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Enzyme-etching technique to fabricate micropatterns of aligned collagen fibrils

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Abstract

A technique to tailor-make pre-coated, pre-aligned bovine collagen fibrils, derived from neonatal cardiomyocytes, on the surface of a glass slide into a designated pattern is reported. The unwanted collagen-coated area was erased by a collagenase solution and the tailored area was retained by attaching a microfabricated polydimethylsiloxane stamp directly to the collagen-coated surface. Using this technique, collagen patterns with designated orientations and with clear pattern boundaries and defined shapes were fabricated.

Keywords

Collagen fibrils; Enzyme etching; Microfabrication; Micropattern; Myofibrils; Neonatal cardiomyocytes

Introduction

The extracellular environment is crucial to the distribution of cytoskeletal and cellular functions. Many surface-modification techniques have been developed to direct cell attachment and the cytoskeleton for in vitro study of cell functions. For example, a cytoskeletal arrangement has been controlled by setting the boundaries of the coated extracellular matrix (ECM) components through surface patterning (Bray et al. 2008) or by regulating the fibril configurations on the surface (Lee et al. 2006). Microcontact printing (μ CP) (Chen et al. 1997; Singhvi et al. 1994) and protein alignment (Lee et al. 2006; Simpson et al. 1994) are the two major techniques developed to accomplish extracellular environment modifications.

In the conventional μ CP method, proteins are first coated on polydimethylsiloxane (PDMS) stamps and then transferred to the target surface (Kane et al. 1999). The μ CP technique can be used to create designated boundaries for the coated ECM components that mimic the in vivo geometric confines of the extracellular environment; however μ CP cannot simultaneously control alignment of the fibers within the coated pattern (Pan et al. 2013; Parker et al. 2008). Alignment of fibers, particularly type I collagen fibers that are coated on the substrate, is an important in vivo-like feature of the extracellular environment. Alignment of fabricated collagen fibrils has finer in vivo-like features (Friedrichs et al. 2007) than the micropatterns generated through μ CP (Bray et al. 2008). This alignment can be achieved using the protein-fiber-alignment technique (Lee et al. 2006; Saeidi et al. 2011), including flowing (Lanfer et al. 2008), spinning (Saeidi et al. 2011), and magnetic force (Guo and Kaufman 2007). Although the restriction to microchannels of flow-generated collagen-fibril alignment creates well-defined boundaries that parallel the aligned fibrils (Guo and Kaufman 2007), boundaries that are perpendicular to the aligned fibrils cannot be controlled through the presently available techniques.

Here, we describe a technique to combine the advantages of μ CP and aligned collagen fibrils through enzyme etching of the pre-aligned collagen-fibril patterns. This technique can tailor the pattern of the pre-coated and pre-aligned collagen fibrils on glass slides to the designated geometric confinement with preset angles between the collagen fibrils and the pattern boundaries. We describe how the distribution of myofibrils in neonatal cardiomyocytes (NCMs) was simultaneously effected by the geometry of the collagen islands and the aligned collagen fibrils were studied.

Materials and methods

Fabrication of PDMS stamps

Polydimethylsiloxane (PDMS) stamps were fabricated through standard photolithography and soft lithography (Ruiz and Chen 2007). In brief, the features of the PDMS stamps were designed with 2D drawing software (e.g., AutoCAD) and printed to make a mask. The features were then transferred from the mask to an SU-8 layer spin-coated on a silicon wafer through UV exposure, and the mold for the PDMS-stamp fabrication formed after SU-8 development. The SU-8 mold with the silicon wafer was placed in a Petri dish with the SU-8

side facing up. A mixture of SYLGARD 184 silicone elastomer kit (10:1, Dow Corning Corporation) was then poured into the Petri dish to form a layer about 2 mm thick and degassed in a vacuum container for 30 min to remove bubbles. The Petri dish was then baked at 65 °C overnight. The PDMS was carefully peeled from the SU-8 mold. The portion of the PDMS with the designed features was trimmed and used as the stamp.

Microcontact printing (μ CP)

In the conventional μ CP method, the required chemical patterns are printed on the restricted areas via transferring chemicals from the PDMS stamp to the surface (Kane et al. 1999). In our approach, we generated collagen patterns of different shapes by etching the unwanted collagen from the glass slides pre-coated with aligned collagen fibrils derived from a bovine collagen solution (aligned-crimped, AdvancedBioMatrix inc.) using a collagenase solution (Worthington, 1 mg/ml in phosphate buffered saline solution). To prevent etching of the collagen that formed the pattern, the PDMS stamp (Fig. 1a, A) was pressed on the glass slide at the collagen-coated surface (Fig. 1a, B), thus sealing the surface of the slide where collagen patterns would be generated (dark area in Fig. 1b) when the slide was dipped into the enzyme solution. The area that was not sealed by the PDMS stamp (light area in Fig. 1b) was wetted with the enzyme solution. Two magnets were used to press the PDMS stamp firmly to the glass slide (Fig. 1c). To ensure that all the unwanted collagen (light area in Fig. 1b) was etched and degraded completely, the air between the PDMS stamp and the surface of the glass slide was removed by placing the Petri dish in a vacuum chamber for 2 h (Fig. 1e). The Petri dish was then held at 37 °C (Fig. 1f) another 6 h. The magnets were then removed, and the PDMS stamp was carefully detached from the glass slide. The glass slide was rinsed quickly with deionized (DI) water. The collagen remaining on the glass slide was then activated according to the procedure described in the product manual (Fig. 1g). Briefly, the glass slide was dipped into de-ionized water (30 s), then into PBS (5 min), and then into DI water again (30 s) and was soaked in 75 % (v/v) EtOH for 2 h; the glass slide was then exposed to air until its surface was completely dry (Fig. 1h).

Collagen islands

Collagen islands on the glass slides were pre-incubated in Dulbecco's Modified Eagle Medium (DMEM) for 45 min at 37 °C in a CO₂ incubator. The DMEM was then removed and freshly isolated neonatal cardiomyocytes (NCMs) in culture medium (DMEM, 20 % v/v fetal bovine serum) were plated on the glass slides. In the control groups, freshly isolated NCMs were plated on two types of glass slides with untreated pre-coated collagen fibrils: (1) glass slides with pre-coated, pre-aligned bovine collagen fibrils that were commercially available (aligned-crimped, AdvancedBioMatrix Inc.); (2) glass slides coated with non-aligned bovine collagen fibrils. The latter were prepared in our laboratory: 200 mM HEPES (pH 9), 10 $\times$ minimum essential medium (Sigma-Aldrich Co.), and PureCol bovine collagen solution, Type I, 3 mg/ml (AdvancedBioMatrix) were mixed at 1:1:8 (by vol.) and the mixture was placed on ice. The mixture was then randomly spread on the glass slides with a cell scraper. After holding at 37 °C for 1.5 h, the glass slides were rinsed with PBS and were ready for use. The cultured NCMs were fixed at day 3, and then α -actinin was fluorescently labeled with antibodies [primary antibody: anti- α -actinin (Sarcomeric) produced in mouse, Sigma-Aldrich Co.; secondary antibody: Alexa Fluor® 488 Donkey Anti-Mouse IgG (H+L),

Life Technologies], and F-actin was labeled with phalloidin-TRITC (Sigma-Aldrich Co.). Samples were imaged under a confocal microscope.

Results and discussion

Temporal control of the enzyme-etching process, in which the assembled PDMS glass slide was immersed in the enzyme solution and incubated at 37 °C, is critical for generating the designed collagen pattern. We first conducted experiments to determine the optimum enzyme-etching time: We fabricated PDMS stamps using masks with long stripes to generate collagen strips of 20 µm width and gaps of 20 µm between neighboring collagen strips. The experiments demonstrated that if the etching time was too brief, the collagen areas exposed to the enzyme solution would not be completely removed, and the collagen islands would have imprecise boundaries (Fig. 2a, b). But if the PDMS-glass slide assembly was soaked too long, the collagen areas protected by the PDMS stamp would be damaged by the etching effect, and the alignment of the collagen fibrils would be destroyed (Fig. 2c, d). The etching time was adjusted according to the morphology of the etched collagen pattern until the generated collagen islands had the designed sizes and demonstrated clean boundaries (Fig. 2e, f).

For the collagen pre-coated glass slides (aligned-crimped, AdvancedBioMatrix) used in this study, 6 h was the preferred enzyme-etching time to generate precisely patterned collagen strips. Using the determined enzyme-etching conditions, collagen patterns of different shapes were generated and imaged under a Zeiss 200 M phase microscope. Figure 3 demonstrates that the collagen areas not protected by the PDMS stamp were completely removed by the enzyme solution (Fig. 3, arrowheads), and the protected areas retained sharp boundaries (Fig. 3, arrows). The shapes of the micropatterned collagen islands matched very well with that of the PDMS stamp. The morphology of the collagen pattern was acquired via an atomic force microscope (AFM, MFP-3D Asylum, contact mode). Figure 4 shows that the alignment of the collagen fibers remained after the enzyme-etching process, suggesting that the internal structure of the collagen islands was well protected by the PDMS stamp.

To study how the pre-aligned collagen fibril patterns tailored by the enzyme-etching process regulate the distribution of myofibrils in cardiomyocytes, we cultured freshly isolated NCMs on the collagen islands. In control group 1, NCMs were plated on the commercially available, aligned-collagen glass slides (Fig. 5a). In control group 2, the NCMs were plated on the glass slides coated with non-aligned collagen fibrils (Fig. 5b). Most of the rod-shaped NCMs orientated along the direction of the aligned collagen fibrils when they were cultured on the aligned collagen fibrils (Fig. 5a), while NCMs spread randomly when they were cultured on the non-aligned collagen fibrils (Fig. 5b). After freshly-isolated NCMs were plated on collagen islands (Fig. 5c, circle 112 µm diam.; 5d, rectangle 166 µm × 60 µm; 5e, triangle 152 µm in side-length; and 5f, square 100 µm in side-length) and cultured for 3 days, they were fixed and α-actinin and F-actin fluorescently labeled (Fig. 5c–f). Fluorescence imaging showed that when the NCMs were in the center of the island (Cell 1, Fig. 5c), their myofibrils orientated along the direction of the collagen fibrils. However, when they spread to the boundaries of the collagen islands, their myofibrils orientated along the boundary (yellow arrows in Fig. 5c–f). This boundary effect was demonstrated by the

rectangular, triangular, and square islands shown in Fig. 5d–f, in which the myofibrils of the NCMs that spread to the boundary of the islands aligned along the boundary regardless of the collagen alignment.

The process of enzyme-etching described here is similar to the traditional μ CP method in which the gold layer to be retained is protected by the printed alkanethiol before chemical etching (Kumar and Whitesides 1993). In our method, we did not protect the pre-coated collagen layer by chemical-agent printing which may affect collagen structure and subsequent cell culture. Instead, the collagen fibrils to be preserved were physically protected by pressing the PDMS stamp on the surface. The unwanted collagen fibrils were removed by soaking the assembled PDMS-glass slide in enzyme solution. Through this method, the fine features, particularly the fiber alignment, inside the generated collagen patterns were preserved when the pre-coated aligned collagen fibril layer was tailored to different shapes. In the present research, NCMs were randomly plated to the surface of the collagen-coated glass slides. In future work, we will take advantage of our custom laser micropatterning system (Ma et al. 2012a, b; Pirlo et al. 2011), by plating a single NCM on each collagen island. The spread of the cardiomyocytes on the collagen islands will be recorded in real time, and the regulation of myofibrils by both collagen alignment and geometrical confinement will be investigated jointly.

In summary, We have developed a unique enzyme-etching technique that can generate geometric confinement on pre-coated aligned collagen fibrils with designated angles between the fibrils and the pattern boundaries. The surface of the material can be protected by pressing an elastic stamp, e.g., a PDMS stamp, on the surface, making coating with protective chemicals unnecessary. The generated collagen patterns can be used to study how complicated surface features regulate the orientation of cellular motility and cytoskeletons under in vivo-like collagen alignment.

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Fig. 1.

Procedure for fabrication of enzyme-etched collagen islands. **a** PDMS stamp (**a**) and aligned collagen fibril-coated glass slide (**b**). **b** Sketch of fabricated collagen islands on glass slide. *Dark area*: collagen preserved by PDMS stamp; *light area*: unwanted collagen erased by enzyme solution. **c** PDMS stamp was pressed to the glass slide at the collagen-coated surface. Two magnets were used to ensure contact of the PDMS stamp with the glass slide. Two thick glass plates were inserted between the magnets and the PDMS stamp/collagen-coated glass slide to uniformly distribute pressure exerted by magnets. **d** Assembled PDMS-glass slide was dipped into collagenase solution in a Petri dish. **e** Petri dish was put inside a vacuum chamber to remove air bubbles between PDMS stamp and glass slide. **f** Sample was incubated (37 °C) for 6 h. **g** Glass slide was washed with DI water (30 s) → PBS (5 min) → DI water (30 s) → 75 % (v/v) ethanol (2 h), and slide was air-dried. **h** Enzyme-fabricated collagen islands were ready

Fig. 2.

Enzyme-etched collagen strips. The relationship between the orientation of the collagen fibrils and the enzyme-etched collagen strips is provided at the top of the figure: (*double vertical*) collagen strips parallel to collagen fibrils, (*light up and horizontal*) collagen strips perpendicular to collagen fibrils. **a, b** Collagen layer insufficiently etched by enzyme solution. **c, d** Collagen layer over-etched by the enzyme solution. **e, f** Collagen layer precisely etched by the enzyme solution (scale bar 40 μm)

Fig. 3.

Patterns of aligned collagen fibrils under a phase microscope. Fibrils are oriented in the same direction in all islands. *Arrowheads* areas where collagen was removed by enzyme solution. *Arrows* areas where collagen was preserved by PDMS stamp (*scale bar* 60 μm)

Fig. 4. Patterns of aligned collagen fibrils (phase microscope and AFM). Fibrils are orientated in the same direction (*double-headed arrow at the bottom of the figure*) in all islands

Fig. 5.

Neonatal cardiomyocytes cultured on surfaces coated with collagen fibrils at Day 3. Direction of collagen alignment indicated by *double-headed white arrow* in the *upper left corner* of each image. **a** NCMs cultured on commercially available glass slides pre-coated with aligned collagen fibrils. **b** NCMs cultured on glass slides pre-coated with non-aligned collagen fibrils. **c–f** NCMs cultured on top of the collagen islands. *Island shape* is indicated by yellow markers in *upper right corner* of each image. α-Actinin (*red*), F-actin (*green*), and nucleus (*blue*) were fluorescently labeled. The *yellow arrows* indicate the spreading directions of the myofibrils. (scale bar 60 μm)