Published in final edited form as:

Biomaterials. 2009 December ; 30(36): 6896–6902. doi:10.1016/j.biomaterials.2009.09.012.

Microfabrication of Homogenous, Asymmetric Cell-Laden Hydrogel Capsules

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Abstract

Cell encapsulation has been broadly investigated as a technology to provide immunoprotection for transplanted endocrine cells. Here we develop new fabrication methods that allow for rapid, homogenous microencapsulation of insulin-secreting cells with varying microscale geometries and asymmetrically modified surfaces. Micromolding systems were developed using polypropylene mesh, and the material/surface properties associated with efficient encapsulation were identified. Cells encapsulated using these methods maintain desirable viability and preserve their ability to proliferate and secrete insulin in a glucose-responsive manner. This new cell encapsulation approach enables a practical route to an inexpensive and convenient process for the generation of cell-laden microcapsules without requiring any specialized equipment or microfabrication process.

Keywords

cell encapsulation; hydrogel microcapsules; alginate; diabetes; polypropylene mesh; microfabrication

1. Introduction

Cell encapsulation is a strategy to allow for the transplantation of non-autologous cells without the use of immunosuppressive drugs, which have potentially severe side-effects [1–4]. Transplanted living cells are protected from the host immune system because they are encapsulated in a semi-permeable hydrogel membrane which allows the diffusion of nutrients and cellular metabolic products while excluding antibodies and immune cells [5].

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This technique has potential applications as therapies for many diseases such as diabetes, hormone deficiencies or hepatic failures [4].

Encapsulated islets have been explored as a method to allow transplantation of allogenic or xenogeneic insulin-secreting cells into diabetic hosts [6–11]. Electrostatic droplet generation is the most widely used method in the production of microcapsules containing islets or other insulin-secreting cells [12]. Typically, a laminar liquid jet is broken into droplets by a harmonically vibrating nozzle combined with an electrostatic dispersion mechanism which prevents droplet aggregation [13]. Currently, droplet generator encapsulation systems are commercially available from several manufacturers such as Inotech Biosystem (Rockville, MD) and Nisco Engineering AG (Zürich, Switzerland). The electrostatic droplet generator system is appropriate for the continuous production of hydrogel microcapsules (200–600 μm in diameter) from a polymer/cell mixture of an unchanged composition [13]. However, this apparatus, which requires sterilization of the bioreactor chamber after each use, is not convenient for studies which involve screening a large number of different material formulations [14]. Furthermore, this approach does not allow for generation of non-spherical capsules. Therefore, it is desirable to develop new and convenient methods for fast generation of geometrically controlled, cell-laden microcapsules.

Soft-lithography with PDMS molds has been used to fabricate alginate hydrogel microcapsules in 2D arrays as well as discrete single modules [15–18]. Many living cells, including bacteria and mammalian cells, have been encapsulated in hydrogels with this approach [15–18]. However, a potential challenge for this technique is the difficulty of releasing the microcapsules from the PDMS mold without damaging the cells. Even though methods such as mechanical stretching of the mold, swelling the hydrogel capsules in organic solvent [15] or microtransfer molding [17, 19] have been applied to facilitate capsule release, these processes decrease cell viability and capsules yield [17, 18]. Whitesides *et al.* recently demonstrated the production of cell-laden microcapsules using a PDMS membrane with fully penetrating pores to improve the ease of capsule retrieval [20]. However, this method is still limited by the necessity of a microfabrication process to generate the PDMS membranes and the difficulty of obtaining membranes of large area.

Here, we describe an inexpensive and convenient approach utilizing a commercially available polypropylene mesh for the fabrication of cell-laden hydrogel microcapsules without using any specialized equipment or microfabrication processes. The *in vitro* viability, proliferation and insulin secreting function of the encapsulated cells were characterized. We also demonstrated the fabrication of capsules with different geometries and selective modification of the microcapsule surface using this mesh-based fabrication approach.

2. Materials and Methods

2.1 Fabrication of alginate hydrogel capsules

Thermo-molded plastic meshes were purchased from McMaster-Carr and Industrial Netting. The plastic meshes were cut into pieces of 4×3 cm² and placed in a Chex-all® II instant sealing pouch. These meshes were autoclaved at 121 °C and kept sterile until use. Sodium

alginate (FMC BioPolymer, LF 10/60 LS), with or without materials to be encapsulated, was dissolved in 0.9% NaCl solution at a concentration of 1.5wt%. When encapsulation of microspheres or cells was desired, 1wt% solid solution of 1 μm red fluorescent microspheres (Invitrogen, Eugene, OR) were diluted 500-fold with this 1.5wt% alginate solution while INS-1 cells were suspended in alginate at a density of 1.5×10^6 cells/mL. The resulting mixture was vortexed to obtain a homogeneous mixture which was then centrifuged at $200 \times g$ for three minutes. The air bubbles at the top of the mixture were removed by aspiration before this suspension was poured into a sterile Petri dish. A piece of sterile mesh was lightly dipped onto the cell/alginate mixture with the less reflective surface of the mesh in contact with the suspension. Excess alginate was removed by gently wiping the mesh against the edge of a sterile Petri dish. The mesh was immediately immersed in a solution of 100 mM CaCl_2 and subsequently transferred to HEPES buffer for mild washing. The hydrogel capsules were finally released by holding the mesh with a pair of tweezers and gently agitating it in a collecting solution.

2.2 Asymmetric surface modification of hydrogel capsules

Before the capsules were released from the mesh template into the collecting solution, one side of the mesh containing cross-linked hydrogels were gently placed on the liquid surface of a 4mg/mL solution of 40–70kDa FITC-labeled poly-L-lysine (Sigma Aldrich, St Louis, MO) for 3 minutes. The mesh was then gently washed in HEPES buffer before the capsules were released into a collection medium.

2.3 Cell culture

The rat insulinoma cell line (INS-1) was a gift from the Joslin Diabetes Center (Boston, MA). INS-1 cells at passages of 7–20 were cultured according to a protocol previously described [21]. The cells were cultured in complete medium composed of RPMI 1640 supplemented with 10 mM HEPES, 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Cultures were incubated at 37 $^\circ\text{C}$ in a humidified 95% air-5% CO_2 atmosphere and manipulated under sterile tissue culture hoods. Confluent dish of INS-1 cells was passaged every week at a subculture ratio of 1:3 and fed every 2–3 days. For trypsinization, the adherent cells were exposed to 0.025% trypsin-EDTA solution to yield a cell suspension which was centrifuged at $150 \times g$ at 4 $^\circ\text{C}$ for 3 min. For preparation of frozen cell stock, freshly trypsinized cells were suspended in complete medium containing 10% of dimethyl sulfoxide and kept at -80°C overnight in a Nalgene® Cryo Freezing container before being transferred to liquid nitrogen for long-term storage.

2.4 Viability analysis of encapsulated cells

The viability of INS-1 cells in alginate microcapsules was characterized using the Live/Dead® Viability/Cytotoxicity Assay Kit (Molecular Probe, Carlsbad, CA). Microcapsules were incubated in HEPES buffer containing fluorescent dyes at the concentrations of 2 μM Calcein-AM and 12 μM ethidium homodimer-1 for 45 minutes. The capsules were washed with HEPES buffer and visualized under a Nikon TE 300 inverted microscope under either phase contrast or fluorescent microscopy settings. Green fluorescence was viewed under a FITC filter and red fluorescence under a TRITC filter. Images were photographed using a

CCD camera and Metamorph imaging software (Molecular Devices, Sunnyvale, CA). Confocal images were taken using a Zeiss LSM 510 confocal microscope with an argon excitation source. For live cell imaging, excitation wavelength of 488 nm and a filter set of 505–537 nm were used. Dead cells were imaged using an excitation wavelength of 543 nm and an emission filter set of 559–623 nm.

2.5 Static glucose-stimulated insulin secretion

Five 500 μ l aliquots of microcapsules containing INS-1 cells were cultured over a period of 10 days. At the desired time points, the capsules were subjected to a static glucose stimulation study following a procedure slightly modified from a protocol elsewhere [22]. Briefly, the capsule samples were pre-incubated for one hour in Krebs Ringer buffer Hepes (KRBH) (137 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 mM NaHCO_3 , 16 mM HEPES, 0.1% (w/v) BSA, pH 7.4) containing 2.8 mM glucose. The *in vitro* insulin secretion was then assessed by consecutive incubations of the capsules in 4 mL of KRBH containing 2.8 mM glucose or 16.8 mM glucose for one hour. The samples were kept at 37 °C in humidified air and 5% CO_2 for the duration of the pre-incubation and stimulation. The capsules were washed with HEPES buffer in between two incubations to remove residual insulin. At the end of each incubation, 1000 μ l of the KRBH was removed and frozen at –20 °C for storage before insulin assay. Insulin concentrations were determined using Ultrasensitive EIA assay kits (ALPCO Diagnostics, Salem, NH).

3. Results and Discussion

3.1 Properties of template meshes for successful capsule fabrication

We fabricated the alginate hydrogel microcapsules using a process illustrated in Figure 1. Briefly, the sterile polymer mesh with an array of uniform pores is brought in gentle contact with the alginate solution (1.5% w/v aqueous solution) with or without materials to be encapsulated. After capillary force completely fills the pores with the alginate solution, the mesh is held vertically so that most of the excess alginate is removed by gravity. The mesh surface was subsequently wiped against the flat edge of a sterile petri dish to further remove any excess alginate. This polymer mesh containing uniform pores filled with alginate was immediately dipped into an aqueous solution of calcium chloride which cross-links the alginate polymer chains in to a hydrogel. After mild washing in HEPES buffer, the hydrogel microcapsules are released by holding the mesh with a pair of tweezers and gently agitating it in a collection solution of culture medium. Figure 2A shows large sheets of thermo-molded polypropylene mesh and Figure 2B is a mesh template with an array of uniform pores typically used in this fabrication process. Figures 2C and 2D shows the different pore shapes of the polypropylene meshes from McMaster-Carr and Industrial Netting respectively.

After testing several types of commercial polymer meshes from different suppliers, we determined that the surface properties of the polymer mesh are critical for successful fabrication of individual alginate capsules. Table 1 shows the feasibility of capsule formation and retrieval for a variety of thermoplastic meshes manufactured from nylon, polypropylene and Teflon. Filling of mesh pores by alginate solution was classified as easy (+) for instant

pore wetting and liquid retention or as difficult (–) if the alginate failed to remain in the pores. Capsule retrieval was considered easy (+) if more than 80% of the freestanding microcapsules are released from the mesh pores instantly during the agitation step. If the capsules formed are connected by a thin film of residual alginate, the retrieval process is considered difficult (–). We observed that meshes made from Teflon are too hydrophobic and the alginate solution is unable to fill the pores. This is similar to observations of trapped air bubbles in PDMS molds [15] and membranes [20] due to the hydrophobic nature of PDMS before surface treatment by oxygen plasma. On the other hand, nylon meshes are too hydrophilic and the alginate solution forms interconnected capsules easily, even after the excess alginate is removed by wiping against the edge of the petri dish. Only polypropylene mesh appears to possess appropriate surface property which enables fabrication of individual free-standing capsules. Furthermore, polypropylene has a high heat-resistant ability which makes it desirable for cell encapsulation applications. Its softening and melting temperatures of 152 °C and 161 °C respectively ensure that the mesh property is unaffected during autoclave sterilization at 121 °C.

3.2 Fabrication of hydrogel capsules with different shapes and asymmetrically modified surfaces

Various microcapsule shapes can potentially be useful as building blocks for engineering of 3D tissue constructs or investigating the effect of hydrogel geometries on the viability, proliferation and functions of encapsulated cells [23, 24]. Figure 3A and 3B shows microscopy images of alginate hydrogel capsules fabricated from different mesh templates to produce capsules with rectangular cuboidal and cylindrical shapes. The non-spherical geometries achieved with this mesh template technique complement the spherical capsules produced by conventional droplet generator systems. We are also able to modify a selected surface of the microcapsules by dipping one side of the mesh containing cross-linked hydrogels onto a desired coating material. Figure 3C and 3D show a bead-encapsulating capsule with FITC-labeled poly-L-lysine modifying a selected surface. Figure 3C is the confocal image with only red fluorescence displayed, showing red 1 µm beads encapsulated in a rectangular cuboidal capsule. In figure 3D, the same capsule is visualized with both red and green fluorescence channels, indicating that a green layer of positively-charged FITC-Poly-L-Lysine was selectively coated on one side of the negatively charged alginate capsule. Such asymmetrically modified capsules with two surfaces of opposite charges might have applications in the directed assembly of cell-laden hydrogel capsules for fabrication of 3D tissue constructs [23].

3.3 Fabrication of hydrogel capsules containing insulin-secreting cells

To illustrate the applicability of this technique in the encapsulation of insulin-secreting cells, we chose a cell line (INS-1) derived from x-ray-induced insulinoma in rats [21] to fabricate cell-laden hydrogel microcapsules. Alginate microcapsules containing these cells were fabricated following the procedure shown in Figure 1 using a suspension of INS-1 cells in 1.5% alginate. Since the presence of cells has an important effect on the rheological properties of alginate, a series of experiments were performed to identify optimal maximum cell-packing density (data not shown). Figures 4A and 4B shows cell-laden microcapsules fabricated using polypropylene meshes from McMaster (Item #9265T41) and Industrial

Netting (Item # XN6080) respectively. The initial cell densities in these microcapsules are in the optimal range of $1.5 - 2.0 \times 10^6$ cells/cm³. This range of maximum initial cell density is comparable to that achieved with the conventional droplet generation method [13].

3.4 Assessment of viability, proliferation and function of encapsulated cells

3.4.1 Viability and proliferation—We studied the viability and proliferation behavior of the encapsulated cells cultured *in vitro* for a period of ten days. Figure 4C and 4E shows the phase contrast and fluorescent images of the three-day microcapsules fabricated from mesh XN6080 with live/dead staining. This viability assay differentiates live and dead cells on the basis of intracellular esterase activity and plasma membrane integrity. In this assay, live cells fluoresce green due to the presence of intracellular esterase which converts the nonfluorescent cell-permeable calcein-AM to an intensely green fluorescent product. On the other hand, ethidium homodimer-1 permeates compromised cell membranes and result in the red appearance of dead cells when this dye undergoes enhanced fluorescence upon binding to cellular nucleic acids. After three days in culture, more than 80–90% of the cells were still alive. These viable single cells appeared as individual green dots in Figure 4E while some dead cells stained red were also observed. This small fraction of dead cells possibly arose from the original cell stocks or from minimal cell injury during the encapsulation process. After ten days in culture, the encapsulated cells have also proliferated and formed larger clusters as observed in Figure 4D and 4F. The ten-day hydrogel capsules contained mostly viable cells as shown in Figure 4F. Confocal microscopy was also used to examine the 3D distribution of cells throughout the entire microcapsule. Figure 5A shows a single capsule at high magnification. Figure 5B is the combined projection of multiple images taken at different depths of the same capsule (each optical slice is of 2.5 μ m for a total thickness of 200 μ m). Figure 5C shows a projected image of the capsule along its diagonal confirming that most cell clusters are alive. These cell clusters are about 20–50 μ m in diameter. They are also uniformly viable and homogeneously distributed in all spatial dimensions of the capsules. These results indicate nutrient transport throughout this capsule geometry is sufficient to maintain cell viability and proliferation.

3.4.2 Static glucose-stimulated insulin secretion—We also performed a static glucose challenge to assess the ability of the encapsulated INS-1 cells to maintain their desired glucose-responsive functions. Figure 6 shows the insulin secretion from the cell-laden capsules cultured over a period of ten days in response to two different glucose concentrations. Glucose levels of 2.8 mM and 16.5 mM were used to simulate the physiological basal and hyperglycemic conditions of diabetic patients respectively. At all time points investigated (3 days, 6 days and 10 days) the encapsulated cells were able to maintain the desired glucose-responsive behavior. These cells secreted a higher insulin concentration (1.5 to 3-fold) when subjected to the higher glucose level. The absolute amount of insulin secreted also increases over time confirming that the cells are proliferating in the capsules.

4. Conclusion

Here we have developed an approach for the fabrication of alginate microcapsules containing functional insulin-secreting cells. This technique can be utilized to fabricate non-spherical capsules of different geometries and unlike conventional droplet methods, allows for asymmetric modification of the surfaces of the microcapsules. The surfaces of such capsules can be asymmetrically tailored with functional coatings of different charges or polarities to introduce directionality to the capsules. This strategy potentially allows for the directed assembly of cell-laden hydrogel capsules by controlling their alignments and orientation for the generation of biomimetic tissue constructs with desired microscale architecture and complexity. In addition, the polymer mesh is mechanically and thermally robust. Thus it can be easily handled and sterilized by autoclaving. The entire encapsulation process using the polypropylene mesh can be conveniently carried out inside a sterile tissue culture hood. This mesh-based method offers a complementary approach to the existing droplet generator system for the fabrication of cell-laden hydrogel capsules. We believe that utilizing an inexpensive and commercially available mesh offers straightforward access to tissue engineers and biologists as this avoids any specialized equipment, making this approach more convenient for widespread use in biological laboratories.

Acknowledgments

This research was supported by the Juvenile Diabetes Research Foundation under grant 17-2007-1063 and the National Institute of Health under grant DE016516. T.D is grateful to the Agency for Science, Technology and Research of Singapore for the A*STAR National Science graduate fellowship. Q. X. is grateful to the MIT-Harvard Center of Cancer Nanotechnology Excellence (CCNE) for the postdoctoral fellowship.

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Figure 1. Schematic illustration of the procedure to fabricate alginate microcapsules containing INS-1 cells using thermo-molded polypropylene mesh. The mesh template is dipped into an alginate solution with or without cells and transferred to a 100 mM CaCl₂ solution for cross-linking. Hydrogel microcapsules are released by gently agitating the mesh in cell culture medium.

Figure 2.

(A) Photograph of large sheets of thermo-molded polypropylene mesh utilized in microcapsule fabrication. (B) Light microscopy images of mesh templates with an array of uniform pores. Polypropylene meshes with rectangular (C) and circular pores (D).

Figure 3. Light microscopy image of rectangular cuboidal (A) and cylindrical (B) hydrogel capsules. Confocal microscopy images of a rectangular cuboidal capsule encapsulating red fluorescent microbeads with only red fluorescence displayed (C) and the same capsule with both red and green fluorescences displayed (D). A green layer of FITC-Poly-L-Lysine was coated on one side of the capsule. All scale bars represent 200 μm

Figure 4. Light and fluorescent microscopy images of alginate microcapsules containing INS-1 cells. Microcapsules fabricated using (A) mesh 9265T41 and (B) mesh XN-6080 at cell densities of 1.5×10^6 cells/mL and 2.0×10^6 cells/mL, respectively. Three-day capsules (C) contain single cells which proliferate into larger clusters after ten days (D). Staining of the same capsules with viability markers shows that three-day single cells (E) and ten-day cell clusters (F) maintain good viability. All scale bars represent 300 μm .

Figure 5.

(A) Differential Interference Contrast image of a single microcapsule containing INS-1 cell clusters after ten days in culture. Confocal microscopy of the top view (B) and side view (C) along the diagonal of the same capsule shows homogeneous cell distribution and viability. Dashed line indicates the edge of the microcapsule. All scale bars represent 100 μm .

Figure 6.

Insulin secretion of encapsulated INS-1 cells over a period of ten days. Values are the mean ($\pm$ S.D) from five independent experiments. Cells maintain glucose-responsive function; and insulin secretion also increased over time confirming cell proliferation.

Relationship between the feasibility of microcapsule formation and the properties of a variety of thermoplastic meshes and. Pore filling and capsule release are classified as easy (+) or difficult (-). Assessment of capsule release is not applicable (n/a) if pore filling is unsuccessful. All other meshes were purchased from Industrial Netting, except mesh 9265T41(*) from McMaster-Carr.

Table 1

Sample ID	Mesh Material	Pore shape	Estimated pore dimension (um)	Estimated mesh thickness (um)	Pore filling with alginate	Capsule release
NN-1400	Nylon-6	square	890×890	380	(+)	(-)
NN-1500	Nylon-6	square	710×710	380	(+)	(-)
NN-1800	Nylon-6	square	380×380	510	(+)	(-)
NN-1700	Nylon-6	square	250×250	510	(+)	(-)
9265T41(*)	Polypropylene	rectangular	530×690	300	(+)	(+)
XN-6080	Polypropylene	rectangular	530×690	360	(+)	(+)
XN-6070	Polypropylene	rectangular	630×760	360	(+)	(+)
XN-7110	Polypropylene	circular	650 (diameter)	360	(+)	(+)
ET-8300	PTFE	diamond	1140×630	460	(-)	(n/a)
ET-8120	PTFE	diamond	630×130	200	(-)	(n/a)