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Biomaterials and Tissue Engineering

# Micropatterned cell co-cultures using layer-by-layer deposition of extracellular matrix components

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## Abstract

Micropatterned cellular co-cultures were fabricated using three major extracellular matrix components: hyaluronic acid (HA), fibronectin (FN) and collagen. To fabricate co-cultures with these components, HA was micropatterned on a glass substrate by capillary force lithography, and the regions of exposed glass were coated with FN to generate cell adhesive islands. Once the first cell type was immobilized on the adhesive islands, the subsequent electrostatic adsorption of collagen to HA patterns switched the non-adherent HA surfaces to adherent, thereby facilitating the adhesion of a second cell type. This technique utilized native extracellular matrix components and therefore affords high biological affinity and no cytotoxicity. This biocompatible co-culture system could potentially provide a new tool to study cell behavior such as cell-cell communication and cell-matrix interactions, as well as tissue-engineering applications.

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**Keywords:** Patterned co-culture; Layer-by-layer; Hyaluronic acid; Fibronectin; Collagen

## 1. Introduction

Tissue formation and cellular function in vivo are regulated by diverse biological factors including cell-cell communication, cell-matrix interactions, and soluble factors. The ability to recreate such interactions in vitro may lead to advances in diverse fields, ranging from cell biology to tissue engineering. For example, tissue-engineering constructs that aim to restore and enhance natural tissue function should ideally incorporate features of complex tissues, such as the integration of multiple cell types with appropriate extracellular matrices [1]. The manipulation of the cell microenvironment by the modula-

tion of cell-cell communication and cell-matrix interaction is the first step for building up such constructs for tissue replacement [2].

Many approaches to manipulate the cell microenvironment have been conducted on micropatterned surfaces. These approaches have been based on a number of fabrication strategies such as photolithography, microcontact printing, micromolding, inkjet printing and dip-pen spotting [3-6]. In most approaches, cells have been localized to adhesive regions on a substrate, thus limiting their use to one cell type. More recently, approaches have been developed to pattern two or more cell types in spatially defined co-cultures [7]. These approaches can be used to study the effects of cell shape, cell-matrix interactions, and heterotypic cell-cell contact interactions on various cell functions [8,9]. Many initial studies on patterned co-cultures have involved the selective adhesion of one cell type compared to the adhesion of the other. For example, hepatocyte-fibroblast co-cultures can be fabri-

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1 cated on collagen-patterned substrates by allowing hepa-  
 3 cytes to adhere to the patterned collagen-coated regions  
 5 with fibroblasts only adhering to the non-collagen-coated  
 7 regions [10]. Recently, the development of surfaces that can  
 9 be switched from cell-repulsive to cell-adhesive based on  
 11 specific stimuli has attracted attention [11]. These ap-  
 13 proaches are advantageous since they can be used to form  
 15 patterned co-cultures irrespective of the cell types or  
 17 seeding order. For example, a micropattern of electroactive  
 19 polymers can be switched from hydrophilic to hydrophobic  
 21 to promote cell adhesion [12–14], or thermally responsive  
 23 polymers can be used to change surfaces from cell-adhesive  
 25 to cell-repellant by changing the temperature [15,16].  
 27 Magnetic beads can also be used to assemble magnetically  
 29 labeled cells in specific regions and release them by turning  
 31 off the magnetic force [17]. Although these techniques can  
 33 be used to manipulate a cell's microenvironment, several  
 35 obstacles limit their widespread use. Some of these  
 37 approaches require specialized materials, devices, and  
 39 extensive expertise. In addition, many synthetic polymers  
 41 used in these systems are not optimized for interactions  
 43 with cells and lack biological function associated with  
 45 natural extracellular matrices. These polymers and mag-  
 47 netic beads may influence protein and gene expression and  
 49 have cytotoxic effects [18,19]. Thus, the development of  
 51 easily applicable, biocompatible, and versatile micropat-  
 53 terning approaches for controlling homotypic and hetero-  
 55 typic cell contact is of benefit.

One potential method that may provide avenues for  
 controlling the *in vitro* cell microenvironment is the use of  
 layer-by-layer deposition of biopolymers [20,21]. Layer-by-  
 layer deposition is a simple method to construct polymeric

layers using electrostatic forces and can be used with many  
 natural biopolymers such as polysaccharides and proteins.  
 Hyaluronic acid (HA) is an anionic polysaccharide  
 composed of disaccharide repeat units which can complex  
 with cationic polymers. As an integral part of the  
 extracellular matrix, HA is cell-repellant *in vitro* [22]. We  
 have previously developed a technique that used the layer-  
 by-layer deposition of HA and a cationic polymer, poly-L-  
 lysine (PL), to pattern various cell types [23]. In this  
 approach, the ionic adsorption of PL to HA patterns was  
 used to switch HA surfaces from cell-repulsive to cell-  
 adherent, thereby facilitating the adhesion of a second cell  
 type. The main limitation with the previous approach was  
 that PL was not a desirable extracellular matrix component  
 and was shown to be cytotoxic at high concentrations [24].  
 Collagen is a major structural protein that facilitates cell  
 attachment. Recently, the formation of a stable layer of  
 collagen on HA-coated surfaces has been reported [25,26].

The present study demonstrates that layer-by-layer  
 deposition of HA and collagen can be used to switch  
 surface properties for micropatterning cellular co-cultures.  
 The procedure used in this study consists of simple steps as  
 shown in Fig. 1. HA was micropatterned on a glass  
 substrate by using a soft lithographic method and its  
 capillary force. The exposed region of a glass substrate was  
 coated with fibronectin (FN). Cells were then selectively  
 adhered to the FN-coated regions. The HA-coated surface  
 was complexed with collagen, allowing for the subsequent  
 adhesion of secondary cells. This method may potentially  
 be used for fabricating well-controlled cellular microen-  
 viroments.

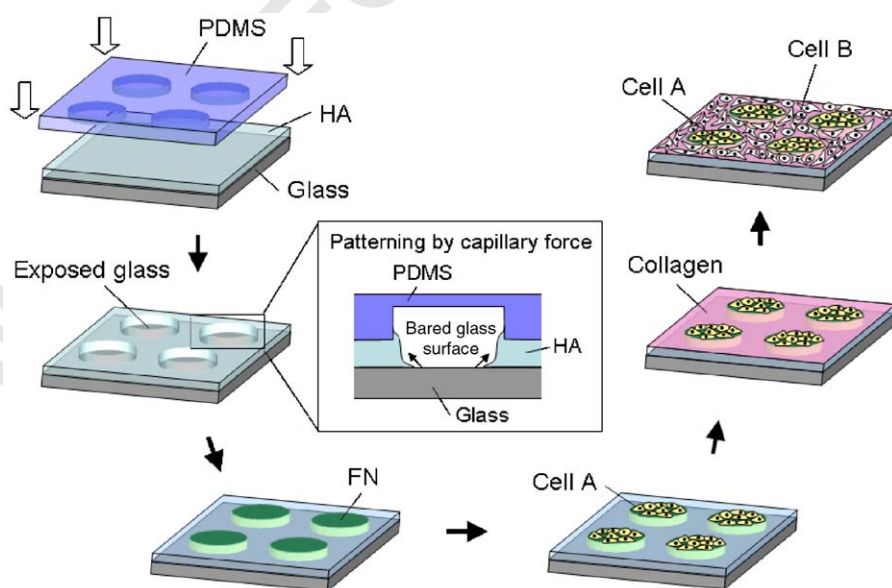


Fig. 1. The scheme for fabrication of the co-culture system using capillary force lithography and layer-by-layer deposition. A few drops of HA solution were spun coated onto a glass slide, and a PDMS mold was immediately placed on the thin layer of HA. HA under the void space of the PDMS mold receded until the glass surface became exposed. The exposed region of a glass substrate was coated with FN, where primary cells could be selectively adhered. Subsequently, the HA surface was complexed with collagen, allowing for the subsequent adhesion of secondary cells.

## 2. Materials and methods

### 2.1. Materials

All tissue culture media and serum were purchased from Invitrogen Corporation, and cell lines were purchased from American Type Culture Collection. All chemicals were purchased from Sigma, unless otherwise indicated.

### 2.2. HA coating and its characterization

A few drops of HA solution containing 5 mg HA/mL in distilled water or phosphate buffered saline (PBS) were spun coated onto a plasma cleaned glass slide at 1500 rpm for 10 s. The stability of the HA film in air or PBS at room temperature was analyzed in terms of the level of adsorption of fluorescein-isothiocyanate (FITC)-labeled BSA for 14 days of incubation.

To test protein adsorption on the HA surface, solutions containing FN (100 µg/mL), FITC-labeled PL (40 µg/mL), or FITC-labeled type I collagen (500 µg/mL) in PBS were prepared. To test for FN adhesion, HA-coated slides were dipped into a solution of FN for 15 min and subsequently rinsed to remove unbound FN. Surfaces were then stained with anti-FN antibody for an additional 45 min, followed by 1 h incubation with the phycoerythrin (PE)-labeled anti-rabbit secondary antibody. To measure the adsorption of other proteins, a few drops of the PL or collagen solutions were evenly distributed onto the HA surface, incubated at room temperature for 45 min, and subsequently rinsed to remove unbound protein. These stained surfaces were analyzed using a fluorescent microscope (Axiovert 200, Zeiss).

### 2.3. Cell culture

NIH-3T3 cells were maintained in 10% fetal bovine serum (FBS) in Dulbecco's modified eagle medium (DMEM). Murine embryonic stem (ES) cells (R1 strain) were maintained on gelatin-treated dishes on a medium comprised of 15% ES-qualified FBS in DMEM knockout medium. AML12 murine hepatocytes were maintained in a medium comprised of 90% 1:1 (v/v) mixture of DMEM and Ham's F12 medium with 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium, and 40 ng/mL dexamethasone, and 10% FBS. All cells were cultured at 37 °C, 5% CO<sub>2</sub> in humidified incubator and passaged every 3 days.

### 2.4. Cell adhesion and viability assay

NIH-3T3 cells were seeded at a concentration of  $1 \times 10^6$  cells/mL on HA surfaces treated with either FN or PL and type I collagen (extracted from rat tails, BD bioscience), and attached cells were counted with hemacytometer after 8 h of incubation. To examine the cytotoxic effects of PL and collagen, the confluent monolayer of NIH-3T3 cells seeded in Petri dishes were treated with several concentrations of PL and collagen for 20 min and 1 h, and the cell viabilities were then measured. To determine the number of viable cells, NIH-3T3 cells were stained with propidium iodide (PI, Invitrogen Corporation) (2 mg/mL) and subsequently analyzed using a FACScan flow cytometer (BD Biosciences). Data was collected and analyzed using the CellQuest software.

### 2.5. PDMS mold fabrication

The silicon master for preparing the poly(dimethylsiloxane) (PDMS) mold was fabricated with SU-8 photoresist (MicroChem Corporation) using photolithography. The patterns on the masters had protruding cylindrical holes of 100 µm in diameter. A PDMS replica against the master was molded by casting the liquid prepolymers composed of a mixture of 10:1 silicon elastomer and the curing agent (Sylgard 184, Essex Chemical). The mixture was cured at 70 °C for 2 h, and the PDMS mold

was then peeled from the silicon wafer, cleaned with ethanol or acetone, and plasma cleaned for 4 min to increase its wettability (PDC-001, Harrick Scientific Co.).

### 2.6. HA patterning using capillary force lithography

Our method for patterning HA using capillary force lithography was described previously [27]. Briefly, a few drops of HA solution containing 5 mg HA/mL in distilled water was spun coated onto a plasma-cleaned glass slide at 1500 rpm for 10 s. The PDMS mold was immediately placed on the thin layer of HA and left undisturbed for at least 12 h. This process produces regions of bare glass at defined areas, as shown in the selective adsorption of FITC-labeled proteins [27]. HA patterns were observed with a light microscope. The surface morphology and thickness of the patterned HA after complete solvent evaporation were analyzed using an atomic force microscope (AFM) (D3100, Veeco Instruments Inc.).

### 2.7. Patterned cell co-cultures

HA-patterned glass slides were incubated with a solution containing FN (100 µg/mL in PBS) for 20 min and then washed. As primary cells for the co-culture, ES cells or AML12 cells were added to the patterned slides at a concentration of  $1 \times 10^6$  cells/mL and allowed to adhere for 8 h. Culture medium was then aspirated, replaced by a solution containing collagen at a concentration of 500 µg/mL or PL at a concentration of 40 µg/mL, and incubated for 20 min. This solution was then aspirated and replaced with secondary cells at a concentration of  $1 \times 10^6$  cells/mL. Depending on the cell types included, primary cell patterns and co-cultures were either maintained with medium used for ES cells or AML12 cells. Cells were incubated and imaged at 3 days of culture using a fluorescent microscope.

### 2.8. Cell staining

Primary cells (ES cells or AML12 cells) and secondary cells (NIH-3T3 fibroblasts) were distinguished from one another by staining with carboxyfluorescein diacetate succinimidyl ester (CFSE) dye (green) and PHK26 dye (red), respectively. Cells were trypsinized and washed with DMEM medium without serum, and incubated in 10 µg/mL CFSE in PBS solution at a concentration of  $1 \times 10^7$  cells/mL or in  $2 \times 10^6$  PKH26 solution of diluent C at a concentration of  $1 \times 10^7$  cells/mL for 10 min at room temperature. Both staining reactions were quenched with the addition of an equal volume of DMEM supplemented with 10% FBS.

## 3. Results and discussion

### 3.1. HA micropattern and its stability on glass surface

As shown in Fig. 2A, HA successfully formed micro-patterns on glass substrates with good edge definition. Fig. 2B illustrates that the initial height of the patterned HA layer was typically ~60 nm using the conditions set in the experiment, which could be controlled by using different concentrations, coating speeds and evaporation rates as reported previously [27,28]. After washing with PBS, a chemisorbed layer of HA of ~3 nm on the patterned surfaces was shown by an AFM and other methods in a previous paper [27,29]. A fluorescent image of collagen adsorbed on patterned HA surface is shown in Fig. 2C. Collagen adsorption on the HA pattern was also observed with AFM and shows that collagen seems to partly retain the fiber structures (Fig. 2D).



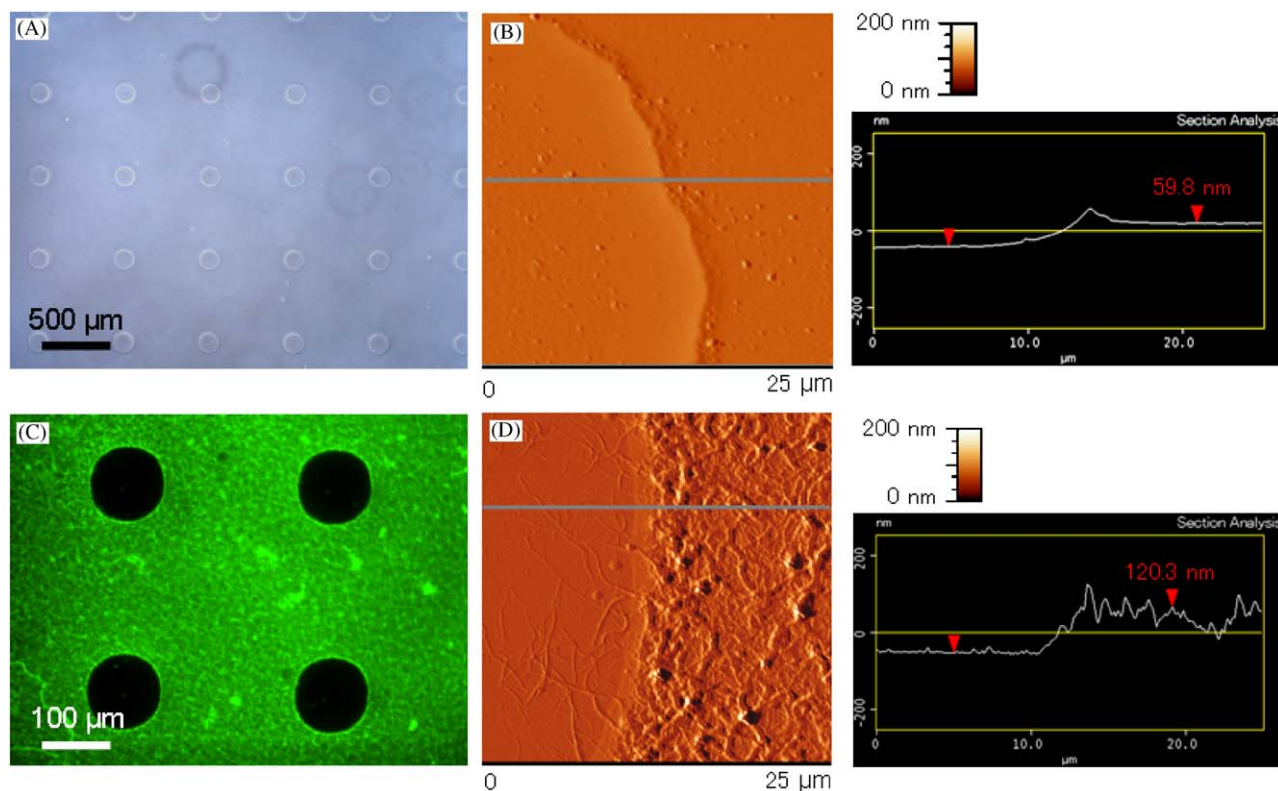


Fig. 2. Micropatterned HA surfaces and subsequent adsorption of collagen. HA patterns were formed with good edge definition, as observed with light microscope (A). The AFM image (B) indicates that the initial thickness of HA layer was typically  $\sim 60$  nm using the conditions in this study. The thickness is a function of HA concentration, spin coating speeds, and the level of rinsing with PBS. Collagen adsorbed onto the thin HA layer, as visualized by using FITC-labeled collagen (C). The AFM image (D) shows that collagen adsorbed on HA seems to partly retain the fiber structures.

To test the stability of HA films on the glass surface, we used an indirect approach in which we analyzed changes to the protein-resistant properties of the films. HA dissolved in water or PBS was cast on glass surfaces, and the stability of the film stored in air or PBS at room temperature was analyzed by measuring the adsorption level of FITC-labeled BSA. As shown in Fig. 3, HA coated surfaces were stable for at least 7 days under all conditions that were tested. Although the HA film was stable after 14 days of exposure to air, the PBS-dissolved HA detached more than 60% after 10 days of exposure to PBS and the water-dissolved HA detached more than 60% after 14 days of exposure to PBS. The glass surface is hydrophilic due to the presence of hydroxyl groups, which allows for direct immobilization of HA. Although the long-term stability of HA films has been sustained using chemical modification of HA on glass surface [30], the stability achieved using direct patterning is sufficient for fabricating patterned cocultures.

### 3.2. Protein adsorption on HA-coated surface

The adsorption of FN, PL, and collagen on the HA-coated flat surface was analyzed by quantifying the fluorescent expression of treated glass slides. As shown in Fig. 4, the adsorption of FN was significantly reduced on

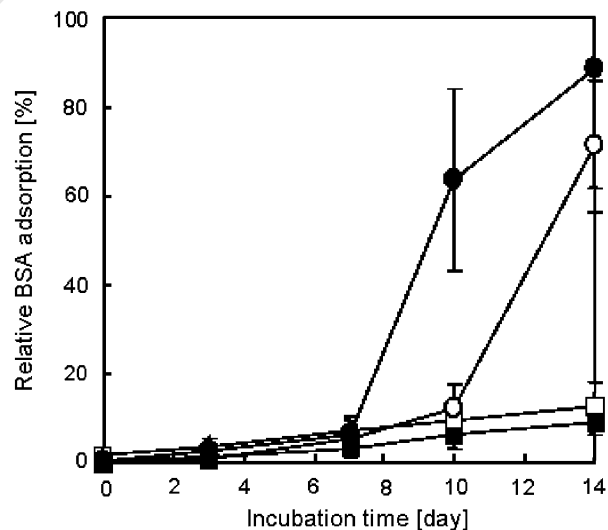


Fig. 3. The stability of the adsorbed HA on a glass substrate was measured by the quantitative analysis of the adsorption of FITC-labeled BSA. The results were normalized relative to a glass control defined as 100%. HA was stable for at least 7 days in all conditions, and HA dissolved either in PBS (■) or water (□), remained stable for at least 14 days in air. HA dissolved in PBS (●) and water (○) detached more than 60% after 10 and 14 days of exposure to PBS. The values indicate the mean of four independent experiments. Error bars indicate SD.

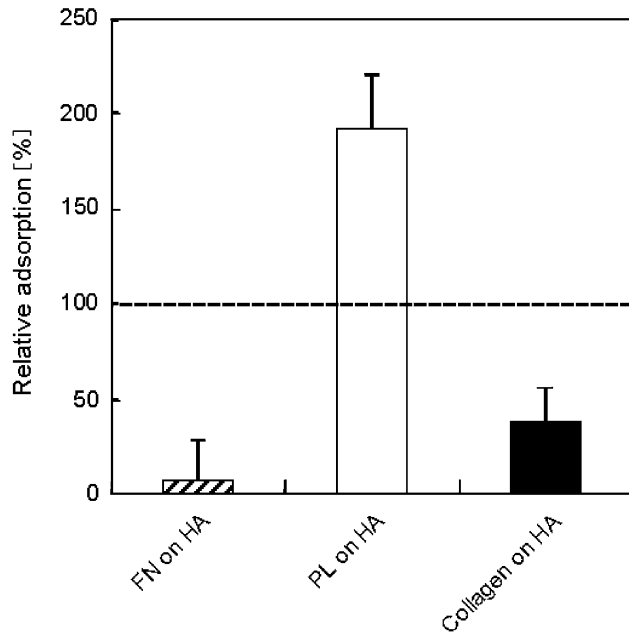


Fig. 4. The protein adsorption on the HA surface was measured by quantifying protein fluorescence intensity. The results were normalized relative to each glass control defined as 100%. The adsorption of FN was significantly reduced on HA-coated glass in comparison to bare glass controls. PL adsorbed onto HA-coated glass at significantly higher amounts than bare glass. Collagen adsorbed 37.1% in comparison to bare glass. The values indicate the mean of four independent experiments. Error bars indicate SD.

HA-coated glass in comparison to bare glass controls. Also, positively charged PL adsorbed to HA-coated glass significantly more than bare glass. Collagen adsorbed 37.1% in comparison to bare glass. Compared with other proteins, including bovine serum albumin and IgG that adhered <10% on HA surface relative to glass [23], collagen displayed higher binding properties to HA surface. The difference in the adsorption of two cationic polymers, PL and collagen, is probably due to the differences in their electric charges, molecular weight, and adsorption processes. PL consists of lysine having an amine functional group whereas collagen consists partially of glycine that has non-ionic side chain, thus PL is more positively charged than collagen. The difference in the adsorption processes can be imaged by the length of time required to reach equilibrium. The adsorption of PL and collagen on HA film reached a plateau value in ~10 min [31] and 1–3 min [30], respectively. The relatively short length of time for adsorption most likely suggests a process with less aggregation and rearrangement of the adsorbent on HA surface, therefore collagen might adsorb randomly in low densities.

### 3.3. Cell adhesion and viability on HA surfaces treated with PL and collagen

To examine the degree of cell adhesion to various surfaces, NIH-3T3 fibroblasts were seeded onto surfaces

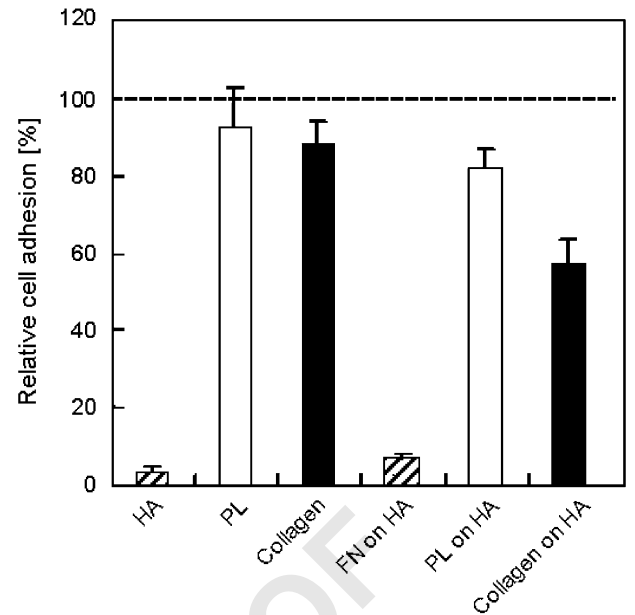


Fig. 5. The cell adhesion on surfaces modified with HA, FN, PL, and collagen. NIH-3T3 fibroblasts were seeded onto these surfaces, and the adhesion of cells was measured. The results were normalized relative to each glass control defined as 100%. Less than 10% of cells adhered onto HA or FN-treated HA surface in comparison to the glass control, which is significantly lower than FN-coated glass surface. Collagen treatment modified the properties of HA surface from being cell-repulsive to cell-adhesive. There were no significant differences in the number of adhered cells between collagen and PL-treated HA surfaces, despite the fact that the adsorption of PL on HA was significantly higher than that of collagen (Fig. 4). The values indicate the mean of four independent experiments. Error bars indicate SD.

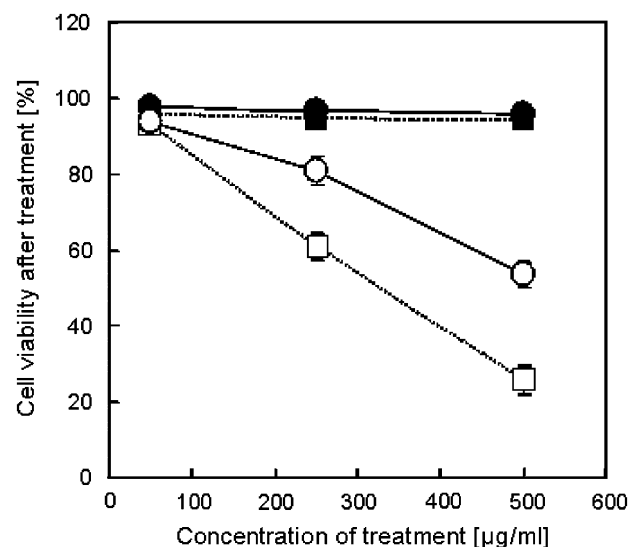


Fig. 6. Cell viability in response to treatment with several concentrations of PL and collagen. The confluent monolayer of NIH-3T3 cells was treated with PL for 20 min (○) and 1 h (□) and collagen for 20 min (●) and 1 h (■). Cell viabilities were measured by FACScan flow cytometer. Cell viability decreased as PL decreased in a time- and concentration-dependent manner. Collagen had no cytotoxic effects even at high concentrations, irrespective of the length of the treatment. The values indicate the mean of four independent experiments. Error bars indicate SD.

1 modified with HA, FN, PL, and collagen, and the  
 2 percentage of the seeded cells that adhered were measured.  
 3 As shown in Fig. 5, <10% of cells adhered to HA alone or  
 4 FN-treated HA surfaces in comparison to glass controls,  
 5 which is significantly lower than FN-coated glass surface.  
 6 As expected, collagen treatment modified the properties of  
 7 HA-coated surfaces from cell-repulsive to cell-adhesive.  
 8 Lower concentrations (250 and 50  $\mu\text{g}/\text{mL}$ ) of collagen than  
 9 those used in this study (500  $\mu\text{g}/\text{mL}$ ) slightly decreased the  
 10 number of cells that adhered (55% and 42%). Interestingly,  
 11 there was no significant difference in the adhesive proper-  
 12 ties between collagen and PL-treated HA surfaces with  
 13 respect to adhesion of NIH-3T3 fibroblasts, despite the fact  
 14 that the adsorption of PL on HA was significantly higher  
 15 than that of collagen. This may be caused by the high  
 16 affinity of cells for collagen, since fibroblasts express many

collagen-binding integrin domains [32]; collagen is also an  
 integral part of the natural extracellular matrix [33].  
 Another contributing factor can be the cytotoxic effects  
 of PL [24]. The viability of cells treated with PL decreased  
 over time in a concentration-dependent manner as shown  
 in Fig. 6, which was similar to what other researchers have  
 reported [19]. Collagen had no cytotoxic effects even at  
 high concentrations, irrespective of the length of the  
 treatment. The same results were obtained using ES cells  
 and AML 12 hepatocytes on the cell adhesion and viability  
 experiments (data not shown). These results demonstrate  
 that collagen may be a more suitable material for switching  
 surfaces properties of HA-coated substrates and encour-  
 aged us to examine the use of collagen in developing  
 patterned co-cultures for various applications.

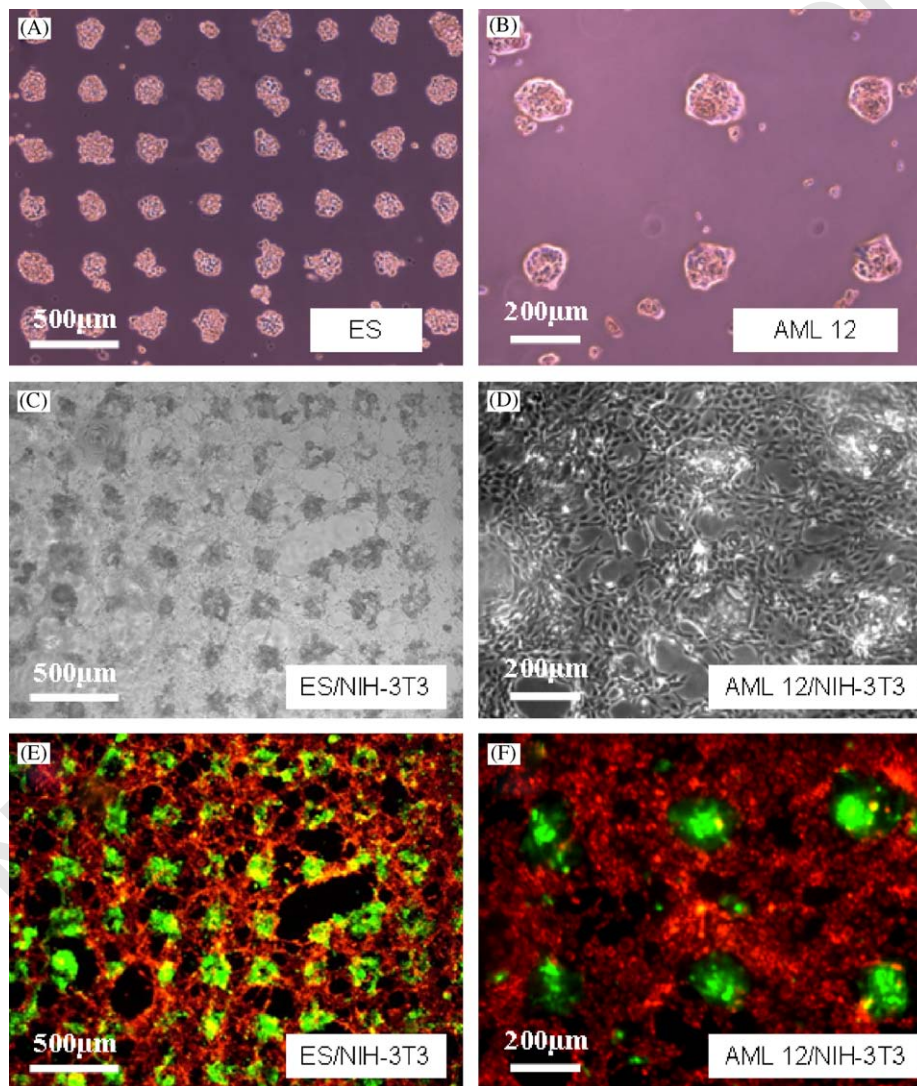


Fig. 7. Patterned cell culture and patterned co-culture on HA/collagen surface. ES cells (A) and AML 12 hepatocytes (B) selectively adhered to the FN-coated region on HA-patterned surface during the incubation of 8 h. The HA surface including the primary cells was treated with collagen and seeded with NIH-3T3 fibroblasts. After 3 days of culture, ES cells formed dense spherical aggregates and were clearly distinct from the surrounding fibroblasts monolayer (C). The co-culture of AML12 hepatocytes and NIH-3T3 fibroblasts was difficult to distinguish under light microscope (D). Fluorescently stained primary cells (green) and secondary cells (red) were visualized for ES/NIH-3T3 (E) and AML 12/NIH-3T3 (F) co-cultures at 3 days of culture.



### 3.4. Patterned cell co-culture on collagen/HA surface

To pattern primary cells, HA-patterned surfaces were treated with FN for 15 min so that FN adsorbed in bared glass spots. ES cells or AML12 hepatocytes were subsequently seeded on the surface and incubated for 8 h. Fig. 7A and B show that both cell types deposited preferentially to the FN-coated 100  $\mu\text{m}$ -diameter exposed islands. ES cells formed multilayer aggregates potentially as a result of strong ES cell–ES cell interactions.

To pattern secondary cells, the HA-patterned surface and the seeded primary cells were treated with collagen at the concentration of 500  $\mu\text{g}/\text{mL}$  for 20 min, then washed and seeded with NIH-3T3 fibroblasts. Although ES cells formed dense spherical aggregates and were clearly distinct from the surrounding fibroblast monolayer (Fig. 7C), hepatocyte/fibroblast co-cultures were difficult to distinguish under light microscope (Fig. 7D). Fluorescence staining with a cytoplasmic tracer (CFSE-green) and a membrane labeling dye (PHK26-red) were used to visualize the co-cultures, and served to validate the patterns in both co-culture systems as shown in Fig. 7E and F. Similar to our previous system using HA and PL [23], the patterned co-cultures could be achieved independent of the cell types and the seeding orders of cells on various pattern sizes (data not shown).

We anticipate that the co-culture of ES cells with other mature cell types could facilitate their differentiation into specific cell types [34]. For example, co-cultures with dermal fibroblasts or keratinocytes facilitated the differentiation of ES cells into cells of a neural lineage or of an endothelial lineage, respectively [35]. Our patterned co-culture system may be useful for investigating the effect of the size of embryoid bodies and the degree of heterotypic cell–cell interactions on the ES cell differentiations.

To our knowledge, this is the first paper to report the generation of patterned co-culture on a switchable surface fabricated with natural extracellular matrices. The fabrication process is simple and does not require any chemical modification or special equipment. Through molecular interactions of HA and collagen, a technique was developed for fabricating a switchable surface without the use of potential cytotoxic materials. This may be important for biological studies and for designing tissue-engineering constructs. Because other extracellular components such as laminin, proteoglycans, and other types of collagen can bind to HA, FN, or collagen with their specific binding sites or electrical charges, the approach presented here may also be applicable for building up more complex biomimetic surfaces. We believe that this simple and versatile biological co-culture system could open new research opportunities including further studies on cell–cell communication, cell–matrix interactions, differentiation, and apoptosis.

### 4. Conclusion

A novel technique for preparing micropatterned co-cultures utilizing three major extracellular matrices, HA, FN, and collagen is described. HA had high resistant properties toward FN adsorption and high affinity to collagen. The ionic adsorption of collagen switched the HA surface from being cell-repulsive to cell-adherent without cytotoxic effects, thereby enabling distinctly localized co-cultures. This simple and biocompatible method may be a useful tool for fabricating controlled cell microenvironments for fundamental biological studies and tissue-engineering applications.

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