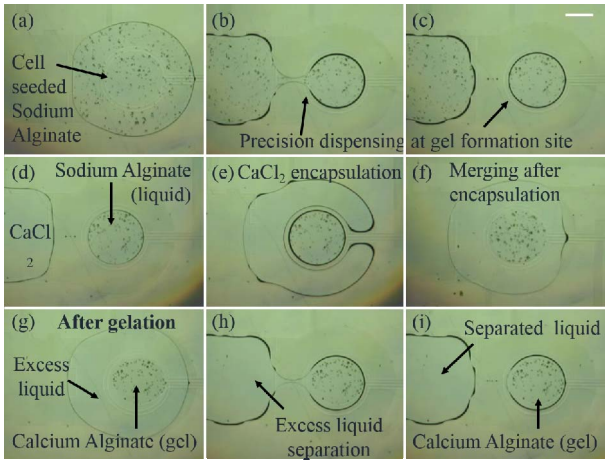

Figure 3: Sequence of alginate gel formation starting with precision sodium alginate dispensing at gel formation sites (a)-(c). This is followed by encapsulation with calcium chloride (d)-(e) and subsequent merging (f) through actuation of the separator ring electrode. After gelation, calcium alginate hydrogels are formed and excess liquid is removed from the calcium hydrogel as shown in (g)-(i). Scale bar in (c) represents 0.25 mm

To demonstrate the targeted chemical delivery abilities of this proposed screening platform, four MCF-7 seeded calcium alginate hydrogels were formed on chip as per the encapsulation design. While gelation was taking place, different concentrations of DMSO (0, 12.5, 25 and 50 %) were formed on chip as per the method described in the methods and material section. Once the calcium alginate hydrogel gelation was complete, excess liquid was removed and discarded to waste and the 4 different DMSO concentration drops were delivered to 4 gels and incubated for 30 minutes. Fig. 4 shows one set of results from such an experiment where cells were seeded at a high density to better allow visualization of the variation in cell. Based on the fluorescence images, a clear difference in viability of cells is observed with viability decreasing as DMSO concentration increases. Due to the high cell density used, this particular data set was not quantified and was only used for visualization purposes.

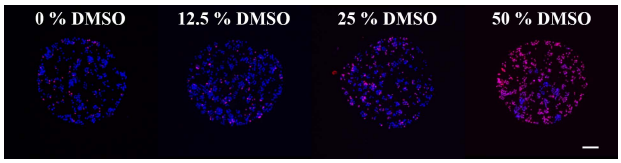

Figure 4: Fluorescent images showing effects of on chip DMSO delivery at 4 different concentrations (0%, 12.5%, 25% and 50%) to MCF-7 cell seeded calcium alginate hydrogel post. Blue dots represent live cells while red dots represent dead cells. Scale bar = 100 micron.

In order to determine the repeatability of this experimental procedure, 3 sets of the same experiment were carried out with cells seeded at lower density (in order to facilitate counting of cells). The results of the replicate experiments are shown in Fig. 5. The 0% DMSO concentration serves as the control gel to show the viability of cells formed on chip. The 12.5% DMSO gels showed a very slight decrease in viability compared to the control gels and this is to be expected since it is well known that exposure to low concentrations ~10% DMSO for short durations of time have a negligible effect on viability.

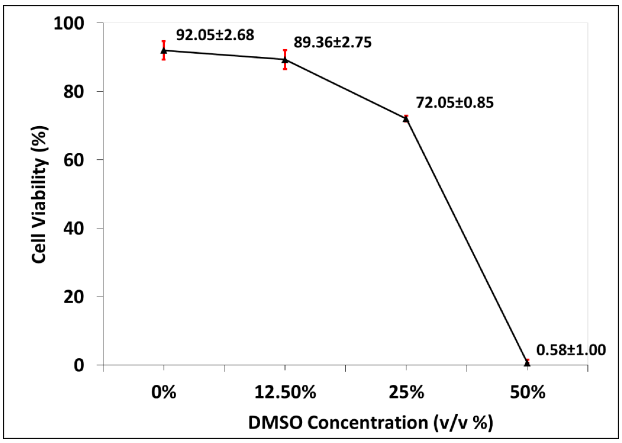

Figure 5: Plot showing quantified data for the effects of DMSO delivery at 0%, 12.5%, 25% and 50% concentrations on cell viability after delivery and incubation for 30 minutes to separate gel posts. Error bars (in red) represent ±1 standard deviation, n=3.

The gels exposed to 25% and 50% concentrations of DMSO show a marked decrease in viability with 25% DMSO concentrations showing a viability of 72.05% ± 0.85 while the 50% DMSO concentrations killing off all

the cells in the 30 minute exposure time.

In order to demonstrate the ability to note changes in response of cells to DMSO over time, cell seeded alginate gels were formed on chip. While gelation was allowed to happen, different concentrations of DMSO (0%, 12.5% and 25%) were prepared on the same chip through serial dilution. Once gelation was complete and excess liquid was separated, the 3 different DMSO concentration drops were delivered to the tissue posts and the device was incubated. Fluorescent images were taken at 30 minutes intervals to measure the effect of DMSO over a span of two hours on cell laden hydrogels.

Fig. 6 shows the resultant viabilities obtained at each time step for each gel post exposed to DMSO concentration. From the graph, it can clearly be seen that there is minimal decrease in viability of the cells in the control gel exposed to 0% DMSO. However a decrease in cell viability could be seen over the first 60 minutes for the 12.5% DMSO treated gel followed by no major increase in cell death at 90 minutes and 120 minutes. The 25% DMSO treated gel on the other hand showed a consistent increase in cell death at every 30 minute interval for the first 90 minutes with the cell death rate starting to taper off at 120 minutes.

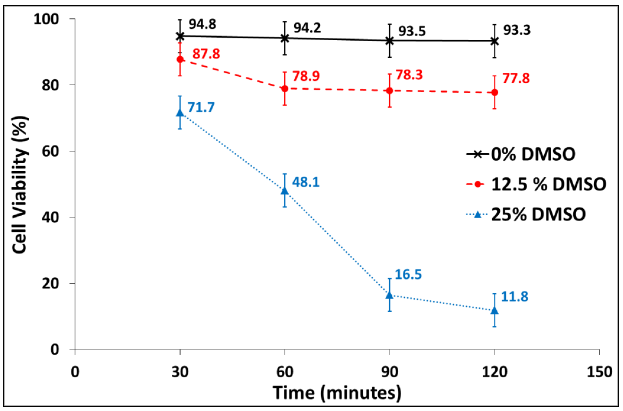

Figure 6: Plot showing time lapse variation in cell viability % during a single experiment (n=1) as hydrogels are exposed to varying DMSO concentration (0%,12.5% and 25%) for 30,60,90 and 120 minutes. Error bars represent ±5% error to account for errors in cell counting.

Such time-lapse experiments provide valuable insight into the effect of chemicals over time on cells without having to carry out multiple experiments. Being able to monitor the variation in cell death during the same experiment over time reveals that cell death in the 12.5% DMSO post increases only over the first 60 minutes and after that remains more or less constant. This kind of observation relating the nature of cell death with both time and concentration would be hard to make using conventional experimental setups without having to carry out multiple experiments. The EWOD DMF platform provides a simple and convenient means to take rapid images of multiple gel posts over multiple time frames with ease allowing investigators the ability to observe the transient effect of chemicals on cells cultured in a 3D environment.

CONCLUSION

An encapsulation design to allow the formation of uniform alginate hydrogels on EWOD DMF was developed. This encapsulation design was tested for use as a 3D cell culture platform for chemical screening and the differential effects of DMSO delivery on cell viability in alginate hydrogels were demonstrated. A time-lapse experiment was also demonstrated showing how the effects of different chemical concentrations on cells could be tracked temporally. This showcases the potential for EWOD DMF to be used for hydrogel based cell culture and chemical screening.

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