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Bio-inspired configurable multiscale extracellular matrix-like structures for functional alignment and guided orientation of cells

Won-Gyu Bae [a, 1], Jangho Kim [b, 1], Yun-Hoon Choung [c], Yesol Chung [d], Kahp Y. Suh [a], Changhyun Pang [e], Jong Hoon Chung [d, **], Hoon Eui Jeong [f, *]

[a] Interdisciplinary Program of Bioengineering, Seoul National University, Seoul 151-742, Republic of Korea
[b] Department of Rural and Biosystems Engineering, Chonnam National University, Gwangju 500-757, Republic of Korea
[c] Department of Otolaryngology, Ajou University School of Medicine, Suwon, 443-721, Republic of Korea
[d] Department of Biosystems & Biomaterials Science and Engineering, Seoul National University, Seoul 440-746, Republic of Korea
[e] School of Chemical Engineering, Sungkyunkwan University (SKKU), Suwon 440-746, Republic of Korea
[f] Department of Mechanical Engineering, Ulsan National Institute of Science and Technology (UNIST), Ulsan 689-798, Republic of Korea

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ABSTRACT

Inspired by the hierarchically organized protein fibers in extracellular matrix (ECM) as well as the physiological importance of multiscale topography, we developed a simple but robust method for the design and manipulation of precisely controllable multiscale hierarchical structures using capillary force lithography in combination with an original wrinkling technique. In this study, based on our proposed fabrication technology, we approached a conceptual platform that can mimic the hierarchically multiscale topographical and orientation cues of the ECM for controlling cell structure and function. We patterned the polyurethane acrylate-based nanotopography with various orientations on the microgrooves, which could provide multiscale topography signals of ECM to control single and multicellular morphology and orientation with precision. Using our platforms, we found that the structures and orientations of fibroblast cells were greatly influenced by the nanotopography, rather than the microtopography. We also proposed a new approach that enables the generation of native ECM having nanofibers in specific three-dimensional (3D) configurations by culturing fibroblast cells on the multiscale substrata. We suggest that our methodology could be used as efficient strategies for the design and manipulation of various functional platforms, including well-defined 3D tissue structures for advanced regenerative medicine applications.

1. Introduction

Living tissues are complex ensembles of multiple cell types that are surrounded by a complex extracellular matrix (ECM) that provides well-defined multiscale structures to regulate cellular morphology and function through biochemical and mechanical signals. Therefore, engineering a complex ECM is one of the most important challenges to regenerate or replace complex and functional living tissues and organs in regenerative medicine [1–6]. To fabricate ECM-like platforms, three-dimensional (3D) porous scaffolds fabricated by gas foaming, salt leaching, and rapid prototyping have been proposed, whereas they failed to mimic the well-defined nanostructures of protein fibers such as fibrillar collagens [2–5]. Recent nanoengineering technologies have provided important approaches for the fabrication of ECMs like-nanotopographies that can regulate or improve functions of cells for advanced regenerative medicine [7–16]. For example, two dimensional (2D) matrices that mimic the nanotopographies of the ECMs in various tissues were developed using nanofabrication technologies such as capillary force lithography, electrospinning, and peptide self-assembling [1–17].

Despite the potential importance of engineering ECMs, a large gap still exists in the ability to fully mimic the topographies of complex ECMs for cell and tissue engineering. Even though the native ECM is composed of very complex and well-defined nanostructures of protein fibrils (such as collagen and fibronectin) in

* Corresponding author.
** Corresponding author.
E-mail addresses: jchung@snu.ac.kr (J.H. Chung), hoonejeong@unist.ac.kr (H.E. Jeong).
[1] These authors contributed equally to this work.

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specific orientations, as well as hierarchically organized protein fibers with microscale sizes (i.e., from tens of nanometers to several hundreds of micrometers), most studies have dealt with fabrication of very simple nanotopographies (e.g., nanogrooves or nanoposts), while frequently neglecting a microscale hierarchical topography of the ECM because of existing technical limitations for multiscale fabrication [18,19]. In other words, the current simple fabricated patterns cannot provide cells and tissues with the precisely defined biophysical cues of native physiological microenvironments composed of nano- and microscale topographies, which may eventually create a major obstacle for constructing functional tissues or organs. Thus, it is very important to develop a methodology for the creation of a truly biomimetic ECM-like architecture that regulates the structure and function of cells and tissues for advanced tissue engineering and regenerative medicine applications [20].

In this study, we present a simple but robust method, based on both capillary force lithography (CFL) and an original wrinkling technique (see Fig. 1 in Ref. [21]), in which we fabricate precisely controllable multiscale hierarchical structures using polyurethane acrylate (PUA) without any deformation in the periodic wrinkles of a polyethyleneglycol-diacrylate (PEG-DA) mold and a UV/O-treated polydimethylsiloxane (PDMS) sheet. Using the multiscale hierarchical platforms that can mimic the nano- and microtopography, as well as defining the structural orientation of the complex ECM, precisely controlled cellular morphology and orientation was demonstrated. We also propose a new approach to generate native-like ECM fibers using our platforms inspired by decellularization technology.

2. Materials and methods

2.1. Fabrication of hierarchically multiscale patterned substrata

To fabricate nanopatterned PUA mother mold, a droplet of UV-curable PUA (Minuta Tech., Korea) precursor solution with photoiniator was dropped on the silicon master pattern, where linear grooves (250 nm groove and 250 nm spacing) are patterned by conventional photolithography and then a flexible, transparent polyethylene terephthalate (PET) film was brought into contact with the liquid mixture. Subsequently, it was exposed to UV light ($\lambda = 250–400$ nm, 100 mJ/cm^2) for 30 s. After UV curing, the cured PUA replica was peeled off from the master mold using tweezers and additionally exposed to UV light for 12 h to completely annihilate the residual of reactive acrylate groups [22,23].

To prepare multiscale patterned substrata, PDMS pre-polymer (Sylgard 184 Silicon elastomer, Dow corning) was mixed with 10% curing agent, and poured into a petri dish until enough thickness (~1 cm), and baked at 75 °C for at least 2 h for perfect curing without any residues. After peeled off from the petri dish, the PDMS was oxidized in an UV/O treatment system (Yuil ultraviolet system, Korea) at the dose of ~15 mW/cm^2 for 300 s. Thereafter, a small quantity of adhesion promoter, 3-trimethoxysilylpropyl methacrylate (TMSPMA) (10% v/v) was poured onto the UV/O-treated PDMS sheet and spread evenly using a spin coater set as 3000 RPM, following which the sheet was baked at 75 °C for 1 h to dry the promoter (Step 1) (see Fig. 1). Then, a few droplets of PEG-DA (Sigma–Aldrich) precursor solution with 1% (v/v) photoinitiator were dropped and covered with the prepared PUA mother mold. The assembly was pressed at 10 bar for 1 h. After UV curing for 5 min, the PUA mother mold was peeled off carefully using a tweezers (Step 2) (see Fig. 2 in Ref. [21]). And then, based on the study of wrinkle phenomena, the compressive strain (~10%) was applied using the custom-designed strain apparatus (see Fig. 3 in Ref. [21]). Finally, a few droplets of PUA precursor was dropped on the PEG-DA multiscale patterns and covered with PET film. Subsequently, the assembly was exposed to UV light for 20 s. After UV curing, PUA multiscale mold was peeled off from the PEG-DA multiscale patterns (Step 3) (Fig. 1), and additional UV-curing was performed for more than 12 h to completely annihilate the residual of active acrylate groups (see Fig. 4 for final multiscale patterns in Ref. [21]). Finally, additional UV-curing was performed for more than 12 h to completely annihilate the residual of reactive acrylate groups [22,23].

2.2. Preparation and observation of ex vivo human ECM

Skin tissues were obtained from patients during chronic otitis media surgeries under the approval of the Institutional Review Board of the Ajou University School of Medicine (Suwon, Korea). The tissues were fixed overnight with a solution containing 2% glutaraldehyde, 0.1 M sodium cacodylate, and 3 mM calcium chloride (pH 7.4) at 4 °C. The tissues were rinsed three times with PBS. The specimen was perfused with 1% osmium tetroxide and placed on a tissuerotator for 30 min. The sample was then rinsed in PBS three times. The tissues were serially dehydrated in 50%, 70%, 90%, 95%, and 100% of acetone. Each specimen was treated with hexamethyldisilazane (HMDS), air dried, and placed on a stub for sputter-coating with gold. The tissue was then observed with a FESEM (JEOL, JSM-5410LV, Japan).

2.3. Cell culture on hierarchically multiscale patterned substrata

NIH3T3 fibroblast cells were grown in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Milan, Italy) at 37 °C in a 5% CO_2 atmosphere. For immune histochemical analysis, adhered cells on samples were fixed with a 4% paraformaldehyde solution (Sigma–Aldrich, Milwaukee, WI, USA) for 20 min, permeabilized with 0.2% Triton X-100 (Sigma–Aldrich, WI, Milwaukee, USA) for 15 min, and stained with TRIT conjugated phalloidin (Millipore, Billerica, MA, USA), 4, 6-diamidino-2-phrnykinodole (DAPI; Millipore, Billerica, MA, USA), or fibronectin antibody (Sigma–Aldrich, WI, Milwaukee, USA) for 1 h. Images of the stained cells were taken using a fluorescence microscope (Zeiss, Germany). For SEM imaging cells on the substrates, cells adhered to the sample surfaces were fixed with modified Karnovsky's fixative consisting of 2% paraformaldehyde and 2% glutaraldehyde (Sigma–Aldrich) in a 0.05 M sodium cacodylate buffer (Sigma–Aldrich) for 4 h. The samples were washed with 0.05 M sodium cacodylate buffer 3 times for 10 min and fixed with 1% osmium tetroxide (Sigma–Aldrich). The samples were then washed with distilled water and dehydrated with graded concentrations (50, 70, 80, 90, and 100% v/v) of ethanol. Then, the samples were treated with hexamethyldisilazane (Sigma–Aldrich) for 15 min. Finally, the samples were coated with gold prior to cell shape observation by FESEM (JEOL, JSM-5410LV, Japan). The quantitative analysis was analyzed using a custom-written MATLAB script using the images by obtained.

3. Results and discussion

3.1. Design and fabrication of multiscale wrinkle substrates as a biomimetic ECM

Fig. 1a shows a schematic diagram of the fabrication of a multiscale wrinkle-patterned mold. In step 1, a slab of PDMS was treated with UV/O and coated with an adhesion promoter, 3TMSPMA. The slab, 1 cm in thickness, was thick enough to endure a large compressive strain for inducing a wrinkling effect in the following step. In step 2, a few droplets of a PEG-DA precursor