

ALGINATE ENCAPSULATION OF CELL-LADEN BEADS FOR MICROFLUIDIC TUMOR SPHEROID CULTURE.

C. Bayly, L. Yu, K. C. Cheung.

BioMEMS laboratory, Department of Electrical and Computer Engineering, - University of British Columbia, 2355 East Mall, Vancouver, British Columbia, CANADA

ABSTRACT

We present a method for the generation of alginate core-shell beads, in which differing alginate composite materials may be incorporated into the alginate of the core and shell. We then investigate the capacity of these beads to address the problematic out-of-bead and monolayer cell proliferation that occurs during microfluidic culture of alginate-encapsulated tumor spheroid cultures. When using cell-laden alginate for the core bead, the two-step internal gelation procedure yielded alginate core beads with a size distribution CV (coefficient of variation) of 0.081, while the size distribution of the core-shell beads have CV of 0.057. Encapsulation in an alginate shell, however, did not stop but only lengthened the amount of time required for out-of-bead proliferation to occur. While 1.3 % collagen was successfully incorporated into the alginate cores of core-shell beads, its presence did not cause the cells to proliferate preferentially in the core bead. These core-shell beads, while of limited use themselves, nonetheless demonstrate the capacity of the encapsulation method to generate a variety of core-shell beads with differing core and shell compositions, which would have been otherwise been challenging difficult to make.

INTRODUCTION

Tumor spheroid – based drug assays provide superior tumor models to the two-dimensional cancer cell cultures. Compared with monolayer cultures, it has been demonstrated that tumor spheroids exhibit superior resistance to a variety of anti-cancer drugs, and behave more similarly to actual tumors[1]. There has therefore been significant interest in the incorporation of tumor spheroid cultures into high throughput assays for anticancer drugs. Microfluidic methods have been pursued as a promising option for the development of such assays, as its miniaturized nature can be amenable to high throughput screening. The cell-laden bead approach uses a chip to suspend cancer cells in hydrogel microbeads, which are then cultured into tumor spheroids (ideally in bead generation chips). This setup is advantageous in that it generates cultures with a defined volume for spheroid growth, and the PDMS (polydimethylsiloxane) cell culture chips permit the diffusion of oxygen, nutrients and waste [2,3]. Alginate hydrogel is amenable to these systems, as it maintains sufficient stiffness to be trapped and cultured on-chip. One challenge with the on-chip culture of cell-laden alginate microbeads has been the tendency of the cells to either fall or proliferate out of the alginate bead, resulting in rapid monolayer proliferation throughout the microchip. The bead encapsulation method described here was devised as a way to generate core-shell alginate beads, in which the composition of the cell-laden core could be tuned to stop out-of-bead proliferation. Core shell beads with two different cores, alginate and a collagen-alginate mixture, are prepared and their effect on out-of-bead proliferation is investigated.

MAIN FUNCTIONAL PRINCIPLES

The key advantage of the encapsulation method used here is that it keeps the core bead generation step distinct from the addition of the shell, which permits greater control over the volume of the core and a greater variety of possible core bead compositions. The alginate core-shell bead generation procedure relies on the method of internal gelation (REF!). Alginate gels via calcium-mediated crosslinking. Internal gelation works by having calcium, bound in the insoluble form of CaCO_3 , mixed into an ungelled alginate solution of high or neutral pH. When the pH is lowered, the calcium carbonate is broken down into water, CO_2 and Ca^{2+} , initiating gelation. In bead generation procedures which use streams of oil to break an alginate flow into droplets, the oil is acidified, and as the alginate droplets spend longer more time in the oil, more acid will diffuse into the alginate, causing Ca^{2+} release and alginate gelation. Once gelled, the beads can be collected in a tube of culture media, which can be centrifuged to bring the beads down into the aqueous phase. These core beads can then be encapsulated in an additional layer of alginate.

The encapsulation process is similar to the core-bead generation process. The pregelled alginate beads are resuspended in a small volume of 3% alginate (1/4 of the total volume of beads). This is because the alginate's viscosity keeps the beads from rubbing against each other causing deformation and clogage. This beads and then introduced onto a microfluidic chip where the beads are flow-focused into single file by two alginate/ CaCO_3 flows. Then the whole flow-focusing ensemble is pinched off into larger beads by two oil flows. The ungelled alginate surrounds the bead, and then slowly gels into a shell in the as it remains in contact with the acidified oil. The microfluidic chip for core-shell bead production works as diagrammed below: Pregelled beads in alginate are introduced at one end of the center channel. This stream is soon focused by two streams of alginate- CaCO_3 solution, forcing the beads into single file down the center. This 'composite stream' of beads and alginate with CaCO_3 is then pinched off into droplets by two streams of oil perpendicular to

the center channel. Another oil inlet is located after the pinch-off intersection, adding extra oil to keep the droplets well-spaced. After ~ 30 seconds in the chip and in microfluidic tubes, the beads are deposited into the container of culture media.

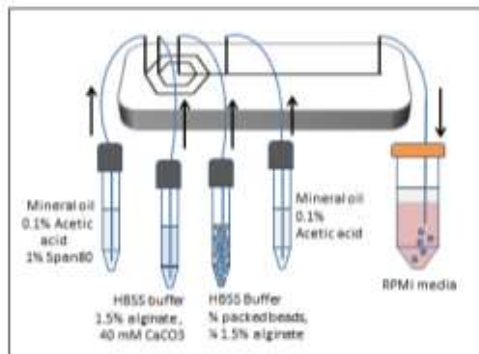


Figure 1: Alginate encapsulation



Figure 2: flow focusing

EXPERIMENTAL METHOD

The molds for the microfluidic chips were fabricated using soft lithographic techniques, in which features were patterned onto a silicon substrate using the photoresist SU-8. This SU-8 and silicon structure served as a mold master, onto which liquid polydimethylsiloxane (PDMS) could be poured and then cured at 60C. After 2 hours of curing, the PDMS structure was peeled off the silicon mold and bonded with a thin PDMS membrane, forming closed channels. Chips were baked for at least 4 hours following plasma bonding to help the chip lose some of its hydrophilicity. Inlets and outlets were punched with a 75 mm punch.

MCF-7 cancer cells were cultured in RPMI media supplemented with 10 % Fetal Bovine Serum and 1% Penicillin/streptomycin, at 37°C and 5% CO₂. After washing twice with Washed PBS, the cells were trypsinized with only enough 0.025 mM trypsin + EDTA to cover the cells themselves. Cells were then resuspended in 1 ml of RPMI media, to which was added 500 ul of 7% BSA fraction VIII. Cells were then centrifuged at 99 g, washed in HBSS buffer, centrifuged again, and then resuspended in a solution of 3% alginate and 40 mM CaCO₃, to a final concentration of 6×10^6 cells /ml. The oil solution for pinching the alginate solution into droplets was prepared by mixing 35 mL of mineral oil with 0.35 g of stabilizer Span80, and 1 µl/ml acetic acid. The oil solution for inlet 2 was prepared by mixing oil and acetic acid, also at 1 µl/ml.

Solutions were introduced into the chip in the following order: Oil solutions were placed in at the same time, and then the cell-alginate-CaCO₃ solution was added. Pressures were adjusted as necessary to obtain stable bead production. Droplets pinched off remained in the chip and tubing for ~ 30 seconds until they reached the collection tube. At 15 minute intervals, the contents of the collection tube (containing oil, beads and media) was centrifuged at 500 rpm to bring the rest of the beads from the oil into the media. Beads were then washed twice with media, and incubated overnight. In a flask of culture media at 37C and 5ppm CO₂. beads were then centrifuged 50 g for 3 minutes, and resuspended in HBSS. They were then pelleted again, and this time, once the supernatant was removed, the total volume of the beads was measured, and then 1/4 the bead volume of 3% alginate was added to the beads. This bead-alginate solution was then reintroduced into the 4-inlet microfluidic chip such that they were pinched off inside larger droplet of liquid alginate at the oil/alginate intersection. The outer shell layer again gelled via the internal gelation technique, and core-shell beads were gathered in the collection tube. After pelleting and washing the beads, some were introduced into a microfluidic culture chip, via pipette. The remaining beads were cultured in flasks with RPMI supplemented with FBS and penicillin-streptomycin.

Collagen-alginate core beads were produced in a way similar to that of the alginate core shell beads, except that neutralized collagen was added to the alginate mixture to a final concentration of 1.3%. this mixture was then kept on ice always, and was transported to the microfluidic chip using a jacketed cooling tube, through which ice water was always flowed. The oil added after the intersection was not acidified at all, permitting the collagen to retain its gellation properties (which are sensitive to low pH). Droplets were flowed into a second chip heated at 37 C, and then to a third chip into which acidified oil was added. The result was a homogenous dispersion of particles throughout the alginate bead.

RESULTS AND DISCUSSION

Alginate core beads were coated with an additional layer of alginate via the bead encapsulation method described above. This procedure generated core-shell beads containing viable cells (confirmed by subsequent cell division) within the core-beads only. While core-beads were of monodisperse size (Figure 3, CV= 0.081) the shell thickness was far more variable (CV = 0.057). This variation was caused by buildups occurring in the microfluidic tubes, a phenomenon to be addressed in future work. First, the sufficiency of structural integrity of the core-shell beads was verified by introducing them

into a culturing chip. Though fragile, if treated with care they were found to be strong enough to be caught and held in the bead-traps. The core-shell beads, along with unencapsulated core beads for reference, were cultured in flasks for one week to determine whether the extra alginate layer would inhibit out-of-bead proliferation. Figure 4 shows alginate core beads and alginate core shell beads five days after culture, at which point cell proliferation out of the core bead surfaces is clearly visible. The alginate encapsulation was found to keep the cells within the alginate beads for a longer period of time, although after 9 days they began to proliferate outside of the bead as well.

Since collagen has been found to enhance cell proliferation [4], cell-laden alginate core beads doped with 1.3% collagen were also prepared also encapsulated in alginate, to determine whether the cells would proliferate preferentially in the collagen-doped alginate core. The fabrication of these required that the alginate-collagen solution be chilled during the pinch-off procedure, then heated in a second microfluidic chip, before oil with additional acid was added to ensure internal gelation of the alginate also occurred. While the method was successful in producing cell-laden collagen-alginate core beads, which were subsequently encapsulated in alginate (see fig ???), the presence of collagen did not significantly influence the growth behavior of the cells, which behaved similarly to the those is pure alginate beads.

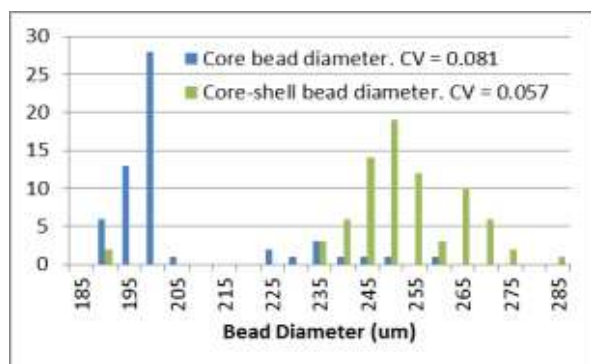


Figure 3. Core and core-shell beads size distribution

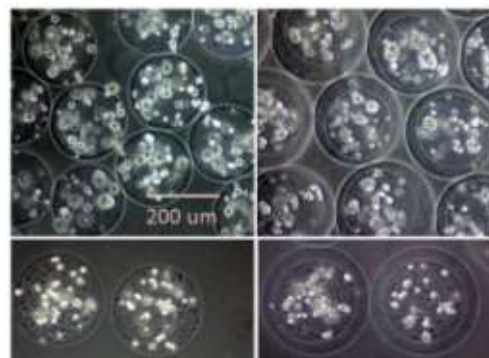


Figure 4. A1. alginate core beads and. A2. alginate core-shell beads after 5 days of culture. B1 Alginate collagen core beads and B2. Alginate-collagen core-shell beads after 1 day culture

CONCLUSION

The presented method can be used to generate core-shell beads of alginate an alginate-collagne mixture, with the goal of making cell-laden alginate beads more compatible with on-chip culture. Encapsulation of cell-laden beads with alginate did not stop, but only postponed out-of-bead proliferation, which still permits spheroids to grow larger before proliferating out-of-bead. The method further showed its utility in enabling the generation collagen/alginate core bead encapsulated in alginate. However, the presence of collagen did not significantly affect the proliferation rate or direction. Future work will include the incorporation of additional materials into the core and shell to further constrain cell growth to the core bead, modifying the chip design to decrease shell size variation, most notably, generation of beads with a matrigel core, which MCF7 cells proliferate rapidly[5]

ACKNOWLEDGMENTS

This work was funded by the Natural Science and Engineering Research Council of Canada, the Canada Foundation of Innovation , and the Canadian Breast Cancer Research Alliance. Dr. Marcel Bally at the BC Cancer Research Centre kindly provided the MCF7-GFP3 cells.

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CONTACT

K.C.Cheung, tel: +1-604-827 4114; kcheung@ece.ubc.ca