Electronic Supplementary Information (ESI) for RSC Advances

The Construction of Cell-Density Controlled Three-Dimensional Tissues by Coating Micrometer-Sized Collagen Fiber Matrices on Single Cell Surfaces

Chun-Yen Liu, Michiya Matsusaki and Mitsuru Akashi

Department of Applied Chemistry, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

chun-yen@chem.eng.osaka-u.ac.jp

The ESI contains the following sections:

1. Materials

Human dermal fibroblast cells were purchased from CAMBREX (East Rutherford, USA). Collagen Type I solution (from bovine corium, 0.3 wt%, 5 mM acetic acid) was purchased from Nippi Co. Ltd. Dulbecco's modified eagle medium (DMEM), 10% formalin solution (4% formaldehyde in water containing methanol) and 1M NaOH were from Wako Pure Chemical Industries, Ltd (Osaka ,Japan). Fetal bovine Serum (FBS) was purchased from Biowest (Miami, USA). The antibiotics were purchased from Nacalai Tesque (Kyoto, Japan). Bovine plasma fibronectin (FN) was purchased from Sigma-Aldrich (St. Louis, USA). FITC-labelled type I collagen was purchased from Collagen Gijyutsu Kensyukai, Ltd. (Tokyo, Japan).

2. Methods

2.1. Cell Culture

Normal human dermal fibroblast (NHDF) cells at passage 5-9 were cultured in DMEM with 10% FBS and 1% antibiotics at 37 ^{o}C in a humidified 5% CO_2 incubator. During each passage, NHDF cells were treated with 3 mL of 0.1% trypsin for 20 min. to detach from the culture mediums. The detached cells were then collected in a 15 mL plastic tube containing 4 mL of DMEM and centrifuged under 1000 rpm for 5 min. Supernatant of the centrifuged solutions were sucked out and then fresh DMEM was added into the plastic tube for calculating cells numbers or further experiments.

2.2. Coating Procedures

Firstly, 120 μl of 5x DMEM, 60 μl of FBS, 20 μl of ddH_2O, 8 μl of 1M NaOH were mixed together. Then 600 μl of DMEM with NHDF cells was added into the prepared solutions. After mixing, 400 μl of 0.03wt% collagen solution (10% FITC-collagen for unlabeled collagen) was added into the solution and pipette with pipetteman softly. Then move to 37 ^{o}C oven for certain coating time. After coating step, the solutions were poured into 15 mL of plastic tubes and washed with PBS for 2 times to get rid of unreacted molecules. Then the washing solutions stayed for 20 min till the precipitation of the coated cells. Collected the coated cells and used for further experiments. About 20 collagen coated cells were selected to calculate thickness of coated collagen fibers and cell diameter from CLSM images. Based on results, distribution curve of thickness and diameter were generated and fitted with Gaussian peak in Origin Pro 8 (OriginLab, USA).

2.3. Coating Factors

In order to fabricate collagen gel layers onto cell surfaces, different conditions

were tried through a series of experiments. There are a lot of different factors which affect the coating results, such like collagen concentrations, temperature, rotation speed, cell density, or coating time, etc. Different factors mentions above were examined. The results are shown in the following.

2.3.1 Temperature

In this part, 1 x 10^6 NHDF cells coated with 0.03 wt% collagen solutions under 25 ^oC and 37 ^oC for 30 min. As shown in **Figure S1 (a)**, cells coated with collagen under 25 ^oC revealed inhomogeneous coated layers onto the surface of cells. Contrary to that, cells coated with collagen solution under 37 ^oC for 30 min showed well coated results (**Figure S1 (b)**). Form the results, we suggest that 37 ^oC environment is needed for the coating procedures.

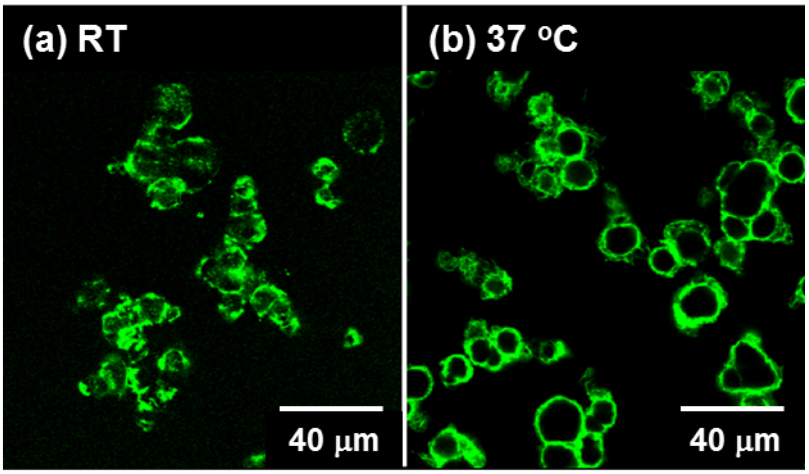

Figure S1. Confocal laser scanning microscope (CLSM) images of NHDF coated with 0.03 wt% FITC-collagen solutions at (a) room temperature and (b) 37 ^oC with 50 rotation speed for 30 min. The experiment was repeated more than three times.

2.3.2. Collagen Concentration

Three different collagen concentrations were applied: 0.3 wt%, 0.15 wt%, and 0.03 wt%. Commercial available 0.3 wt% collagen type I (from bovine corium) solution was diluted with 5 mM acetic acid to 0.15 wt% and 0.03 wt%. Then all these three different concentration of collagen solutions were mixed with 1 x 10^6 NHDF cells followed by the coating procedures mentioned above. All these three groups coated with collagen for 15, 30 and 60 min and then the washing steps. As shown in **Figure S2**, aggregation of the coated cells occurred when cells coated with higher concentration of collagen solutions (0.15 wt% and 0.3 wt%). Cells coated with 0.03 wt% collagen solutions revealed homogenously collagen layers on the surface of cells. In order to fabricate spherical collagen coated cells, 0.03 wt% collagen solution was chosen as optimized concentration for coating procedures.

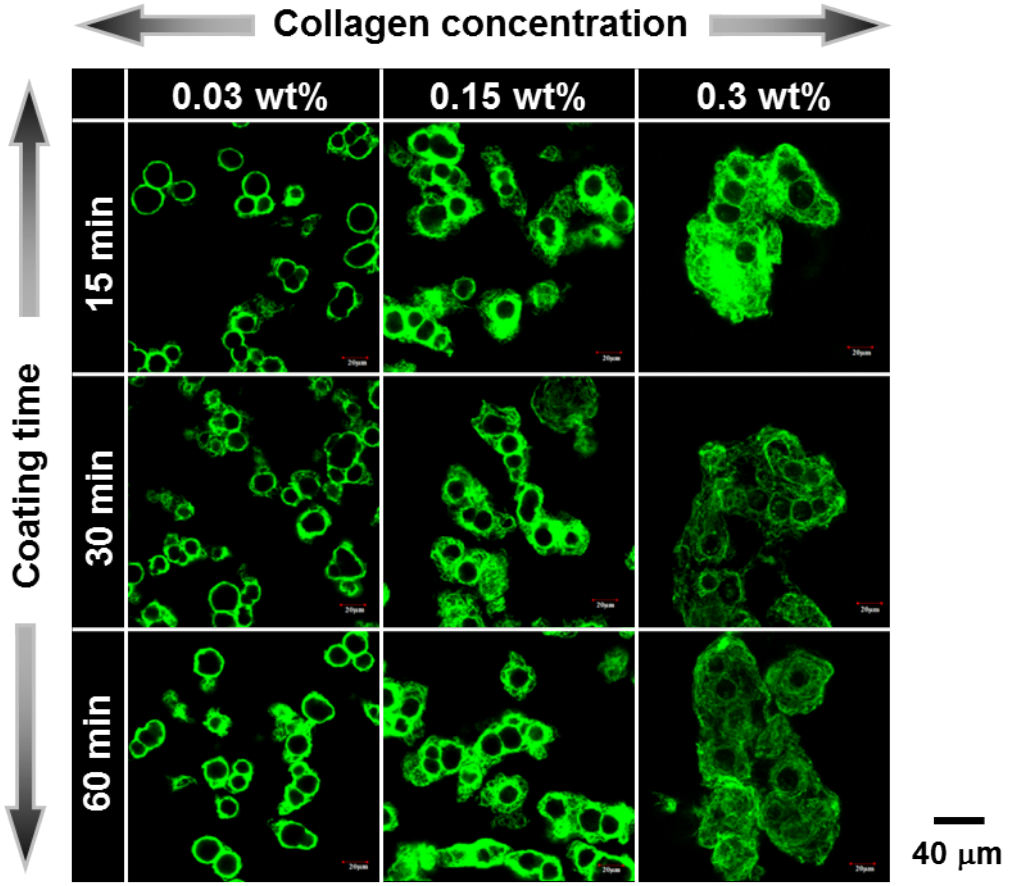

Figure S2. CLSM images of NHDF cells coated with 0.03, 0.15, and 0.3 wt% FITC-collagen solutions for 15, 30, and 60 min. The experiment was repeated more than three times.

2.3.3. Coating Time

We examined the effect of coating times to the formation of collagen layers onto the surface of cells: 0.03 wt% collagen solutions mixed with DMEM containing 1 x 10^6 cells under 37 ^oC for 15, 30, 60, and 90 min. From the results, collagen layers were coated onto the surfaces of cells after 15 min coating. Other coating time, 60 and 90 min also revealed homogenous collagen coated layers after coating and washing steps. From the confocal laser scanning microscope (CLSM) images, almost the same thickness of collagen coated gel layers were fabricated (**Figure S2**).

2.3.4. Rotation Speed

In this experiment, different rotation speed was examined: 0, 10, 25, and 50 rpm. As same as previous conditions, 1 x 10^6 NHDF cells were coated with 0.03 wt% collagen solutions under 37 ^oC for 30 min with 0, 10, 25, and 50 rpm rotation speed. As shown in **Figure S3**, with lower rotation speed, collagen coated cells aggregated to each other easily when rotation speed slower than 50 rpm. Accordingly, 50 rpm

rotation speed was chosen as optimized condition for the coating procedures.

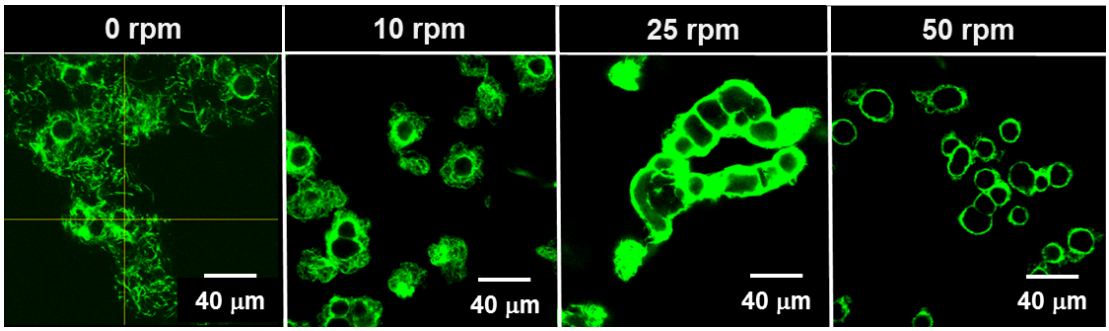

Figure S3. CLSM images of NHDF cells coated with 0.03 wt% FITC-collagen solutions at 0 to 50 rpm for 30 min incubation. The experiment was repeated more than three times.

2.3.5. Cell Number

Here, we would like to discuss the effect of cell number to the coating results. As shown in the **Figure S4** four different densities were examined. From the results, thinner and homogenous coated collagen layers on the surface of single cells were fabricated when the cell density was higher than 5 x 10^5 cells in each coating vial. Contrary to that, when the cell density lower than 5 x 10^5 cells in each coating vial, thicker coated layers were fabricated, but some aggregation occurred under lower cell density. In order to fabricate homogenous coated collagen layer onto the surface of single NHDF cells, higher cell density is needed.

From these experiments, we set at least 5 x 10^5 cells coat with 0.03 wt% collagen solutions under 37 ^oC with 50 rpm rotation speed for 2h as the optimized coating conditions for further experiments.

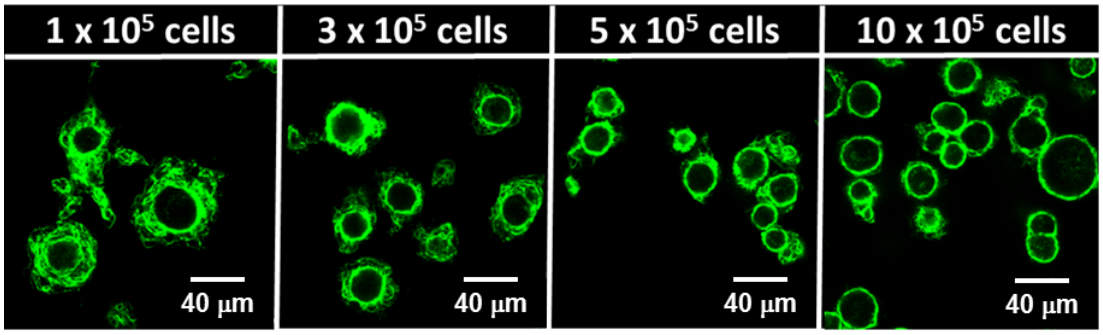

Figure S4. CLSM images of NHDF cells coated at (a) 1 x 10^5 cells, (b) 3 x 10^5 cells, (c) 5x 10^5 cells, and (d) 10 x 10^5 cells with 0.03 wt% FITC-collagen solutions under 37 ^oC with 50 rpm rotation speed for 2h. The experiment was repeated more than three times.

2.4. Effect of Cell Existence to Coating

From previous reports,[1] there is a kind of transmembrane receptor called "integrin", which can recognize collagen molecules and then catch the collagen molecules tightly due to their strong association constant at 6.7 x 10^4 M^{-1} by using its $\alpha 2\beta 1$ receptor. In order to realize the specific recognition between collagen molecules and integrins, collagen coating processes were performed with or without NHDF cells for 5 min of incubation. As shown in **Figure S5 (a)**, very few amount of small aggregation were observed, whereas initial collagen microfiber formation was found clearly on cell surfaces even after 5 min of incubation (**Figure S5 (b)**). These phenomena suggest that integrin receptors on the cell membrane can accelerate specific adsorption of collagen molecules on cell membranes and self-assembly formation of collagen microfibrils.

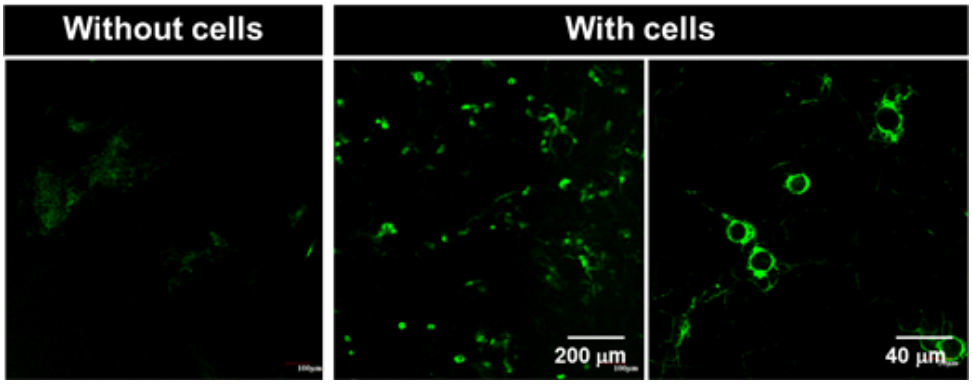

Figure S5. CLSM images of 0.03 wt% FITC-collagen solutions after 5 min of incubation at 37 °C without (left) or with NHDF cells (right). The images in with cells are lower (left) and higher (right) magnifications, respectively. The experiment was repeated more than three times.

To understand importance of integrin receptors for specific formation of collagen microfibrils onto cell surfaces, whole collagen coating processes for 90 min were performed with or without NHDF cells. Since collagen microfibril formation usually occurred after neutralization of solution, heterogeneous aggregations were observed after 90 min of incubation (**Figures S6 (a) and (c)**). However, there were few remaining nonspecific aggregations after washing and centrifugation processes. On the other hand, almost collagen microfibers were formed selectively on cell membranes when NHDF cells were added in the solution (**Figures S6 (b) and (d)**). There are very few amount of nonspecific aggregation in solution. These data also demonstrate that integrin receptors can work as an initiator to specifically fabricate collagen microfibrils on cell surfaces.

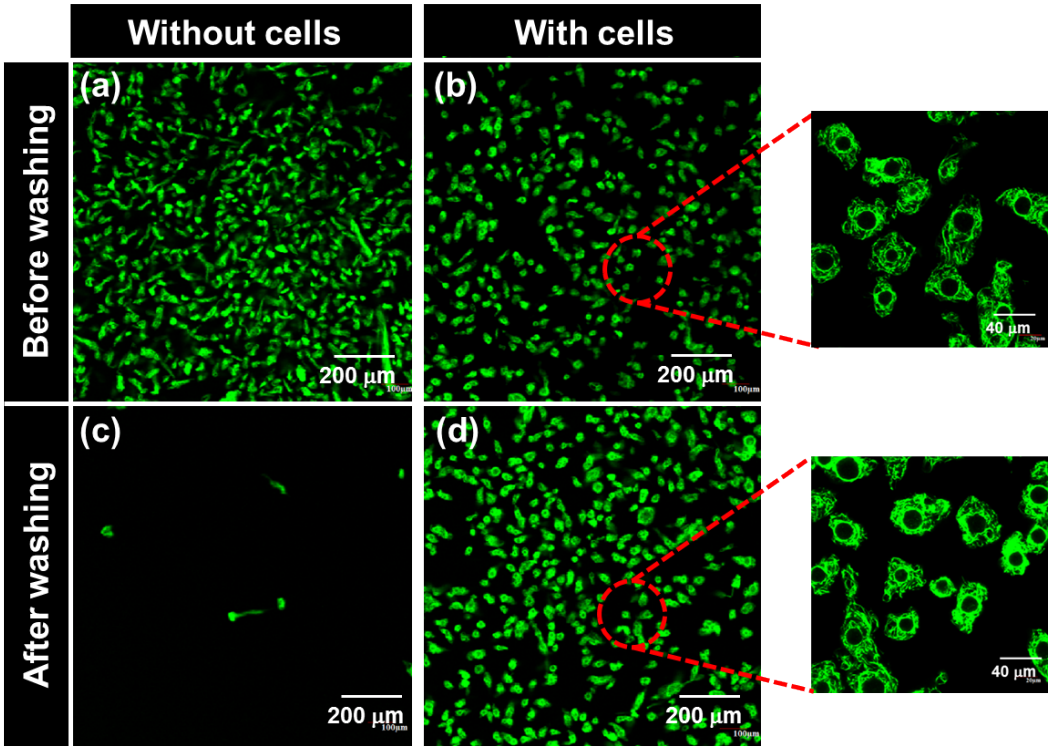

Figure S6. CLSM images of 0.03 wt% FITC-collagen solutions after 90 min of incubation at 37 °C without (a) or with NHDF cells (b). (c) and (d) are CLSM images of (a) and (b) after washing processes, respectively. The experiment was repeated more than three times.

2.5. Coating Effect to Cell Proliferation

To evaluate whether collagen coatings affect cell viability, morphology, and growth or not, the coated cells with one, two, and three times were cultured for 4 days. The 1 x 10^5 NHDF cells were seeded onto 6 microwells with DMEM containing 10%FBS, and cultured for 4 days with medium change in every day. Cell viability and living cell number were counted by trypan blue staining method.

As shown in **Figure S7 (a)**, all coating conditions did not affect cell viability. However, cell growth curves were delayed with increasing the coating number (**Figure S7 (b)**). Phase contrast images of one time coating samples in **Figure S7 (d)** showed high spreading morphologies as same as the uncoated control sample (**Figure 7 (c)**). On the other hand, multiple coated cells indicated the delay of spreading and growth probably due to taking time for breaking the collagen shells (**Figure S7 (e) and (f)**). However, the cells indicated good spread and growth morphologies after breaking the shells, suggesting harmless property of the collagen microfiber coating method.

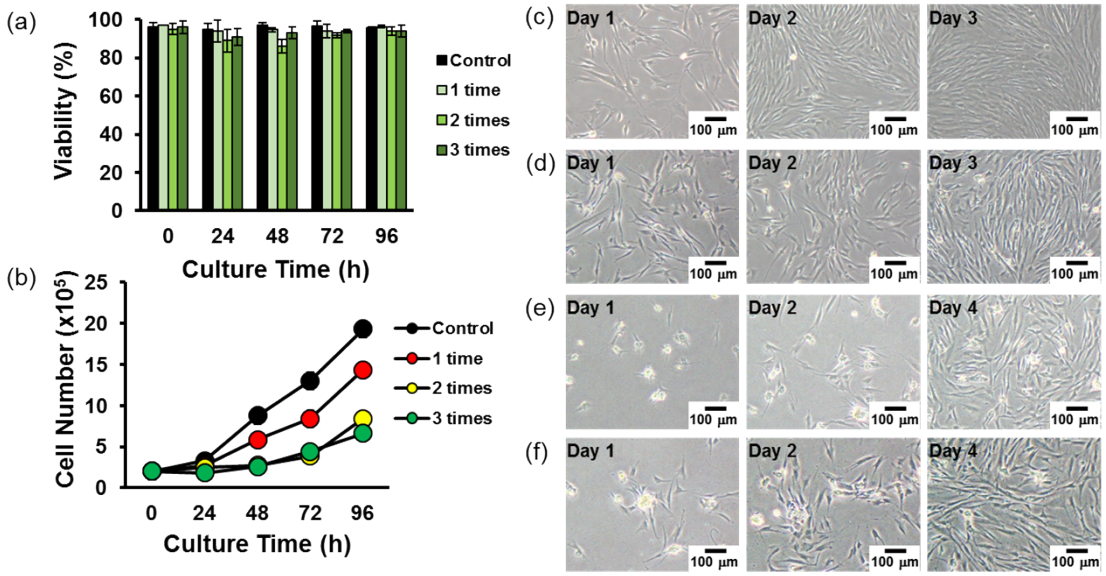

Figure S7. Viability (a), proliferation curve (b), and phase contrast images (c-f) of NHDF cells with or without coating at 0.03 wt% collagen for one, two, and three times coating (n=3).

2.6. Coating Effect to Fabrication of 3D-Tissues

Construction of 3D-tissues using collagen-coated cells was performed, and the constructed structures were compared with the 3D-tissues constructed by our previous method, FN-G nanofilm coating.

Cell culture insert was treated with 0.2% fibronectin for 15 min in 37 ^{o}C incubator. The 6 x 10^5 cells of collagen-coated or uncoated NHDF were seeded in the insert, and then they were cultured in DMEM containing 10% FBS for 24 hours. The fabricated 3D tissues were fixed by 10% formalin buffer for 24 hours. The fixed tissue constructs were peeled off from the culture insert using tweezers, and then paraffin-embedded blocks containing the constructs were fabricated. The blocks were cut into 4-μm-thick sections, and the specimens were stained with hematoxylin/eosin (HE).

Figure S8 shows HE staining image of the sectioned samples using uncoated NHDF cells. The image clearly showed heterogeneous morphology of the surfaces and cracks inside structures, due to lack of cell-cell interaction because of no-ECM matrix on cell surfaces. On the other hand, collagen coated cells revealed obviously thick constructs with over 80 μm thick and lower cell density in Figure 3 (a). This structure was much thicker than 3D-tissues constructed by previous FN-G nanofilm coating due to thicker micrometer-sized collagen fiber coating.

Figure S9 shows HE staining images of 1, 5, and 10 layered (L) NHDF tissues obtained by one time coating. This image clearly suggested controllability of tissue

thickness.

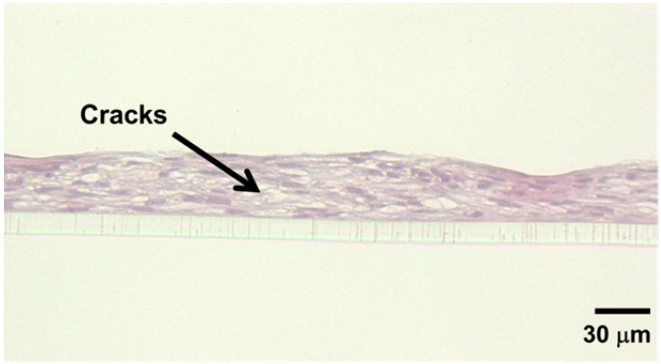

Figure S8. HE staining image of the sectioned samples using uncoated NHDF cells. The experiment was repeated more than three times.

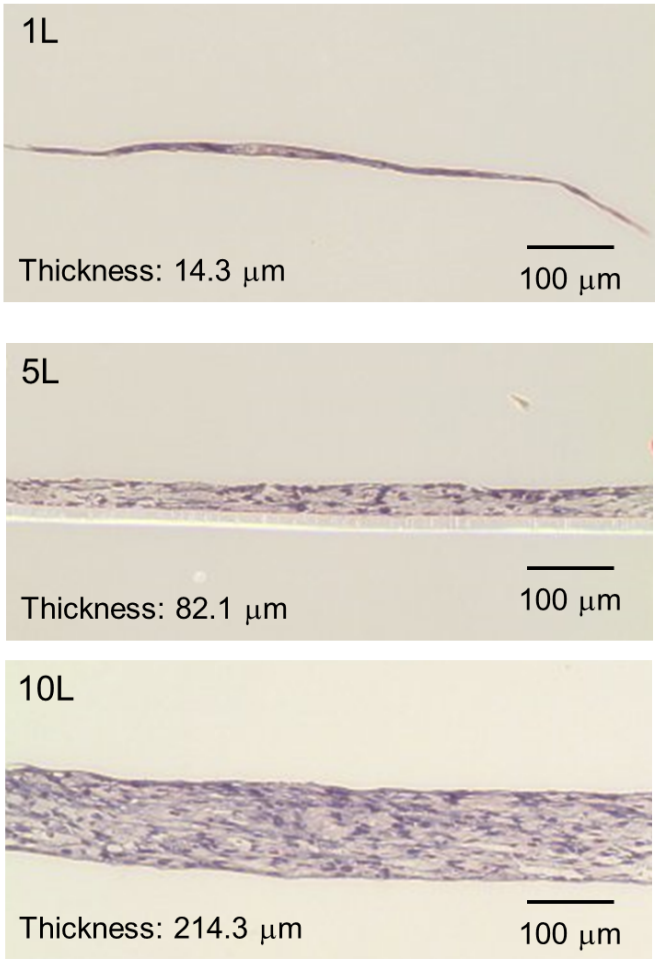

Figure S9. HE staining images of 1, 5, and 10L NHDF tissues by one time coating. The experiment was repeated more than three times.

2.7. Reference

1. [1] M. J. Humphries *et al.* J. Biol. Chem. 1997, **272**, 12311. [2] S. Hattori *et al.* J.

Biochem. 1999, **126**, 54. [3] S. Irie *et al., J. Biochem.* 1999, **125**, 676.