PATTERNING OF MULTILAYERED MICRO HYDROGELS ON PDMS SUBSTRATES PARTIALLY EMBEDDING CaSO₄ POWDERS
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ABSTRACT
Here we present a simple method for fabricating hydrogel micropatterns with multilayered structures. PDMS regions embedding CaSO₄ powders were patterned on planar PDMS substrates, by introducing PDMS prepolymer with CaSO₄ powders into bare PDMS microwells. Simply by pouring a sodium alginate (NaAlg) solution on the micropatterned PDMS plate, hydrogel patterns are formed because of the supply of calcium ions from the PDMS matrix. We successfully prepared multilayered and micropatterned hydrogels, employing several strategies to selectively introduce CaSO₄ powder-containing PDMS. In addition, we cultured two types of cells within the layered hydrogels and examined the effect of coculture on cellular functions.

KEYWORDS: Hydrogel, Patterning, Multilayer, Cell culture

INTRODUCTION
Micropatterned hydrogels are useful as platforms for conducting efficient drug screening, constructing shape-controlled micro tissues and organs, and fabricating biomolecule microarrays. Several studies have been proposed for fabricating micropatterned hydrogels such as photolithography, inkjet printing, and microcontact printing [1, 2]; however, methods that allow us to easily produce multilayered, patterned hydrogels have not been fully developed. In this study, we propose a facile method to prepare multilayered alginate hydrogel patterns on PDMS by utilizing calcium supply from the PDMS substrate.

EXPERIMENTAL
The procedure for fabricating multilayered hydrogel patterns is shown in Fig. 1. First, patterned PDMS substrates were prepared by introducing PDMS prepolymer containing CaSO₄ powders into PDMS microwells. Then an aqueous solution of NaAlg was poured on the PDMS substrate, to form patterned hydrogels thanks to the calcium ions supplied from the CaSO₄ powder-containing PDMS region. By repeating the deposition of NaAlg solution with different compositions, multilayered hydrogels are fabricated.

In the experiment, we used CaSO₄ powders with the diameter smaller than ~50 μm. PDMS plates having CaSO₄ powder-containing regions were prepared by introducing the PDMS prepolymer with the powders at a concentration of 30% (w/v) into the microwells (Fig. 2). The PDMS surface was then coated with poly-L-lysine (PLL) to order to improve the adhesion of alginate hydrogels on the channel surface. Next, an aqueous solution of 1% NaAlg was poured onto the PDMS plate, to form Ca-alginate hydrogels with shapes corresponding to the PDMS patterns. After a certain period of time, non-gelled NaAlg solution was removed by dipping the entire device in a buffer solution.
Then, linearly patterned PDMS plates were fabricated by employing a microchannel method, as shown in Fig. 3. PDMS prepolymer with 30% (w/v) CaSO₄ powders was introduced into the dead-ended PDMS microchannel, which was physically contacted with a planar dish (Fig. 3), using the vacuum-driven pumping scheme; the microchannel was degassed and then the PDMS prepolymer with CaSO₄ powders was introduced into microchannel by pouring the prepolymer solution on the inlet.

Next, for the purpose of obtaining more precisely patterned hydrogels, we employed sodium citrate powder-incorporating PDMS plates. We expected that non-specific hydrogel formation is decreased on the PDMS surface because citrate can chelate calcium ions (Fig. 4).

Finally, we demonstrated the coculture of hepatocytes (HepG2 cells) and fibroblasts (NIH-3T3 cells) as a biological application. HepG2 and NIH-3T3 cells were individually suspended in 1% NaAlg solutions at concentrations of 2.0×10⁷ and 6.0×10⁷ cells/mL, respectively, and then double layered hydrogel structures were formed in which these cells were encapsulated on different levels of the hydrogel. Cells were cultured in DMEM supplemented with 10% FBS at 37°C for 3 days, and their functions were analyzed using quantitative PCR.

RESULTS AND DISCUSSION

Fig. 5 shows the obtained multilayered hydrogel, when a NaAlg solution was poured onto a PDMS plate with an array of circular CaSO₄ powder-containing region. After the first deposition process (Fig. 5 (a)), a hydrogel layer with a uniform thickness of ~100 μm was generated, but hydrogel patterns were not formed probably because of the remaining CaSO₄ powders on the entire PDMS surface. Multilayered hydrogel was formed by repeating the deposition of NaAlg solution, with a thickness of 300-400 μm.

**Fig. 2:** Photographs showing the preparation process of the micropatterned PDMS with CaSO₄ powder-containing regions. (a) A PDMS plate having microwell structures was prepared by replica molding. (b) PDMS prepolymer with CaSO₄ powders was then introduced into the microwells and (c) the remained prepolymer was swept away using a glass slide. (d) Photograph and (e) micrograph of the patterned PDMS after curing.

**Fig. 3:** (a, b) Schematic images showing the fabrication process of patterned PDMS using a microchannel structure.

**Fig. 4:** Schematic image showing the cross-sectional images of hydrogel patterns using sodium citrate powder-embedding PDMS plates.

**Fig. 5:** Fluorescence micrographs showing the cross-sectional images of (a) monolayer, (b) 2 layers, and (c) 3 layers of alginate hydrogel. (d) Fluorescence and (e) bright field micrographs showing the cross section of the multilayered alginate hydrogel, prepared by using circular array of CaSO₄-containing regions.
We then attempted to fabricate distinct hydrogel micropatterns using a PDMS plate with a microchannel structure. We introduced PDMS prepolymer with CaSO$_4$ powders into the dead-ended channel. After pouring the NaAlg solution onto the patterned PDMS plate, we confirmed that micropatterns of the hydrogels were actually formed, with a shape corresponding to the microchannel structure (Fig. 6 (a-c)). In addition, we confirmed that the pattern size was decreased when the Na-citrate/CaSO$_4$ patterns on a PDMS plate were used (Fig. 7).

Finally, we cultured hepatocytes (HepG2 cells) and fibroblasts (NIH-3T3 cells) on different levels of double layered hydrogel structures. As a result, we successfully prepared multilayered Ca-alginate hydrogels embedding two types of cells. In addition, we confirmed that the functions of HepG2 cells were upregulated for the coculture condition compared with the single culture of hepatocytes (Fig. 8).

**Fig. 6:** Micrographs showing (a) upper view and (b, c) cross-sectional view of the formed hydrogel with a straight pattern.

**Fig. 7:** Hydrogel patterns using (a) 1% (w/v) and (b) 2% (w/v) sodium citrate powder-embedding PDMS plates.

**Fig. 8:** Gene expressions of HepG2 cells within the patterned hydrogels analyzed by real-time RT-PCR. CYP1A2: cytochrome P450 1A2, CYP3A4: cytochrome P450 3A4, ALB: albumin. GAPDH was used as the housekeeping reference. Hep: hepatocytes only; Hep+3T3: coculture of hepatocytes and NIH-3T3 cells.

**CONCLUSIONS**

We have successfully prepared multilayered hydrogel patterns using the patterned PDMS substrates. The presented technique for producing the multilayered micro hydrogels would be useful for tissue engineering, cell-based drug screening, and preparation of biomolecule arrays for biochemical sensing.

**ACKNOWLEDGMENTS**

This study was supported in part by Grants-in-aids for Scientific Research (23106007 and 25750171) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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