Cellular Multilayers

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Fabrication of Cellular Multilayers with Nanometer-Sized Extracellular Matrix Films**

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Tissues and organs consist of a complex organization of cells, extracellular matrix (ECM), and signaling molecules. In particular, blood vessels and skin are a highly organized hierarchical layer composed of various types of cells and ECM layers. The construction in vitro of three-dimensional (3D) cell–polymeric material composites has created notable advances in tissue regeneration.[1] However, an effective methodology to fabricate a 3D multilayer composed of cells and an ECM layer with the appropriate components and thickness has not yet been achieved. Recently, new technologies such as a cell sheet,[2a] magnetic liposomes,[2b] and a chitosan membrane[2c] have been reported to fabricate layered tissues. Although these methods are intriguing, complicated manipulation is required and the thickness of the ECM layer is not controllable.

We focused on a layer-by-layer (LbL) technique, which is an appropriate method to prepare nanometer-sized films on a substrate through the alternate immersion into interactive polymer solutions.[3] The preparation of nanometer-sized multilayer films composed of ECM components on the surface of the first layer of cells provides a cell-adhesive surface for the second layer of cells. Rajagopalan et al. demonstrated a bilayer structure composed of hepatocytes and other cells by preparing a polyelectrolyte multilayer consisting of chitosan and DNA on the hepatocyte surface.[4a] However, chitosan cannot dissolve in neutral buffer solutions and fabrication of polyelectrolyte multilayers onto the cell surface is limited owing to the cytotoxicity of polycations.[4b,c] Furthermore, a highly organized cellular multilayer with more than three layers will be required for the creation of functional artificial tissues that are similar to natural tissues. The use of natural ECM components for nanofilms is significant because the typical ECM presents with cell-adhesive moieties such as RGD (arginine-glycine-aspartic acid) and other amino acid sequences for cellular functions.[5]

In the present study, fibronectin (FN) and gelatin were selected to prepare nanometer-sized ECM films (nano-ECM film) on the cell surface. FN is a flexible multifunctional glycoprotein that plays an important role in cell attachment, migration, differentiation, etc.[6a,b] FN is well known to interact not only with a variety of ECM proteins, such as collagens (gelatins) and glycosaminoglycans, but also with the $\alpha_5\beta_1$ integrin receptor on the cell surface.[6c] Recently, we reported FN-based protein multilayers composed of FN and ECM components, such as gelatin, heparin, and elastin, constructed by LbL assembly.[7] Although FN and gelatin have a negative charge under physiological conditions, they interact with each other because FN has a collagen binding domain.[6b] The preparation of FN–gelatin nanofilms on the surface of the first layer of cells will provide a suitable cell-adhesive surface that is similar to the natural ECM for the second layer of cells. Herein, we report well-organized, four-layered architectures of mouse L929 fibroblast cells and the appropriate thickness of FN–gelatin nanofilms. Furthermore, xenogeneic human cellular multilayers like blood vessel were successfully constructed.

The fabrication of 3D cellular multilayers composed of cells and FN–gelatin nano-ECM films was performed according to the process shown in Figure 1. Before the demonstration of the cellular multilayer, the LbL assembly of FN and gelatin on the cell surface was analyzed quantitatively by using a quartz crystal microbalance (QCM) as the assembly

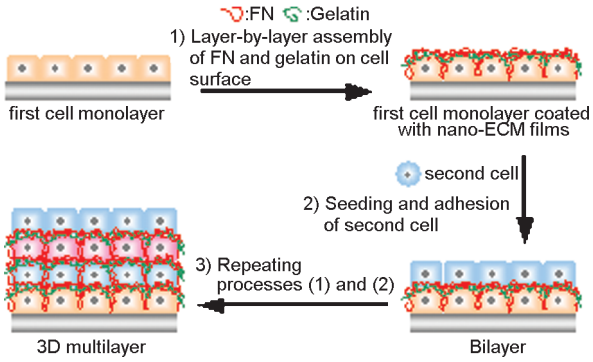

Figure 1. Fabrication process of 3D cellular multilayers composed of cells and nano-ECM films. The nano-ECM films were composed of FN and gelatin, and the outermost surfaces of all films were FN, which allowed cell adhesion.

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substrate and with a phospholipid bilayer membrane as a model cell membrane (Figure 2a). A phospholipid bilayer composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA) was prepared and adsorbed on the base layer, a four-step assembly of poly(diallyldimethylammonium chloride) (PDDA) and poly(styrenesulfate sodium) (PSS), according to Krishna et al.[8] The alternate immersion of the QCM substrate with the phospholipid bilayer into a Tris-HCl buffer solution (pH 7.4; Tris = tris(hydroxymethyl)aminomethane) of FN followed by a Tris-HCl solution of gelatin resulted in a stepwise decrease in the frequency, indicating the formation of FN–gelatin multilayers. The mean thickness of the LbL assembly after one, seven, and 23 steps was calculated to be 2.3, 6.2, and 21.1 nm, respectively. To evaluate the FN–gelatin nanofilms on the cell surface directly, the LbL assembly was performed by using rhodamine-labeled FN (Rh-FN). The Rh-FN–gelatin nanofilms were assembled in seven steps on monolayered mouse L929 fibroblasts labeled with the fluorescent probe, cell tracker green. This assembly was confirmed by confocal laser scanning microscopy (CLSM; Figure 2b and c). The top and cross sections of the CLSM 3D merged images suggested a homogeneous assembly of FN–gelatin nanofilms on the cell surface (Figure 2d). For the quantitative studies on the thickness of the multilayers on the cell surface, the fluorescence intensity of Rh-FN was estimated by a line scan (Figure 2e). The fluorescence intensity of the Rh-FN–gelatin nanofilms increased linearly upon increasing the LbL assembly step number, similar to the frequency shift of the QCM analysis, indicating a clear increase in the film thickness on the cell surface. These results demonstrated the preparation of FN–gelatin nanofilms on the cell surface.

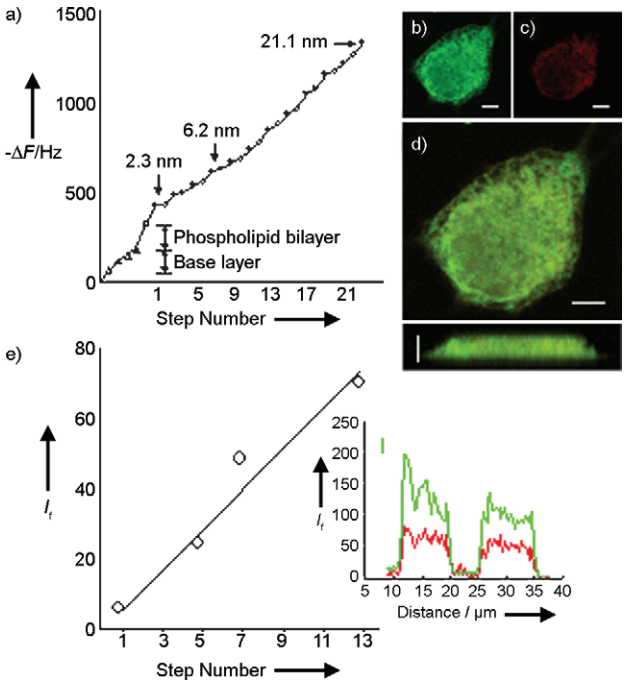

Figure 2. a) QCM analysis of the LbL assembly of FN and gelatin onto a phospholipid bilayer (DPPC/DPPA 4:1). Open and closed circles show the assembly steps of gelatin and FN, respectively. The base layer was a four-step assembly of PDDA and PSS. CLSM images of monolayered L929 fibroblast cells (b) and Rh-FN–gelatin nanofilms (c). The cells were labeled with cell tracker green and coated with a seven-step assembly of Rh-FN and gelatin. d) Top and cross-section-merged images of parts (b) and (c). The scale bars in parts (b) and (c) are 5 μm, and the horizontal and vertical scale bars in part (d) are 5 and 6.6 μm, respectively. e) Relationship between the LbL assembly step of Rh-FN–gelatin on the L929 cells and the fluorescence intensity of the Rh-FN. Inset: Fluorescence intensity of the cells (green) and the seven-step-assembled Rh-FN–gelatin nanofilms (red) by line scan. $-\Delta F$ = frequency shift, I_f = fluorescence intensity.

We fabricated a bilayer of mouse L929 fibroblast cells with or without FN–gelatin nanofilms by using a cover glass as a substrate. When seven-step-assembled FN–gelatin nanofilms were prepared on the surface of the first L929 cell layer, the cells of the second layer were then observed on the first cell layer (Figure 3a). Fluorescence microscope observation clearly showed overlapping nuclei, suggesting adhesion of the

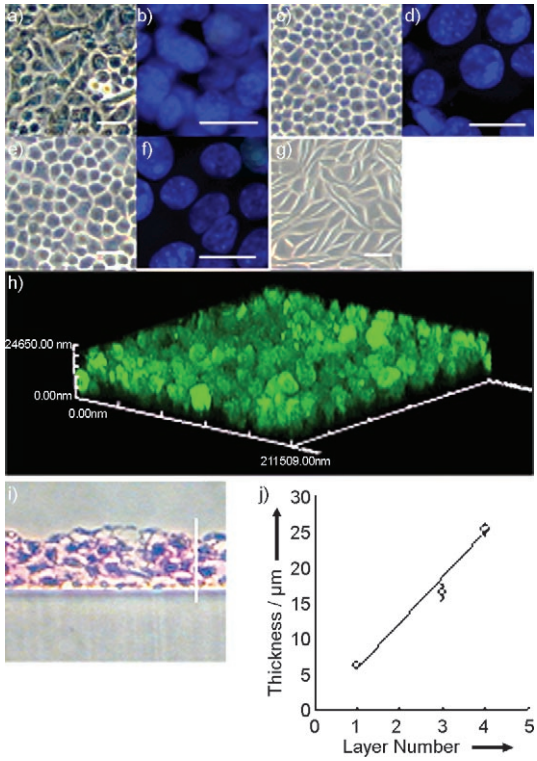

Figure 3. a, c, e) Phase-contrast (Ph) and b, d, f) fluorescence (Fluo) microscope images of L929 fibroblast cell bilayers with a seven-step-assembled (a, b), without a seven-step-assembled (c, d), or with a one-step-assembled (e, f) FN–gelatin nanofilm on the surface of the first L929 cell layer. g) Ph image of a L929 cell monolayer as a control. The fluorescence images were obtained by nuclei labeling with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The scale bars in parts (a–g) are 20 μm (Ph) and 10 μm (Fluo). h) 3D reconstructed CLSM cross-section image of four-layered L929 cells. The cells were labeled with cell tracker green. The image area is 211.5 × 211.5 μm^2, and the height is 24.7 μm. i) Hematoxylin and eosin (HE) staining image of four-layered L929 cells. The scale bar is 30 μm. j) Relationship between the L929 cell layer number and the mean thickness estimated from 3D CLSM images ($n = 3$).

second cell layer onto the surface of the first cell layer (Figure 3b). However, when the nanofilm was not prepared or the one-step-assembled nanofilm (only FN) was assembled on the first cell layer, then the bilayer architecture was not observed by phase-contrast (Figure 3c and e) or fluorescence images (Figure 3d and f). In these cases, the second cell layer also adhered onto the substrate in areas where there was no first cell layer, and all cells were closely compacted. The mean thickness of the FN–gelatin nanofilm after one and seven steps was estimated to be 2.3 nm and 6.2 nm by QCM analysis (Figure 2a). In other words, 2.3 nm of FN film was inadequate and at least approximately 6 nm of FN–gelatin nanofilm was required as a stable adhesive surface for the second cell layer. The L929 cell assembly was repeated four times with seven-step-assembled FN–gelatin nanofilms on each cell layer and then CLSM observation was performed. As shown in Figure 3h, laminated cells were observed and the thickness was estimated at approximately 25 μm by a 3D reconstructed CLSM cross-section image. As the height of the adhered L929 cell monolayer was 6.6 μm (Figure 2d), the observed thickness suggested a four-layered cellular architecture. The mean thickness of one-, three-, and four-layered cell assemblies was estimated at 6.3 ± 0.2, 16.4 ± 1.2, and 25.3 ± 0.9 μm, respectively. HE staining images also clearly indicated the four-layered structure of L929 cells (Figure 3i), thus indicating the successful fabrication of cellular multilayers with the desired laminated numbers (Figure 3j). The four-layered architecture of L929 cells was stably organized and easily peeled from the substrate. Scanning electron microscopy (SEM) observation of the peeled four-layered architecture indicated a thickness of about 24 μm, which is in good agreement with the results of the 3D CLSM and HE staining images (see the Supporting Information).

The methodology can be applied to fabricate xenogeneic cellular multilayers. The bilayer architecture, like that in blood vessels, which are composed of human smooth muscle and endothelial cells, was successfully prepared by fabricating seven-step-assembled FN–gelatin nanofilms on the surface of smooth-muscle cells (Figure 4a). The heterogeneous monolayer, however, was obtained in the absence of the nanofilms (Figure 4b). These results suggest fabrication in vitro of various human cellular multilayers, such as blood vessels or skin, by this system.

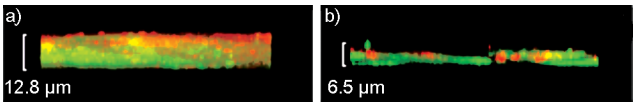

Figure 4. 3D reconstructed CLSM cross-section images of bilayered human umbilical artery smooth-muscle cells (UASMC) and human umbilical vascular endothelial cells (HUVEC) with (a) or without (b) seven-step-assembled FN–gelatin nanofilms on the surface of UASMC. UASMC and HUVEC were labeled with cell tracker green and orange, respectively.

In summary, we demonstrated that cellular multilayers with more than three layers were successfully fabricated in vitro by preparing FN–gelatin nanofilms on the surface of each cell layer. It was seen that approximately 6 nm of FN–gelatin nanofilm was required to act as a suitable cell-adhesive surface similar to the natural ECM. The four-layered cellular architecture was well organized, even after peeling from the substrate. Furthermore, the xenogeneic human cellular multilayers were successfully formed. The FN–gelatin nanofilms and cellular assemblies did not show any cytotoxicity (see the Supporting Information). To the best of our knowledge, this is the first example of cellular multilayers constructed by controlling the thickness of the ECM nanofilms on the cell surfaces. The evaluation of long-term viability of the cellular multilayers is now in progress. The present methodology may be applied as one of the biomedical applications of LbL assembly to fabricate various cellular multilayers composed of target cells and ECMs.

Experimental Section

LbL assembly of FN and gelatin: A 9 Hz QCM plate (USI System, Japan) with polished gold electrodes and a diameter of 4.5 mm was used as the substrate. The QCM was alternately immersed into a 50 mM Tris-HCl buffer solution (pH 7.4) of FN (0.2 mg mL^{-1}) and a 50 mM Tris-HCl buffer solution of gelatin (0.2 mg mL^{-1}). Each immersion was for 15 min and at 37 °C. A sample was taken and rinsed with 1 mM Tris-HCl buffer solution (pH 7.4), dried with nitrogen gas, and the frequency of the sample was then measured. The frequency shift (ΔF) was related to the amount of assembly (Δm) in accordance with the Sauerbrey equation[9]: $-\Delta F\,[\mathrm{Hz}] = 1.15\,\Delta m\,[\mathrm{ng}]$. The phospholipid bilayer was prepared in accordance with Krishna et al.[8] Briefly, a unilamellar vesicle of DPPC containing 20 wt% of DPPA was prepared by mixing the reagents in chloroform followed by solvent evaporation and continuous sonication of the obtained mixtures in deionized water (pH 6.5). The QCM was alternately immersed in an aqueous solution of PDDA (1 mg mL^{-1}) and an aqueous solution of PSS (1 mg mL^{-1}) four times to prepare the base layer and then subsequently immersed in the unilamellar vesicle solution to form the phospholipid bilayer.

Fabrication of cellular multilayer: Preparation of the FN–gelatin nanofilm on the cell surface was performed in the same manner as that of the above-mentioned LbL assembly. Briefly, a cover glass as the substrate was immersed in 50 mM Tris-HCl buffer solution (pH 7.4) of FN (0.2 mg mL^{-1}) for 15 min, and mouse L929 cell (8×10^4 cell cm^{-2}) was seeded on the substrate and incubated in Eagle's minimum essential medium (MEM; 10% fetal bovine serum (FBS)) for 6 h at 37 °C. The monolayered L929 cells on the substrate were alternately immersed into a 50 mM Tris-HCl buffer solution (pH 7.4) of FN (0.2 mg mL^{-1}) and one of gelatin (0.2 mg mL^{-1}) for 15 min and at 37 °C. The substrate and the monolayered cells were then rinsed with 50 mM Tris-HCl buffer solution (pH 7.4). After the seven-step assembly of FN and gelatin, mouse L929 cells (8×10^4 cell cm^{-2}), as the second layer, were seeded and incubated for 6 h at 37 °C. The FN–gelatin assembly and the cell seeding were repeated four times to fabricate the four-layered cellular architectures. For fluorescence labeling, L929 cells were incubated with 10 μM cell tracker green (Molecular probe, USA) in Eagle's MEM (serum free) for 40 min before the seeding. CLSM observation was performed by using a FV1000 with FV10-ASW software (Olympus, Japan). For the human bilayer structure, the human UASMC and HUVEC (CAMBREX, USA) at passages 5 to 7 were used in the present study. UASMC was cultured in smooth-muscle basal medium (SmBM; CAMBREX, USA) containing human epidermal growth factor (hEGF), human fibroblast factor basic (hFGF-B), GA-1000, FBS, and insulin, and HUVEC was cultured in endothelial basal medium-2 (EBM-2; CAMBREX, USA) containing hFGF-B, vascular endothelial growth factor (VEGF), R3-IGF-1 (IGF-1 = insulin-like growth

factor 1), ascorbic acid, FBS, hEGF, and GA-1000. The bilayer structures composed of UASMC (4×10^4 cell cm^{-2}) as the first layer and HUVEC (6×10^4 cell cm^{-2}) as the second layer were fabricated by the same manner as that of the L929 cells. For CLSM observation, UASMC and HUVEC were labeled with cell tracker green and orange, respectively, before the fabrication of cellular multilayers.

SEM: The four-layered L929 cells on the cover glass was immersed in 10% formalin aqueous solution for 10 min, then subsequently immersed into ethanol for dehydration followed by *tert*-butyl alcohol for 2 h. The four-layered cellular architectures were peeled from the substrate and freeze dried for 3 h. SEM observation was performed by using a HITACHI S-800 electron microscope.

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[1] a) R. Langer, J. P. Vacanti, *Science* **1993**, *260*, 920 – 926; b) K. Y. Lee, D. J. Mooney, *Chem. Rev.* **2001**, *101*, 1869 – 1879; c) M. P. Lutolf, J. A. Hubbell, *Nat. Biotechnol.* **2005**, *23*, 47 – 55.

[2] a) J. Yang, M. Yamato, C. Kohno, A. Nishimoto, H. Sekine, F. Fukai, T. Okano, *Biomaterials* **2005**, *26*, 6415 – 6422; b) A. Ito, Y. Takizawa, H. Honda, K. Hata, H. Kagami, M. Ueda, T. Kobayashi, *Tissue Eng.* **2004**, *10*, 833 – 840; c) C. C. Co, Y.-C. Wang, C.-C. Ho, *J. Am. Chem. Soc.* **2005**, *127*, 1598 – 1599.

[3] a) G. Decher, *Science* **1997**, *277*, 1232 – 1237; b) *Handbook of Polyelectrolytes and Their Applications*, *Vol. 1* (Eds.: S. Tripathy, J. Kumar, H. S. Nalwa), American Scientific Publications, Los Angeles, **2002**; c) Z. Tang, Y. Wang, P. Podsiadlo, N. A. Kotov, *Adv. Mater.* **2006**, *18*, 3203 – 3224; d) A. P. R. Johnston, C. Cortez, A. S. Angelatos, F. Caruso, *Curr. Opin. Colloid Interface Sci.* **2006**, *11*, 203 – 209.

[4] a) P. Rajagopalan, C. J. Shen, F. Berthiaume, A. W. Tilles, M. Toner, M. L. Yarmush, *Tissue Eng.* **2006**, *12*, 1553 – 1563; b) D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, *Biomaterials* **2003**, *24*, 1121 – 1131; c) M. Chanana, A. Gliozzi, A. Diaspro, I. Chodnevskaja, S. Huewel, V. Moskalenko, K. Ulrichs, H.-J. Galla, S. Krol, *Nano Lett.* **2005**, *5*, 2605 – 2612.

[5] H. K. Kleinman, D. Phlip, M. P. Hoffman, *Curr. Opin. Biotechnol.* **2003**, *14*, 526 – 532.

[6] a) K. M. Yamada, *Annu. Rev. Biochem.* **1983**, *52*, 761 – 799; b) R. O. Hynes, *Fibronectins*, Springer, New York, **1990**; c) E. Ruoslahti, M. D. Pierschbacher, *Science* **1987**, *238*, 491 – 497.

[7] Y. Nakahara, M. Matsusaki, M. Akashi, unpublished results.

[8] G. Krishna, T. Shutava, Y. Lvov, *Chem. Commun.* **2005**, 2796 – 2798.

[9] G. Sauerbrey, *Z. Phys.* **1959**, *155*, 206 – 222.

