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Fabrication of Collagen Gels That Contain Patterned, Micrometer-Scale Cavities**

By *Min D. Tang*, *Andrew P. Golden*, and *Joe Tien**

This communication describes a procedure that uses microtransfer molding[1] and sacrificial digestion to generate collagen gels that contain patterned, micrometer-scale cavities. Hydrogels—in particular, that are composed of type I collagen—are well suited to applications in cell biology: The mechanical properties of hydrogels can approximate those of living tissues, while the hydration of these gels enables compatibility with cell culture. Gels that consist of type I collagen,

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the most abundant structural protein in tissues, can support the attachment and spreading of cells within or on the gel,[2,3] and can also activate signal-transduction pathways within adherent cells.[4] To fabricate structures that can serve as in-vitro models of complex mammalian tissues, we and others have examined the use of soft lithography[5] and other patterning techniques towards the creation of micrometer-scale structures in gels.[6,11] Recently, we have developed methods to extend soft lithography to the micromolding of gels, and have demonstrated the fabrication of patterned collagen gels that incorporate distinct, segregated populations of cells.[5] Here, we describe methods that are employed to create collagen gels that contain patterned arrays of cavities, and demonstrate the localization of cells at the surfaces of these cavities. It is believed that this work will enable the in-vitro fabrication of structures that begin to resemble epithelial tissues in geometry.

Our strategy consisted of two steps: Firstly, we used microtransfer molding[1,5] to generate composites of gels in which the collagen completely encapsulated a patterned, sacrificial gel (Fig. 1A). Secondly, we digested the enclosed gel to create cavities of a defined shape within the collagen. Introduction of a pressure difference caused the liquid to flow through the collagen and cavities; this flow enhanced exchange of materials between the cavities and the surrounding gel. This strategy required a sacrificial gel that could be digested selectively in the presence of the type I collagen. We chose Matrigel, a mixture of basement membrane proteins derived from mouse sarcoma,[12] as the sacrificial gel; treatment with the enzyme dispase under mild conditions (~ 2 U mL^{-1}, 1.5–2.5 h) breaks down Matrigel rapidly and completely, while digesting the type I collagen minimally.[13] Because Matrigel supports the growth of many types of cells,[14,15] we hypothesized that its use would not lead to the formation of toxic byproducts after digestion. It is unclear if collagen that has been exposed to dispase supports cell culture to the same extent as untreated collagen does; if necessary, purification of dispase to enhance its specificity of digestion is possible.

Figure 1A outlines the experimental procedure. Microtransfer molding of liquid Matrigel precursors on a layer of collagen, and gelation of the precursors by heating to 37 °C, generated separate microstructures on the surface of the collagen gel (Fig. 1A). Subsequent addition of liquid collagen precursors, and their gelation, encased the patterned Matrigel fully in collagen. Slow addition of the liquid collagen precursor (held at 4 °C) onto the patterned Matrigel/collagen composite (held at 37 °C) is crucial to avoid sharp gradients in temperature that can damage the molded structures. Figure 1B shows images of hexagonal structures of Matrigel (100 μm on a side, 100 μm thick) embedded in collagen and the resulting cavities after treatment with dispase. To demonstrate that the structures that form after digestion are truly cavities, we molded an array of Matrigel that incorporated a suspension of iron powder (average size of iron particle ~ 10 μm). Before digestion, the iron particles were immobilized firmly in the Matrigel (Fig. 1B, bottom, left). After
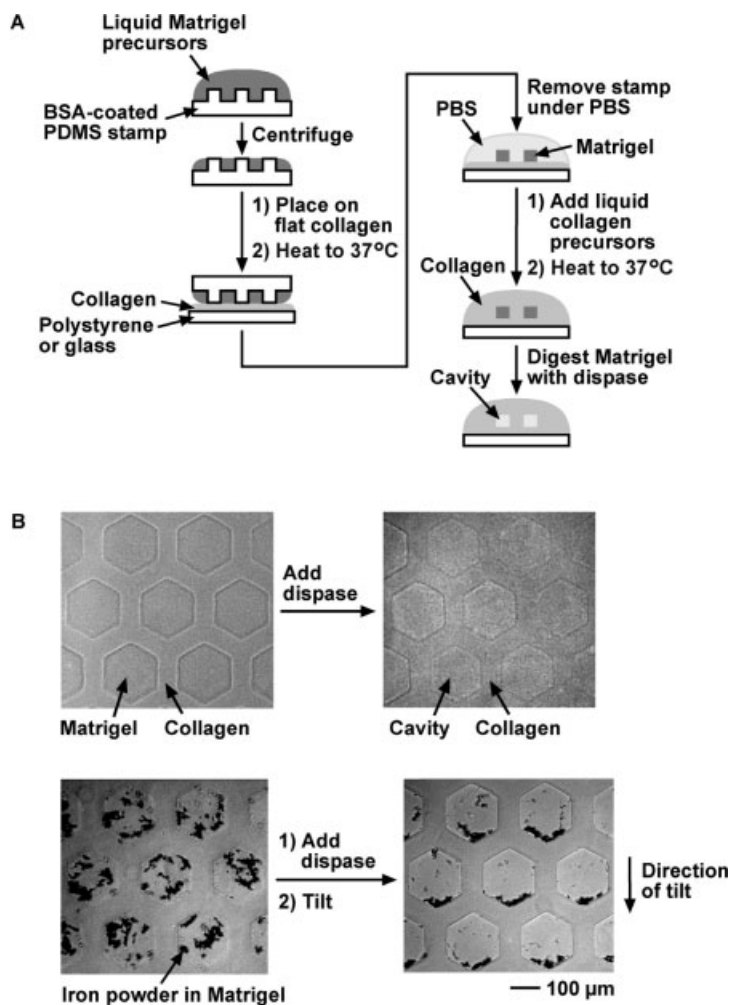
Figure 1. Fabrication of arrays of micrometer-scale cavities in collagen. A) Schematic diagram of the procedure. Shaded regions denote gels or their liquid precursors, cavities after digestion, and phosphate-buffered saline (PBS). After microtransfer molding, the gels were hydrated by immersion in PBS. B) Phase-contrast images of collagen gels that encapsulate hexagonal arrays of molded Matrigel, before and after selective digestion of Matrigel with dispase (2 U mL^{-1} in PBS, 37 °C, 1.5 h). Top: A hexagonal array (100 µm on a side, 100 µm thick). Bottom: A hexagonal array that incorporated iron powder (average size of iron particle ~10 µm) within Matrigel. After digestion was completed, tilting of the array (~90°, 10 min) allowed the released, free-floating iron particles to settle at the corners of the hexagonal cavities. Some iron particles were bound at the surface of the cavities. Images of each pair were taken from different representative regions of the same sample.

the stamp and substrate. For the liquid gel precursors used here, we found that centrifuging the Matrigel-filled stamp (50–200 g, 5–10 min) helped to reduce the thickness of this film. This step removed excess liquid Matrigel from the surface of the stamp, and allowed this liquid to dewet from the raised regions of the stamp. To determine whether a thin film of Matrigel persisted between molded features, we incorporated fluorescently labeled proteins (IgG or type IV collagen) into the liquid Matrigel before molding (Fig. 2B, $t = 0$ h). By fluorescence microscopy, an interconnecting film of Matrigel does not appear to be present after removal of the stamp.

Our experimental procedure places liquid precursors (of Matrigel or collagen) directly onto preformed gels (a collagen film or molded Matrigel/collagen composite). Since typical diffusion constants in dilute gels for ~100 kDa proteins (the approximate size of proteins in liquid collagen or Matrigel precursors[12,16]) are ~10 µm^2 s^{-1},[17] we expect proteins from these liquid precursors to diffuse ~1 µm into the underlying gels before gelation of the precursors is complete. This diffusion may help to bond the newly formed and pre-existing gels; it also restricts the resolution of this procedure to ~1 µm.

To use these structures in tissue engineering and basic cell biology, it is important to be able to introduce compounds to, and remove compounds from, the cavities. In the procedure outlined in Figure 1A, diffusion limits the rate of transport of molecules into and out of the cavities. To enhance transport in these gels, we used a gravity-induced pressure difference (~5 mm H$_2$O pressure) to introduce the flow of aqueous solution through the gel and cavities (Fig. 2A); under this pressure difference, the velocity of flow through a typical sample with dimensions of 1 mm × 1 cm × 1 cm was ~100 µm min^{-1}. To demonstrate the flow-enhanced transport of materials from the cavities, we incorporated fluorescently labeled IgG (~150 kDa) into Matrigel before molding, and used fluorescence microscopy to track the release and transport of the antibody out of the cavities after digestion. In the absence of imposed flow, the decay in fluorescence in the hexagonal Matrigel structures was extremely slow. We suspect that non-specific binding of the antibody with fibrils in Matrigel is sufficient enough to immobilize the antibody in the gel. Even after digestion of the Matrigel, diffusion of the antibody out of the cavities was limited (Fig. 2B, top).

In contrast, flow rapidly transported the initially bound protein out of the hexagonal cavities (Fig. 2B, bottom). Two hours after the introduction of flow, the intensity of fluorescence decreased by > 50 %; in the absence of flow, < 10 % of the signal was lost after 2 h. Six hours after the introduction of flow, the cavities lost nearly all of their fluorescence. At all times under flow, the initially uniform distribution of fluores-

digestion, the iron particles migrated freely within the boundaries of the hexagons; tilting the sample caused the iron particles to aggregate at the corners of the hexagonal shapes (Fig. 1B, bottom, right). This result suggests that the hexagonal structures no longer contained a mechanically stable gel after digestion, and that the boundaries of these fluid-filled cavities were identical to the boundaries initially defined by transfer molding.

Microtransfer molding often results in the formation of a thin (~100 nm) film[1] between molded features, presumably from incomplete exclusion of the liquid precursor between
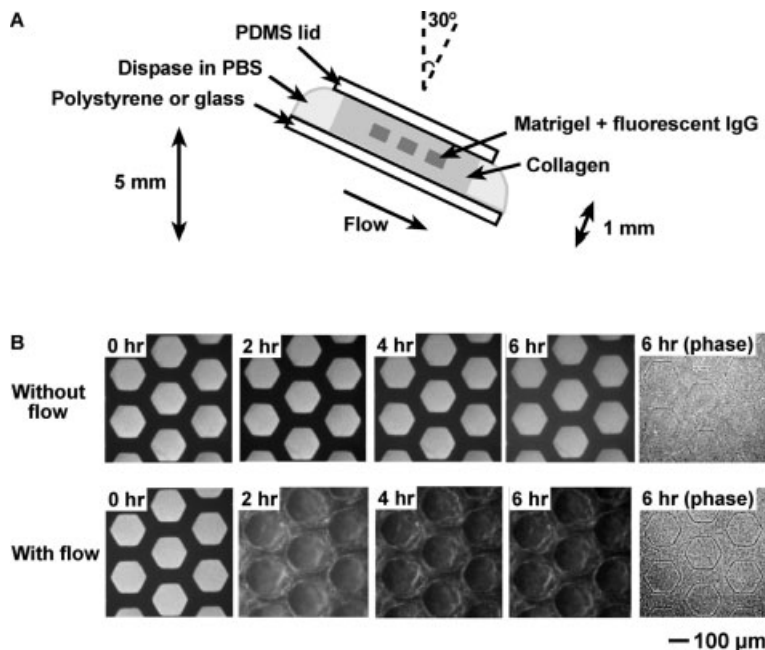
Figure 2. Flow-enhanced transport of fluorescent proteins out of cavities in collagen gel. A) Schematic diagram of experimental setup to introduce flow. The gels were supported on a polystyrene Petri dish or a glass cover slip, and were secured at two sides by two PDMS strips (1 mm thick; not shown in the side view) and at the top by a flat PDMS lid (1 cm × 1.5 cm). Tilting of the dish at an angle of ~30° created a pressure difference of ~5 mm H$_2$O; PBS was added at one end of the gel and replenished regularly. B) Time-lapse fluorescence images of a hexagonal array of Matrigel (containing fluorescent IgG at 200 μg mL^{-1}) in collagen gel, either in static PBS (top), or under flow (bottom). Dispase (2.5 U mL^{-1} in PBS) was added at $t = 0$ h and replaced by fresh PBS at $t = 2.5$ h. In the sample subjected to flow, the non-uniform fluorescence intensities outside the hexagons (bottom, 2–6 h) indicate the transport of fluorescent antibody out of the cavities in the direction of flow. Phase-contrast microscopy—top and bottom, 6 h (phase)—shows that the shapes of the hexagonal cavities were not noticeably altered by continuous flow.

cence of the hexagons (Fig. 2B, bottom) developed an outward "halo" that surrounded the cavities, and that polarized in the direction of flow. Although flow can enhance the transport of molecules into and out of the cavities, it does not alter the shapes of the cavities (Fig. 2B, 6 h). Thus, we have demonstrated that application of a pressure difference across the collagen gel can generate a directional flow that transports materials out of the cavities efficiently, and that preserves the geometry of the cavities.

We envision that the methods described here will find broad applicability in the tissue engineering of micrometer-scale epithelial structures. As an initial demonstration, we adapted those methods to fabricate a topologically unusual structure—a toroidal cavity—seeded by endothelial cells. Here, micromolding of Matrigel that contained a suspension of bovine pulmonary artery endothelial cells (BPAECs) (~10^7 cells mL^{-1}), and encapsulation by collagen, formed a composite of gels in which BPAECs were immobilized in the volume of a torus (Fig. 3). Treatment with dispase (2 U mL^{-1} in serum-containing media,

1.5 h) digested the Matrigel and released the cells, and they gradually settled within the cavities. Figure 3 shows images of these cells trapped at the surfaces of the toroidal cavities.

To determine whether the cells remained viable after this procedure, we diffused a fluorescent stain (Calcein AM, 5 μM) into the cavities (Fig. 3); this stain labels viable cells only. The high levels of fluorescence suggest that a sizeable population of the cells endured the experimental procedure of micromolding and digestion. Cells in these gels were able to be spread in the cavities within ~10 h after the dispase was replaced with fresh media. We have obtained similar results with other epithelial cell lines, including Madin–Darby canine kidney (MDCK) cells and normal rat kidney (NRK) epithelial cells. Delivery of non-epithelial cells into cavities is also possible, but these cells eventually migrate into the gel. Long-term (>1 day) culture of bovine pulmonary arterial endothelial cells (BPAECs) inside the cavities often resulted in invasion of collagen gel and/or mild contraction of the gel. We suspect that additional experimental conditions (e.g., the presence of shear stress, the introduction of agents to reduce cytoskeletal tension) will be needed to stabilize these cellular structures.

The use of micromolding to fabricate a composite of two distinct gels, and selective enzymatic digestion of one gel, represents a general strategy for the fabrication of cavities of defined shapes within gels. Many orthogonal pairs of gels and enzymes (e.g., agarose and agarase,[18] hyaluronic acid and hyaluronidase[19]) exist, and may be adapted to form cavities within gels other than type I collagen. This strategy enables the formation of gels that contain inner surfaces with arbitrary geometries of feature size >1 μm; gels
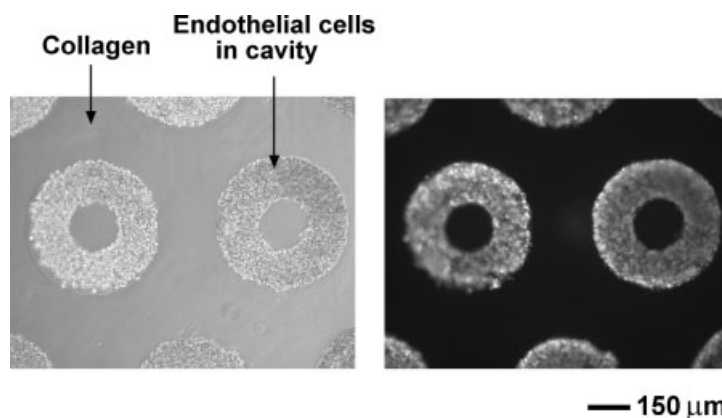

Figure 3. Cells encapsulated in toroidal cavities in collagen gel. Left: Phase-contrast image of an array of cavities with BPAECs in collagen gel, supported on a Petri dish in culture media. Right: Fluorescence image of the cells after diffusing a fluorescent viability stain (Calcein AM, 5 μM, 30 min in serum-free media) into the cavities.

with these topologies are difficult to fabricate with other lithographic techniques, such as photolithography.[10] Combined with our previous work,[5] which described methods to mold patterned composites of gels, the techniques discussed here allow the formation of structures that incorporate distinct populations of cells within or on the surface of a gel. These lithographic techniques thus enable the formation of micrometer-scale tissues in vitro that contain separate epithelial and mesenchymal compartments.

Experimental

Stamps were cast from patterned lithographic masters using polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning), as described previously [20]. Stamps were coated by adsorption of a monolayer of bovine serum albumin (fatty acid-poor BSA, Calbiochem; 1 % in PBS, > 1 h) to allow distortion-free detachment of molded gels [5]. To fabricate collagen gels with defined cavities, we first added liquid precursors of Matrigel (BD Biosciences; ~ 0.2–0.4 $\mu L\,mm^{-2}$) to the patterned surface of a treated stamp, and centrifuged the stamp (for isolated features, 200 g, 10 min, 4 °C; for interconnected features, 50 g, 5 min, 4 °C) to remove excess liquid Matrigel precursor from the raised regions of the stamp. The stamp was brought vertically into contact with a flat layer (~ 100 μm thick) of collagen gel (rat tail collagen type I, BD Biosciences, 3.66 mg mL^{-1}; pre-cooled to 4 °C) on a Petri dish or a glass cover slip, and heated to 37 °C at 100 % humidity (40–60 min) to gel the molded Matrigel. We removed the stamp by carefully adding excess PBS; the surface tension of PBS caused the stamp to detach spontaneously from the underlying gels. We then slowly flushed the surrounding PBS with liquid collagen precursors two to three times until the molded Matrigel was completely immersed in liquid collagen precursors; heating the sample to 37 °C at 100 % humidity (30 min) allowed the added collagen precursors to gel and thereby encase the Matrigel. Immersion of the composite of gels in dispase (GIBCO; 2–2.5 U mL^{-1} in PBS or culture media, 1.5–2.5 h, 37 °C) digested the Matrigel. The digestion was stopped by washing with fresh PBS or media.

To incorporate iron powder in Matrigel, iron powder (Polysciences, average size of iron particles ~ 10 μm) was coated with BSA (1 %, 1 h), and mixed with liquid Matrigel precursors at ~ 1:5 volume ratio. To incorporate cells in Matrigel, cells were trypsinized, washed with PBS, concentrated by centrifugation (300 g, 2 min), and mixed with liquid Matrigel precursors at ~ 1:2 volume ratio (~ 10^7 cells mL^{-1}). The iron particles or cells in liquid Matrigel precursors were allowed to settle in the relief features of the stamp (5–10 min, 4 °C) before centrifugation and removal of excess liquid Matrigel precursors. To generate fluorescent features of Matrigel, Alexa Fluor 488-conjugated goat IgG (Molecular Probes, 200 μg mL^{-1}), and Oregon Green 488-conjugated human collagen type IV (Molecular Probes, 250 μg mL^{-1}) were mixed with liquid Matrigel precursors.

Bovine pulmonary artery endothelial cells (BPAECs) (Cambrex BioScience) were cultured in Dulbecco's modified eagle's medium (DMEM) that contained 10 % calf serum (CS, GIBCO). Madin–Darby canine kidney (MDCK) cells (ATCC) were cultured in minimum essential medium that contained 10 % CS. Normal rat kidney (NRK) epithelial cells (ATCC) were cultured in DMEM that contained 10 % fetal bovine serum (GIBCO). Media were supplemented with 1 % penicillin–streptomycin–glutamine (GIBCO). Cells were cultured at 37 °C, 5 % CO$_2$, and 100 % humidity.

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Directed Growth of Ordered Arrays of Small-Diameter ZnO Nanowires**

By *Eric C. Greyson, Yelizaveta Babayan,* and *Teri W. Odom**

The controlled growth of optically functional nanometer-scale materials is of importance to several interesting scientific problems. These include two-dimensional (2D) photonic crystals, single-nanowire lasers, and chemical- and light-based sensors.[1–3] The ability to define the position, size, and density of nanostructures on surfaces enables detailed studies of the properties of individual structures and the collective properties of their assemblies. For example, one-dimensional (1D) arrays of equally spaced metallic nanoparticles are predicted

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