One-Step Construction of Thick Multilayered Tissues by Fabrication of Layer-by-layer Nanofilms onto Cell Surface

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Statement of purpose: *In vitro* construction of layered tissues has attracted much attention in tissue engineering. Up to date, cell sheet engineering and magnetic liposome have been employed for layered tissue construction, but these methods can't control the thickness of extracellular matrix (ECM) and three-dimensional (3D) configuration of cells. Recently, we reported hierarchical cell manipulation technique by fabrication of fibronectin and gelatin (FN-G) nanofilms onto cell surfaces by the layer-by-layer (LbL) assembly.[1)] The 6 nm of FN-G nanofilms on the surface of the first layer of cells provided a suitable cell adhesive surface that is similar to the natural ECM for the second layer of cells.[2)] We successfully prepared 3D-blood vessel constructs[3)] and clarified the effect of 3D-structures on cellular functions.[4)] Although this method was effective to control cell types one by one in the obtained 3D-layered tissues, it had a limitation for a maximum layer of two-layers per day due to waiting for stable cell adhesion. For example, development of ten-layered (10L-) constructs required for at least five days. To fabricate thicker 3D-tissues in one step, we developed a novel one-step fabrication technology of approximately 10L-tissues by LbL assembly. By preparing FN-G nanofilms on a dispersed single cell surface and subsequent incubation in a cell culture insert, 3D-tissues with seven or eight layers were obtained after 24 hours of culture (Figure 1a). Furthermore, cellular configuration was easily controlled in the obtained tissues. This approach can be an innovative technique for tissue engineering.

Methods: The 5 x 10^6 cells/mL human dermal fibroblast cells (hFCs) were alternatively incubated with 0.2 mg/mL FN ($M_w = 4.6 \times 10^5$) and G ($M_w = 1.0 \times 10^5$) in 50 mM Tris-HCl (pH = 7.4) for 1 min at 30 rpm. After repeating the nine steps of immersion, the $(FN/G)_4FN$ films with 6 nm thickness were prepared on the cell surface. The preparation of FN-G films onto the cell surface was confirmed by confocal laser scanning microscopic (CLSM) observation using rhodamine (Rh)-FN and FITC-G. Moreover, the viability and proliferation of the cells coated with FN-G and other LbL films were also evaluated. Finally, the hFCs coated with FN-G nanofilms were seeded into a cell culture insert and cultured for 24 or 48h to construct multilayered tissues in one step.

Results: Fluorescence microscopic observations showed successful preparation of Rh-FN-FITC-G films onto the surfaces of fibroblast cells. The obtained cells with FN-G films onto their surface maintained high viability (> 98 %) and proliferative property. On the other hand, other polyelectrolyte nanofilms such as FN-ε-poly(lysine) (ε-Lys) and ε-Lys-Poly(styrene sulfate sodium salt) (PSS) showed cytotoxicity depending on their thickness because of the condensation and strong adsorption of the cationic components onto the cell surface even using biocompatible FN as a counterpart component. Due to binding domain interaction of negatively charged FN and G, the FN-G films did not show any cytotoxicity.[1)] Hematoxylin and eosin (HE) staining and CLSM images showed the successful construction of 3D-layered architectures composed of the hFCs. Although non-coated cells poorly adhered and layered tissues could not be constructed, FN-G coated cells indicated well adhesion and organization and seven or eight layers were constructed in one step due to adhesive property of the FN-G nanofilms (Figure 1b, c). By repeating seeding of the hFCs stained with cell tracker green and red, eight-layered tissues consisting of distinct two types of four layers were successfully obtained within 2 days.

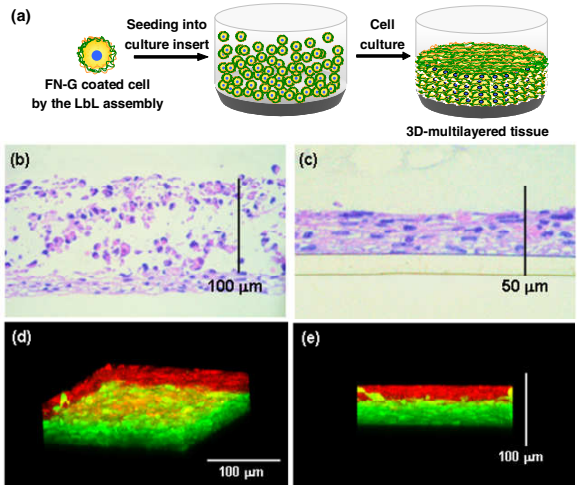

Figure 1. (a) Schematic illustration of 3D-multilayered hFC tissues by preparing FN-G nanofilms onto single cell surface. (b, c) HE staining images of the obtained architectures composed of (b) non-coated cells and (c) FN-G coated cells. (d, e) 3D-reconstructed CLSM cross-section images of multilayers composed of FN-G coated cells labeled with cell tracker green and red, respectively.

Conclusions: One-step construction of multilayered tissues was achieved by fabrication of FN-G nanofilms on single cell surface within 1 or 2 days. This methodology will be useful as a build-up technique for tissue engineering.

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