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Self-loading lithographically structured microcontainers: 3D patterned, mobile microwells

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Abstract

We demonstrate mass-producible, mobile, self-loading microcontainers that can be used to encapsulate both non-living and living objects, thus forming three-dimensionally patterned, mobile microwells.

Studies of cells and their function have traditionally been performed on two-dimensional (2D) cell cultures. However, the omission of the third dimension can greatly impact cell behavior by limiting interactions with the surroundings.¹ Microwells in substrates have been developed to address this, but are typically accessible only from one interface; this limitation is especially pronounced in cell culture when the encapsulated cells may experience nutrient deficient conditions due to well geometry and size.²

Researchers continue to add new functionality to microwells, such as making them compatible with high resolution analysis, adding networking channels to facilitate cell-cell interactions, integrating stimulation/measurement devices, and devising methods to generate dense arrays of wells with individually customizable, variable geometry morphologies.^{3,4} Also, microwell structures for bioreactors have been designed with porous membrane floors that allow for media perfusion through the well.⁵ This allows media to convectively enter the microwell from one interface and exit through another. However, despite the advancements in technology, current microwells essentially only provide a quasi-3D environment, where patterning is limited to 2D and cells are in contact with planar surfaces.³ Truly 3D encapsulation, where porosity is precisely engineered on all faces in 3D, allows for greater interaction between encapsulated cells and surrounding media.

In this communication, we describe photolithographically structured, mobile microcontainers that function like three-dimensionally patterned, mobile microwells. The containers load themselves as they self-assemble from cruciform templates en masse at around 40°C, a temperature low enough to enable parallel loading of biological objects. These containers have porous surfaces and interact with their surroundings in all three dimensions. Additionally, the untethered nature of the cruciforms and containers, coupled with the use of ferromagnetic

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[†]Electronic Supplementary Information (ESI) available: [Movie featuring microcontainers self-loading beads en masse]. See DOI: 10.1039/b000000x/

materials, allows for remote-controlled guidance of both unloaded cruciforms as well as loaded containers.⁷ The assembly of the microcontainers is thermally triggered, without the need for any external connections. Photolithography enables the containers to be fabricated with precisely-engineered monodisperse sizes, shapes, and wall porosity.⁶ Using self-assembly, 2D templates are transformed into 3D structures with porosity in all dimensions. We demonstrate the self-loading of glass beads, L929 fibroblast cells, and *Triops* embryos. The loading of biological contents highlights the utility of the low temperature self-loading process.

Fabrication of the microcontainers was based on stress-driven assembly using thin film hinges and has been described in detail elsewhere.⁸ Briefly, the microcontainers were constructed as 2D cruciform templates above a water-soluble polymeric sacrificial layer deposited onto a silicon or glass substrate. Two steps of photolithography were used to simultaneously pattern large numbers of cruciform templates: One defined the six faces (nickel and gold), and the second patterned a trilayer (chromium/copper/polymer) hinge between faces (to enable rotation of the faces) and at the face edges (to aid in sealing the container after assembly). The cruciforms were released from the substrate by immersion in water and spontaneously assembled into containers when heated above 40°C.

Low temperature actuation enabled easy self-loading of many 500 μm containers at the wafer-scale. Entire 3" wafers of cruciforms patterned with 50 μm pores were placed into an aqueous suspension containing $\sim 250 \mu\text{m}$ glass beads and heated (Supplementary video and Fig. 1), resulting in highly parallel loading of the containers (Fig. 1b). Additionally, since the temperature was low enough to be biologically-compatible, we self-loaded live cells utilizing cruciforms with 50 μm pores (Fig. 2a-f). In this case, the pores were larger than individual cells only to enable visualization of the loaded cells using fluorescence microscopy. Containers with smaller pores can also be patterned using conventional lithographic techniques,^{6,9,10} to permit the movement of media into and out of the containers while retaining the cells within the container. The cruciforms were placed into a dish containing a cell suspension and then heated to 40 °C. We utilized 2.5 mL cell suspensions with a concentration of approximately 2.5×10^6 cells/mL to target loading of ~ 312 cells in the 125 nL containers, under the assumption that the system was homogeneously distributed. After loading, the cells were immediately imaged (Fig. 2 d-f); before they could adhere to the surfaces. By adjusting the microscope focal plane, we verified that cells were present within the container. We also verified that these loaded cells were alive using a LIVE/DEAD[®] assay. The live cells stained green and were seen within the container (Fig. 2f). Dead cells that stained red were not observed, which indicated that the thermal trigger used to self-assemble the containers was not lethal to the cells. Additionally, we have verified that cells can be cultured in such structures for a period of 72 hours.

In addition to cells, crustacean *Triops* (tadpole shrimp) embryos and *Artemia* (brine shrimp) eggs were self-loaded. We loaded $\sim 300 \mu\text{m}$ *Triops* embryos by self-assembling 500 μm containers with larger 250 μm pores (for visualization), either in air or water. Loading was achieved in air by pouring dry *Triops* embryos onto cruciforms, which were heated to self-load (Fig. 2g). Loading was achieved in water by remotely guiding magnetic cruciforms over submerged *Triops* embryos and then heating the water to self-assemble the containers around them (Fig. 2h). We also loaded *Artemia* eggs in salt water by using 500 μm containers with 50 μm pores (data not shown). Due to their smaller size ($\sim 230 \mu\text{m}$) multiple eggs could be loaded within a single 500 μm container. After an incubation period of ~ 24 hours, hatched *Artemia* were observed swimming around the microcontainers, demonstrating that the embryos were still viable after the encapsulation process.

The self-loading process is versatile, and in addition to self-loading on planar substrates, containers can also be loaded in flasks. To enable such loading, cruciforms were released from

their substrate and placed within a tumbling round bottom flask filled with an water:glycerol (1:4 v/v) suspension of 150 μm glass beads (Fig. 3a). The glycerol increased the density and viscosity of the solution thereby reducing the tumbling speed and increasing the propensity of the beads to tumble within the bulk of the solution. By heating the tumbling flask with a heat gun, the cruciforms assembled around and self-loaded the beads within a minute. After loading, the containers were strong enough to be manipulated without opening and could be subsequently imaged in a scanning electron microscope (Fig. 3b-d). The number of beads encapsulated within the container depended on the local concentration of beads in the solution. Hence, the containers could be over-packed (Fig. 3b), or well-packed (Fig. 3c). If more rigorous control over the number of the beads loaded is needed, additional factors such as utilizing an isodense solution and functionalizing the interior surfaces of the container will need to be controlled.

Regarding the size scale of the containers, we note that for the packaging many cells or embryos, the 500 μm size scale is appropriate and relevant^{2,11-12} For example, it has been shown that stem cells packaged in 50-600 μm sized wells can remain undifferentiated for long periods of time without the need for cell passaging.¹¹ Additionally, this size is relevant for cell encapsulation therapy. Using the example of a mouse fibroblast cell, approximately 16 μm in diameter and 2.1 pL in volume,¹³ our 500 μm microcontainers (125 nL capacity) could hold a maximum of approximately 60,000 tightly packed cells. In order to allow for adequate diffusion to all of the encapsulated cells, a more reasonable estimate is approximately 33-50% of the maximum.¹⁴ For other applications, we have also fabricated low-temperature microcontainers with 100 μm dimensions.⁸ However, it is important to note that a reduction in dimensions by a factor of two results in a reduction of capacity by a factor of eight; a balance between the applications' required overall payload considerations and size limitations will determine the desired size of the microcontainers.

We have fabricated self-loading containers with micropores for easy visualization, but it is also possible to fabricate containers with smaller pores. Our self-loading methodology can be utilized in conjunction with several well established nanoscale 2D pore patterning strategies. These strategies include the use of electron beam lithography,¹⁵ imprint lithography,¹⁶ electrochemical methods,¹⁷ and systematic pore closure.⁹ We have previously patterned 100 nm sized pores on high temperature self-assembled 3D containers.⁹ It is also important to note that while it is possible to fabricate such 3D patterned structures using two-photon-absorption photopolymerization,¹⁸ it is challenging to pattern nanopores and scale-up the serial process for mass-production. Additionally, containers fabricated with stereolithography will require subsequent serial loading and sealing steps.

In conclusion, we have demonstrated a low temperature method for enabling truly 3D encapsulation of both living and non-living objects, with porosity that can be precisely engineered on all faces in 3D. Additionally, these 3D containers should facilitate better transport of media and nutrients to the cells contained within. Since the materials used in the fabrication of this microcontainer have passed 72 hour toxicity studies performed in our laboratory,⁹ we believe the microcontainers could be used as miniaturized 3D, self-loading, mobile cell culture wells over that time period. The concept of a 3D microwell with well-defined wall porosity is new and unexplored; we have observed that encapsulated cells and eggs survive the encapsulation process and can be cultured over 72 hours. However, longer term studies are necessary to demonstrate the advantages of 3D perfusion that these microcontainers offer as opposed to quasi-3D (2D patterned, 3D structures) microwells. For applications in cell therapy *in vivo*, additional surface coatings and materials may be needed to increase bioinertness and reduce fouling. However, we believe the lithographic strategy used to construct these 3D microcontainers provides the versatility to enable the incorporation of a wide range of materials and coatings for these applications. We envision these microcontainers

to be utilized in diverse fields, such as cell encapsulation therapy, drug delivery, and in microfluidics. The untethered nature of the microcontainers, coupled with the magnetic traits, enable wireless spatial manipulation. This remote manipulation provides the ability to load objects in hard to reach places, such as within capillaries of microfluidic devices, and may also enable the spatial organization of the loaded microcontainers to form 3D scaffolds for tissue engineering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig.1. Self-loading of 500 μm microcontainers en masse

a) Video capture sequence of the microcontainers on a glass substrate being loaded with beads. As the cruciforms begin to lift-off the substrate and fold upwards, 250 μm glass beads were poured over the microcontainers. The containers close around the beads and encapsulate them.

b) An optical image of a collection of porous microcontainers that self-assembled in a highly parallel manner.

Fig. 2. Microcontainers loaded with biological contents

a) An illustration featuring the procedure used to load cells within the microcontainers. Containers were assembled in the presence of LIVE/DEAD® stained cells and media; upon assembly, cells were encapsulated within the container. b-e) Progressively zoomed-in images of various 500 μm microcontainers loaded with L929 cells, focused within. f) Fluorescent zoom detail focused within a microcontainer containing live cells (green) seen through the 50 μm pores. g) A 500 μm microcontainer loaded with a Triops embryo, seen through the 250 μm pores. h) Video capture sequence showing a microcontainer self-loading a Triops embryo.

Fig. 3. Self-loading of microcontainers in a round bottom flask

a) An illustration depicting an untethered, self-loading microcontainer released in a tumbling flask filled with an aqueous suspension of glass beads. b) A 500 μm , self-loaded microcontainer overfilled with 150 μm glass beads. c) A 500 μm container self-loaded to capacity with 150 μm glass beads. d) Zoom detail of the region highlighted in c) featuring the glass beads.