Facile and Biocompatible Fabrication of Chemically Sol–Gel Transitional Hydrogel Free-Standing Microarchitectures

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We report a facile method to fabricate free-standing, 3D hydrogel microarchitectures of chemically sol–gel transitional hydrogels, which is based on the use of hydrophilic substrate and aerosol of gelling agent without molding (or sandwiching) process. Using proposed methods, we fabricated hydrogel microarchitectures of sheets, meshes, or microunits without morphological distortions on the microscale. These hydrogel microarchitectures could be easily and stably exfoliated from the substrates and cultured (in the case of containing cells). These free-standing hydrogel microarchitectures in sheets, meshes, or microunits can be easily harvested and assembled as a biofabrication unit to generate complex composites with controlled microscale structures.

1. Introduction

Hydrogels are promising biomaterials with intrinsic ability to facilitate the transport of oxygen, nutrients, and other water-soluble metabolites through diffusion and with capability to be tailored to have desirable physicochemical cues to resemble native extracellular matrix (ECM).1,2 These features are especially important when cells are encapsulated in hydrogels that can be used for 3D cell culture scaffolds and cell-delivery vehicles in cell-based therapy.3–5

Hydrogel patterning on a microscale is desirable because engineered hydrogel microstructures can be utilized to adjust in vitro recapitulation of native ECM and morphogenesis of embedded cells.6,7 Furthermore, cellular arrangements can be controlled with the microstructures, which is important to replicate the biological and mechanical functions of in vivo tissues.8 To date, hydrogels of photo-cross-linkable or thermosensitive types have mainly been used for gel microstructuring by utilizing photolithography or micromolding techniques. However, these methods cannot be adapted to other hydrogels of chemically cross-linkable or pH-sensitive types, such as alginate or chitosan.

In previous studies, the construction of gel microstructures using chemically sol–gel transitional hydrogels should have utilized “diffusively semi-permeable physical barriers” such as porous membranes or agarose molds due to rapid gelling property of hydrogels upon contact with gelling agents. Cabodi et al. fabricated microfluidic structures inside millimeter scale alginate slab by injecting the hydrogel precursor into a mold enclosed with semipermeable membrane at the top and delivering a gelling agent by diffusion through the membrane to prevent morphological distortion.9,10 The membrane setup, however, delayed a rapid cross-linking process of the hydrogels (∼1 h). Long gelation time may be disadvantages for the creation of cell-containing microstructures, which could affect cellular viability or metabolism. Franzesi et al. fabricated alginate microstructures by sandwiching alginate solution between a substrate and an agarose mold, which contained and released a gelling agent and subsequently cross-linked the molded alginate.11 These procedures need preparations of not only poly(dimethylsiloxane) (PDMS) molds but also additional setup of agarose molds, which may not be reusable or stable for long-term period. Here we propose a simple direct delivery method of the gelling agent as a nebulized aerosol to cross-link and construct free-standing hydrogel microarchitectures, without molding (or sandwiching) process.

2. Experimental Section

Preparation of Hydrogel Precursor, and Cross-Linking and De-Cross-Linking Agents. Sodium alginate (from brown alga: Sigma-Aldrich) powder was dissolved in phosphate-buffered saline (PBS) with 0.5% w/v ratio overnight at room temperature. As a cross-linking agent for alginate hydrogel, 100 mM calcium chloride (CaCl2) was prepared in distilled deionized water (ddH2O). As a de-cross-linking agent for alginate gel, 160 mM sodium citrate (Na2C6H5O7) was prepared in ddH2O and utilized as a calcium chelator from calcium alginate gel.12 The prepared solutions of sodium alginate, CaCl2, and Na2C6H5O7 were sterilized by germicidal ultraviolet (UV) for 30 min (and/or filtrated through 0.22 µm pore-sized bacterial filter) inside a clean bench before use.

Mammalian Cell Culture. A human liver carcinoma cell line, HepG2, was purchased from American Type Culture Collection as a frozen vial and was cultured and maintained with Dulbecco’s modified Eagle’s media supplemented with 10% fetal bovine serum and 1% streptomycin–streptomycin in a 5% CO2 humidified incubator at 37 °C. Confluent dishes of HepG2 cells were split every 5 days with a ratio of 1:3, and the culture media was exchanged every other day after washing with PBS.

Suspensions of Microbeads and Cells in Hydrogel Precursor. To prepare microbeads suspensions in sodium alginate solution, we added the bead stock solutions of 20 µL to 0.5% w/v alginate solution of 1 mL, which was vortexed thoroughly before use. We used 6 µm green fluorescent isothiocyanate (FITC), and red Rhodamine B fluorescent beads. To prepare cell suspension in alginate solution, HepG2 cells on a tissue culture dish were harvested using trypsin-EDTA and centrifuged at 1000 rpm for 3 min. After supernatant removal, remaining HepG2 cell pellet was resuspended in alginate solution with gentle pipet up-and-down. The cell concentrations in alginate solution were prepared in the range of (0.5 to 5) × 106 cells mL⁻¹.
Preparation of PDMS Substrates. We utilized PDMS substrates to examine the ability of thin hydrogel sheet fabrication using a hydrophilic surface. PDMS is biologically inert and provides excellent optical transparency for observing the overlaid hydrogel sheets on it. To prepare the PDMS substrates, we constructed designed patterns of SU-8 photoresist (PR) on a silicon wafer through standard photolithography. Protruded SU-8 hexagonal patterns had side length of 130 \( \mu \text{m} \), interval between adjacent hexagons of 50 \( \mu \text{m} \), and height of 65 \( \mu \text{m} \). The silicon wafer having the SU-8 PR patterns was silanized by trichloro(1H,1H,2H,2H-perfluoroctyl)isilane and then casted by pouring a degassed 10:1 mixture of PDMS prepolymer and curing agent ethyl alcohol (in ddH2O), rinsed by ddH2O and PBS, and dried inside a clean bench. Subsequently, the PDMS substrates were UV sterilized for 30 min and treated with plasma cleaning for 30 s to derive hydrophilic surface and used for cell works. For this study, prepared PDMS substrates could be reused several times (>10 times) for >6 months with brief tap water washing after use.

Live/Dead Analysis for Cell Viability. A cell viability assay was performed on the cells right after the fabrication of cell-laden hydrogel microarchitectures using a commercially available mammalian cell viability and cytotoxicity assay kit (Molecular Probes, MA). The samples were incubated for 40 min in a solution of 5 \( \mu \text{L} \) of calcine-AM and 20 \( \mu \text{L} \) of ethidium homodimer-1 in 10 mL of PBS. Cellular fluorescence was observed in inverted epiphluorescent microscopes (IX51; Olympus, Japan and Axiosvert 25; Carl Zeiss, Germany) using FITC/RhoA band filters. Alternatively, a trypan blue exclusion assay was conducted to check the cell viabilities using 0.4% trypan blue solution (Invitrogen, CA).

Uniformity Analysis of Alginate Gelation. Methylene blue, which is positively charged, interacts with the carboxylic groups of alginate. The intensity of methylene blue staining visualizes the concentration of alginate molecules.\(^1\) Methylene blue trihydrate (methylthionine chloride, C\(_{16}\)H\(_{18}\)ClN\(_3\)S·3H\(_2\)O; \( M_\text{w} \), 373.9) was purchased from Sigma. Methylene blue staining solution of 1 \( \times \) 10\(^{-5} \) M was prepared in distilled water and used to stain the alginate hydrogel structures with gentle rocking for 15 min. The stained hydrogel structures were inspected using a microscope (IX51; Olympus) and a stereomicroscope (SZX16; Olympus). For side-view analysis of the hydrogel sheets, the stained sheets were located on a polycarbonate film, blade-cut with the film, and examined via the microscope. The staining image results were analyzed by the surface plot using ImageJ software (http://rsb.info.nih.gov/ij/). Additionally, to check the uniform thickness of alginate sheets, double-layered hydrogel sheet composites (Supporting Information) were inspected using a confocal laser scanning microscope (LSM 510 NLO; Carl Zeiss) and rendered in 3D using ImageJ software.

3. Results and Discussion

As shown in Scheme 1A, hydrophilic PDMS with micropatterns was utilized as a substrate, and a controlled volume of hydrogel precursor was dropped and evenly spread on the substrate as a thin film. Hydrophilic substrate at 0\(^\circ\) or low contact angle was desired to form a flat top surface of hydrogel precursor spread. Next, nebulized aerosol of gelling agent, via a nebulizer with ultrasonic transducer, was directly delivered to coat (but not to drench) the top surface of hydrogel precursor, which cross-linked and gelled the hydrogel without morphological distortions of microstructures. By utilizing a nebulizer (a medicated spray) unit, ultrafine mist of 1–5 \( \mu \text{m} \) diameter aerosol droplets can be generated. The quantity of the delivered gelling agent was controlled by the time duration of nebulization in the range of 10 s to 2 min, which could be varied by experimental conditions such as concentration of cross-linking agent, nebulizing performance of nebulizer used, or both. We exfoliated the fabricated hydrogel sheets from substrate as free-standing units by submerging the sheets overlaid on the substrates in a solution such as distilled water, PBS, or cell culture media and then gentle pipetting around the sheets to help the exfoliation. Cell-containing hydrogel sheets were sometimes adhered to and not easily exfoliated from the substrates, so the surface treatment of substrates with bovine serum albumin was performed to exfoliate the cell-containing sheets reliably.\(^1\)

We demonstrated the versatility of this method by generating patterned hydrogel microunits or meshes, as shown in Scheme 1B. Except for substrate (no boundary walls) and an additional step, all procedures were the same as those in Scheme 1A. To make hydrogel microunits or meshes, we removed excessive hydrogel precursor by swiping using a flat hydrophobic PDMS block, and subsequently, hydrogel precursor remained only in microwells or dented hexagonal microlines. After gelling, these patterned hydrogel microunits or meshes were released by gentle pipetting.

As a model for chemically sol–gel transitional hydrogels, calcium alginate was used in this study, which is a naturally driven biocompatible and biodegradable hydrogel widely used for encapsulation of cells, including 3D cell culture and scaffolding for tissue engineering. Figure 1A,D shows fabricated free-standing alginate microarchitectures using Scheme 1A,B (with a hydrogel precursor of 0.5% w/v sodium alginate in PBS and its gelling agent of 100 mM CaCl\(_2\) in ddH2O), respectively. In considering both diffusion limitation and mechanical stability, the alginate sheets (10 \( \times \) 10 mm\(^2\)) were designed and fabricated as \( \sim 150 \mu \text{m} \) thick with repetitive hexagonal patterns with an interval of 50 \( \mu \text{m} \) (Figure 1A, inset), and alginate microunits were replicated using the same hexagonal patterns with side length of \( \sim 130 \mu \text{m} \) and height of \( \sim 65 \mu \text{m} \) (Figure 1D) as the same dimensions of the used PDMS substrate. Figure 1B,E shows microarchitectured alginate sheets and microunits encapsulating 6 \( \mu \text{m} \) red fluorescent microbeads as a simulation of macromolecules or cells, respectively. For the patterning of microbeads (or cells) into microwells during sheet fabrication, gentle sweeping (similar to previously reported method\(^1\)) was performed on the hydrogel precursor film before gelling agent delivery. Additionally, a few standing (or uprighted) microunits showed its side view, which confirmed that the top surface
profile was smooth and flat and the encapsulated beads were distributed throughout the depth of the microunits (Figure 1E). For cellular study or tissue engineering applications, HepG2 as a mammalian cell was encapsulated in the alginate sheets and microunits, as shown in Figure 1C,F, respectively. The cell-containing microarchitectures can be used as cellular building blocks for modular tissue engineering. Recently, assembly techniques for patterned microunits have been developed using microfluidics16 or self-assembly techniques, 17 using mostly photo-cross-linkable hydrogel. We believe that our fabrication technique and microarchitectures could utilize the assembly techniques without using UV and expensive optical setups.

The encapsulated HepG2 cells in the fabricated hydrogel structures were highly viable with viability of 95.57 ± 0.99% (n = 23). A commercially available viability and cytotoxicity assay kit containing calcein-AM and ethidium homodimer-1 (Molecular Probes) was used for the staining. Dead cells show red, whereas live cells show green (Figure 2B). In the patterned sheets, 3D culture morphology was also clearly observed in Figure 2C,D. Microarchitected alginate sheet containing patterned HepG2 cells was released and reattached on a culture dish and cultured for 1 week. Interestingly, patterned cells were proliferated and aggregated not randomly but as they were initially patterned (Figure 2C), so the formed HepG2 aggregations at culture days 4–6 had regular arrangement and uniform size (Figure 2D). These kinds of cellular patterning in free-standing hydrogel sheets should have certain applications to study interactions in cell-to-cell or cell-to-ECM in a defined 3D microenvironment and tissue morphogenesis.

Next, the gelation uniformity of the fabricated hydrogel structures was analyzed using methylene blue staining. The gelation uniformity could affect the 3D microarchitectures of fabricated hydrogel structures. The stronger intensity of the staining indicates the higher concentration of alginate molecules.13 Figure 3A–C shows stereomicroscopic images of the stained free-standing alginate sheets (Figure 3A,B) and meshes (Figure 3C) having hexagonal microarchitectures. The hexagonal meshes have the same dimensions of hexagonal microunits. The sheets and meshes were stably rolled and folded manually using pipet tips in distilled water or PBS. Figure 3D shows a microscopic phase contrast image of the stained sheet with hexagonal microarchitectures. In the sheet, regions of hexagonal micropatterns were thicker than other regions, so the stained intensities were higher in hexagonal regions, whereas the intensities were nearly the same among hexagonal patterns. Figure 3G is a surface plot representation of the image in Figure 3D. The Z axis of a surface plot is the luminance of an image. Hexagonal regions are relatively sunken compared with other regions because of the higher intensity of the staining. Hexagonal regions are relatively sunken compared with other regions because of the higher intensity of the staining. Figure 3E is a staining image of a flat alginate sheet. The surface plot in Figure 3H visualizes a distinctly sunken and uniform area of the flat alginate sheets compared with the other regions, which means the uniform gelation throughout the area of the sheet. Figure 3F is a side view of the sheet in Figure 3E. The hydrogel sheet was placed on a polycarbonate film, blade-cut, and examined. Figure 3I is a surface plot representation of alginate sheet side view (with polycarbonate support) in Figure 3F. The sheet side shows distinctly and uniformly sunken surface plot rather than polycarbonate region, which means that the alginate molecular concentration was also uniform throughout the thickness (~150 µm) of the alginate sheet.

As a potential application of microarchitected hydrogel sheets, we also demonstrated the ability to generate double-layered composites of the sheets (Supporting Information).
Figure 3. Fabricated alginate hydrogel sheets and meshes stained with methylene blue and the inspection of uniform alginate gelation. The stronger intensity of the staining indicates the higher concentration of alginate molecules. (A–C) Stereomicroscopic images of free-standing alginate sheets and meshes having hexagonal microarchitectures. Rolled sheet in panel A, randomly folded sheet in panel B, and rolled mesh in panel C showed the flexibility and mechanical stability of the fabricated hydrogel structures. (D–F) Microscopic phase contrast images of alginate sheets stained with methylene blue: (D) alginate sheet with hexagonal microarchitectures, (E) flat alginate sheet, and (F) the side view of the sheet in panel E covered with a water layer to prevent dehydration of sheet. (G–I) 3D representations of surface plot analysis of panels D–F, respectively. Z-axis of the plot is the luminance of an image. (G) Hexagonal alginate sheets show the lower surface plots in hexagonal regions than other regions because the hexagonal regions have the stronger intensity in panel D. (H) Flat alginate sheet in panel E shows a uniform concentration of alginate molecules throughout the sheet. (I) Surface plot analysis on regions of alginate and polycarbonate support. Alginate molecular concentration was uniform throughout the thickness (∼150 μm) of the alginate sheet.

Figure 4. Hydrogel sheets double-layering using two different schemes of construction. (A–C) Orange (upper layer) and green (lower layer) fluorescent images were merged on phase contrast images. (A) The patterned sides of two hydrogel sheets were directly contacted to each other, shown in the same depth of view. The patterned sides of two sheets were noncontacted, which are composed of two distinctive layers with patterns (B) in depth of focus at upper layer (orange) and (C) in depth of focus at lower layer (green). The gap between the two distinctive layers was 80 μm. (D,E) 3D rendered views of the two schemes: (D) contact mode in panel A and (E) noncontact mode in panels B and C. Orange in panels A–C is shown as red. In a side view of panel E, noncontacted two layers in panels B and C are distinctive. All scale bars are 100 μm.

Figure 4A,B (or C) is two different schemes and corresponding demonstrations of double-layered composites using micropatterned hydrogel sheets. The fabricated sheets in this study were micropatterned at only one side, so the patterned sides of two different layers could be either directly contacted to each other (Figure 4A; contact mode) or composed as two distinctive layers
(Figure 4B,C; noncontact mode). In contact mode, two patterned layers (orange and green merged on phase contrast image in Figure 4A) were observed at the same depth of view by epifluorescent microscope, whereas in noncontact mode, two patterned layers were observed with a 80 µm gap between top (orange) and bottom (green) layers at the same field of view (Figure 4B,C, respectively). Figure 4D,E presents 3D rendered inspections of the two schemes of contact mode (Figure 4A) and noncontact mode (Figure 4B,C), respectively. In Figure 4D,E, the orange color in Figure 4A–C is shown as red. In a side view of Figure 4E, noncontacted two layers in Figure 4B,C are distinctive. These two double-layering schemes of contact and noncontact modes should have certain applications to study 3D cell-to-cell or cell-to-ECM interactions based on the 3D ECM geometries and cell patterns in microscale.

4. Conclusions

These results demonstrate that gelling agent introduction as a nebulized aerosol is a facile method for distortion-free microarchitecture fabrication of chemically sol–gel transitional hydrogels in forms of sheets, meshes, or microunits. In this method, the hydrophilic surface of PDMS substrate was important to minimize the surface tension effects of hydrogel precursors and consequently exclude the molding (or sandwiching) process. The fabricated hydrogel microarchitectures can be easily and stably exfoliated from the substrates as free-standing units, cultured in the case of containing cells, and potentially assembled to generate complex composites. Stacking of two micropatterned hydrogel sheets represents a fabrication strategy for 3D cell-hydrogel composites thorough layer-by-layer assembly. The proposed method can be applied for a variety of applications such as 3D cell culture systems, tissue morphogenesis study, and modular biofabrication of artificial tissues.

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Supporting Information Available. Experimental details and results, including double-layering procedure of hydrogel sheets and cell patterning data. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes


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