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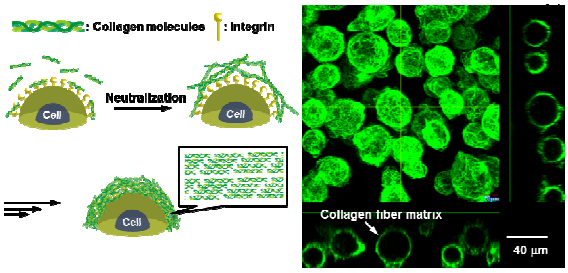
Graphical Abstract



Collagen nanofiber matrices were coated onto single cell surfaces to control cell density in constructed 3D-tissues.

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

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Type I collagen fibers were coated onto single cell surfaces by capitalizing on the formation of collagen microfibers at a neutral pH and exploiting cell membrane interactions between collagen fibers and integrin receptors. Collagen fibers coat thicknesses were controllable from 3 to 30 μm. Multi-layered tissues with controllable thicknesses and cell densities were successfully constructed depending on the thickness of micrometer-sized collagen matrices.

Living tissues or organ modules consist of different types of highly organized cells and extracellular matrices (ECMs) in a hierarchical manner, such as the multi-layered structure of blood vessels and the radial structures of hepatic lobules.[1] Native tissues and organs especially have tissue-dependant cell densities, e.g. skin is composed of high cell density epidermal layers and low cell density dermal layers.[2] Moreover, cartilage has a gradient cell density that varies depending on location.[3] Accordingly, the construction of complex three-dimensional (3D)-human tissues with controlled-cell density remains a central challenge when fabricating replacement tissues in regenerative medicine and in animal-free drug assessment platforms in the pharmaceutical industry.[4] In the past decades, many bottom-up approaches have been reported in the literature describing the fabrication of 3D-tissue constructs, such as cell sheet engineering,[5] magnetic liposomes,[6] cell beads,[7] and cells containing gel layers.[8] However, although these methods are fascinating, the precise control of cell density inside 3D-tissues remains a challenging problem.

We developed a simple and unique bottom-up approach coined "hierarchical-cell manipulation", by preparing nanometer-sized ECM films on cell surfaces.[9,10] Approximately 6 nm-thick fibronectin-gelatin (FN-G) films were fabricated on cell surfaces to promote cell-cell interactions similar to natural ECMs by layer-by-layer (LbL) assembly.[11,12] Various 3D-cell multilayers consisting of single or multiple cell types were successfully fabricated.[13] Furthermore, we reported a rapid construction method using a "cell-accumulation technique", to fabricate over 100 μm sized tissue constructs with blood capillary networks.[14,15] However, it was difficult to control 3D-cell density inside resulting 3D-tissues. Until now, a tissue engineering technology, which can control cell density in 3D-tissues or can give rise to 3D-tissues with cell-density gradients, has not been reported in the literature. Such a novel technology would provide possibilities to construct complex 3D-tissues for tissue engineering, regenerative medicine and pharmaceutical applications.

In the present study, we developed a new method to fabricate micrometer-sized ECM scaffolds on single cell surfaces. In our previous studies, we focused on nanometer-sized ECM films (FN-G films) to promote cell-cell interactions. However, for controlling cell density, we had to also control cell-cell distances, suggesting that micrometer-sized ECMs prefer to separate from neighbouring cells because of their usual cell size which is 10-50 μm. It is expected that cell-cell distances will be controlled by the thickness of coated micrometer-sized ECMs on cellular surfaces, which will be the first report to control cell-cell distance at single cell resolution in 3D-engineered tissues as compared to 3D culture in collagen gel.[16]

To fabricate micrometer-sized ECMs on cellular surfaces, we focused on the fiber formation of type I collagen molecules (Figure 1). Type I collagen molecules usually dissolve under acidic solution (e.g. acetic acid solution), but collagen molecules start to form microfibrils immediately in response to increases in pH to neutrality.[17] Furthermore, it is known that type I collagen molecules can interact with α2β1 integrin receptors on cell surfaces with an association constant of 6.7×10^4 M^{-1}.[18] Accordingly, if microfibrils of type I collagen molecules can be formed on cellular surfaces through interactions with α2β1 integrin receptors, micrometer-sized collagen scaffolds would form on single cell surfaces. Moreover, one would expect that the thickness of the collagen micro-ECMs could be controlled by the coating time.

Certain parameters were optimized to achieve homogeneous collagen fiber formation onto cell surfaces, such as the concentration of collagen solutions, temperature, coating times, and rotation speed. Normal human dermal fibroblasts (NHDFs)

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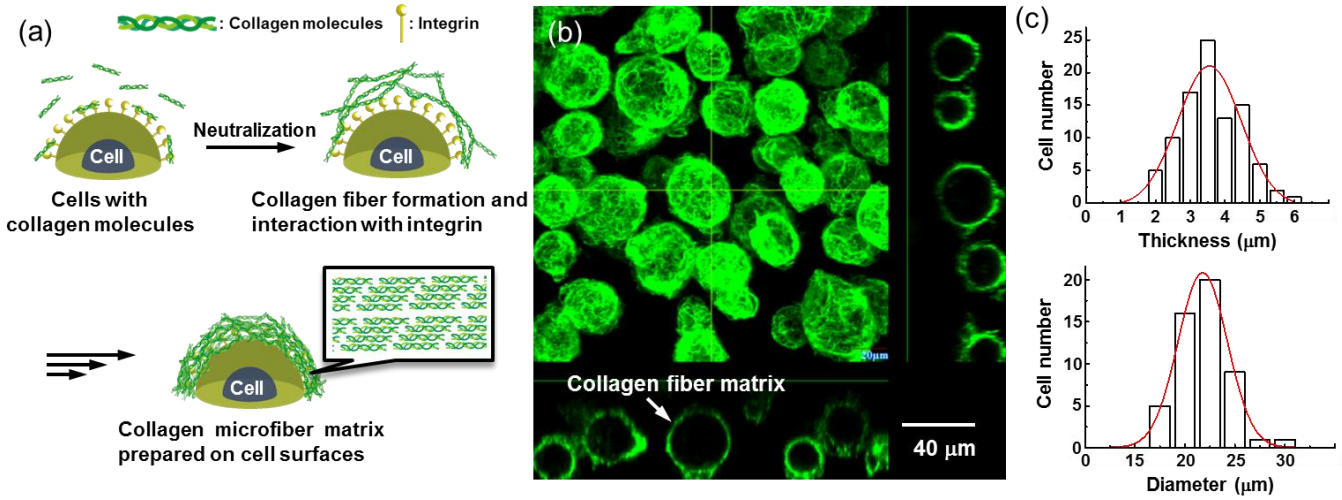

Figure 1. Schematic illustration for coating a collagen microfiber matrix on a trypsinized single cell surface (a). 3D-reconstructed CLSM image of coated cells using 0.03 wt% FITC-collagen solution incubated at 37 °C for 90 min (b). Histograms of thickness and diameter of coated cells (c). Mean thickness and diameter were estimated as 3.3 ± 0.8 μm and 20.7 ± 2.6 μm, respectively (n=20 cells).

were collected after trypsinization, and mixed with collagen solution after neutralization for subscribed time.

First, we evaluated the effect of incubation temperature on the fabrication of homogeneous coating with collagen fibers using fluorescein isothiocyanate (FITC)-labelled type I collagen. A homogeneous coating was observed after incubating at 37 °C for 30 min, whereas room temperature incubations gave rise to heterogeneous cell aggregates (Figure S1 in ESI‡). Room temperature seemed to be low to facilitate collagen fiber formation.

In order to optimize concentrations of collagen solution, various concentrations from 0.3 to 0.03 wt% were used as shown in Figure S2 in ESI‡. A thick matrix was observed at the highest 0.3 wt% condition but cells aggregated independent of coating time. On the other hand, the lowest 0.03 wt% concentration showed thin but homogeneous cell morphology. The thickness and monodispersity slightly increased with increasing coating times, and monodisperse coated cells were obtained when over 60 min of coating times were used with approximately 3 μm thickness as estimated from confocal laser scanning microscope (CLSM).

Rotation speed and cell number during incubation were evaluated in detail. Figure S3 in ESI‡ shows the effect of the rotation speed on the homogeneity of coated cells. A thick coat with cell aggregates was found when slower than 25 rpms was used, whereas thin coats on surfaces of monodisperse cells were observed at 50 rpms. When the cell number was lower than 5 x 10^5 cells in the solution, thicker but unstable collagen matrices were found as shown in Figure S4 in ESI‡. Higher cell numbers especially at 1 x10^6 cells revealed thin but monodisperse fluorescent images.

These results suggested that 1 x10^6 cells in 0.03 wt% type I collagen solution at 50 rpm incubated at 37 °C for over 60 min was optimal for further experiments. Optimal conditions provided collagen fiber matrices on cell surfaces with 3.3 μm and the mean diameter of coated cells was 20.7 μm as shown in Figure 1.

To evaluate the effect of integrin receptors on cell membranes in relation to collagen fiber formation, we compared collagen fiber coating with or without cell conditions (Figure S5 in ESI‡). When the same procedure was performed without NHDFs, fluorescent compounds were barely observed after 5 min incubations. On the other hand, initial collagen fiber formation was evident with the addition of cells even at this initial period. When we continued reactions without cells for 90 min, micrometer-sized collagen aggregates gradually formed because collagen microfibrils usually formed after neutralization of the solution. However, aggregates completely disappeared after washing (Figure S6 in ESI‡). Interestingly, nonspecific aggregates did not form during whole reactions, when 1 x 10^6 NHDFs were added into collagen solutions. These data suggest that integrin receptors on cell membranes can act as initiators to predominately drive the formation of collagen microfibers.

Cell densities and cell-cell distances within native tissues are quite different depending on target organs. Accordingly, a versatile technology which can control cell density is strongly desired. To modulate cell density precisely, we tried to control the thickness of the collagen fiber matrix on cell surfaces by applying multiple coating. In order to visualize the second coated layer, 0.0015 wt% rhodamine-labelled FN (Rh-FN) (5% versus collagen molecule) was added into collagen solutions. When the second coating was performed to the coated cells using Rh-FN containing solution, red fluorescence corresponding to Rh-FN was clearly observed by CLSM (Figure 2). Since the thickness of the second collagen matrix was approximately 10 to 15 μm, the mean total thickness was about 14.9 ± 3.3 μm. We also tried to fabricate a third coated layer using a FITC-collagen solution without Rh-FN. CLSM images clearly revealed three distinct layers consisting of first, second, and third coated collagen fiber matrices. The average thickness of the third layer is about 15 μm, thus the total mean thickness was 30.3 ± 8.0 μm. Two-times coated cells remained monodisperse, but three-times coated cells were slightly polydisperse. However, multiply coated cells showed over 95% viability and good growth properties (Figure S7 in ESI‡). These data suggest the methods described herein can easily control the collagen microfiber thickness of single cell surfaces (see supplementary movies in ESI‡). Furthermore, the data contains important suggestions for the possibility to add other kinds of ECMs into collagen microfiber matrices. In the body, ECMs are typically composed of FN, hyaluronic acid, collagens, and laminin and provide complex biochemical and physical signals.[19,20] The ECM component varies by tissue type, e.g. skin dermal layers predominately contain FN and type I collagen,[2] and cartilage tissue has hyaluronic acid and type II collagen as a main component.[3] Thus, precise modulation of 3D-localization of additional ECM components must be important for the construction of artificial complex 3D tissues. The data in

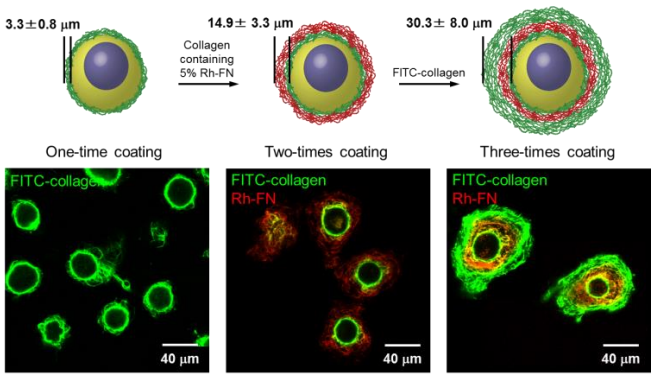

Figure 2. Illustration and CLSM images of the multiple coated cells. The second coating solution contains 5% Rh-FN versus collagen molecule, and all the other condition was completely same. Thicknesses of all samples were average of over 20 cells in each.

Figure 2 clearly indicate the achievement of 3D-localization of FN in the second layer of collagen microfiber tissues on a single cell surface.

Since it is expected to modulate cell density and cell-cell distance in 3D-tissues, we tried to fabricate 3D-tissue constructs using only one-time coated NHDFs. The 6 x 10^5 NHDFs after one-time coating were seeded in 24-microwell inserts and cultured in Dulbecco's modified eagle me medium (DMEM) containing 10% fetal bovine serum (FBS) for 1 day. Histological images with hematoxylin-eosin (HE) staining of the obtained 3D-tissue constructs were compared with those from 3D-tissues constructed using 1 x 10^6 cells of NHDFs by our previously reported "cell accumulation technique" using 6 nm-sized FN-G nanofilms.[14]

When surface coatings were not employed (uncoated cells), heterogeneous and porous structures were obtained (Figure S8 in ESI‡). There were many cracks and the thickness of rough constructs was less than 30 μm. On the other hand, NHDFs coated with 3 μm-thick collagen microfibers clearly revealed thick soft tissues with approximately 80 μm thicknesses (Figure 3a). This thickness was about 2-fold higher than 3D-tissues fabricated by the cell accumulation technique using FN-G nanofilms on NHDFs, although the cell number was 1.67-fold higher (Figure 3b). It was really surprising data because approximately 2 x 10^6 NHDF cells were necessary in the preparation of 3D-tissues with the same 80 μm thicknesses by our previous cell accumulation technique. These data suggest that about 1/3 the cell number was sufficient to fabricate equi-thick 3D-tissues by a collagen microfiber coating method. In order to evaluate in detail, we calculated cell density in both 3D-NHDF tissues. Cell densities obtained via "collagen microfiber coating methods" and "cell accumulation techniques" were about 2.3 x 10^8 and 8.0 x 10^8 cells/cm^3, respectively, suggesting a 3.5-fold lower cell density in 3D-tissues by a collagen microfiber coating method. Cell-cell distances in these 3D-tissues were calculated as 15.6 ± 4.0 μm and 8.8 ± 3.1 μm, respectively (Figure 3). Because collagen fiber coated cells wear 3 μm-thick "collagen cloths", each cell should be separated by about 6 μm distance. This estimate agrees well with the calculated difference of about 6.8 μm between two tissues. These data suggest the successful fabrication of cell-density controlled 3D-tissues by a collagen microfiber coating method. Cell layer number and thickness were easily controlled from 14 μm (1 layer) to 214 μm (10 layers) (Figure S9 in ESI‡).

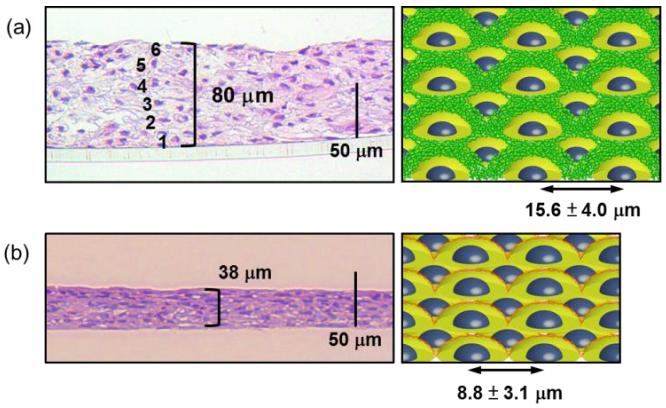

Figure 3. Histological HE staining images (left) and schematic illustration (right) of 3D tissues constructed by one-time collagen microfiber coating (a) and FN-G nanofilm coating (cell accumulation technique)[14] (b). The mean distances between nuclei-nuclei were estimated from 50 nuclei in HE images.

In conclusion, a type I collagen microfiber coating method applied to single cell surfaces was successfully developed by optimizing the reaction temperature, concentration, rotation speed, and cell number. Under optimal conditions, homogeneous and monodisperse fiber matrices were formed on cell surfaces. The thickness of the fiber matrix was easily controlled by the addition of multiple coatings and approximately 30 μm-thick matrices were deposited on cell membranes. Furthermore, additional ECM components were specifically localized in desired layers. When coated cells were seeded in culture inserts, thick 3D-tissues with low cell densities were successfully constructed as compared with our previously reported method. We can fabricate thicker 3D-tissues with lower cell densities using two or three-times coated cells because of thicker collagen matrices. To the best of our knowledge, this is the first report in the literature that describes the construction of cell-density controlled 3D-tissues at single cell resolution that will be useful for tissue engineering and pharmaceutical applications. Inkjet cell printing technique[21] will allow us to control cell-cell distance at single cell level in 3D-tissues.

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