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Three-dimensional culture of epidermal cells on ordered cellulose scaffolds

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Abstract
An ordered cellulose film scaffold, termed a nematic ordered cellulose (NOC) template, had unique surface properties and successfully induced the establishment of a three-dimensional (3D), hierarchical structure of epidermal cells by cell attachment and subsequent culture. Initially, the scaffold surface properties were characterized through contact angle measurements and atomic force microscopy to evaluate appropriate hydrophobicity and orientation of molecular chains for 3D culture. The template surfaces exhibited higher hydrophobicity, in the range of 70–75°, than usual cellulose films and appeared suitable for surface cell adhesion. In fact, epidermal cells successfully attached and proliferated favorably on the NOC templates, similar to development in normal culture flasks. Furthermore, the NOC film, as a semipermeable template, was also employed to allow 3D proliferation of epidermal cell layers in the perpendicular direction. The template proved to be suitable as a 3D cell culture device, resulting in the proposal that the construction processes of these 3D cell layers followed the basic concept of skin formation.

(Some figures may appear in colour only in the online journal)

Introduction

A unique cellulose film template, termed a nematic ordered cellulose (NOC), possessing a functional ability to induce oriented deposition of various objects on its surface has been extensively studied here [1–4]. An NOC film is prepared by uniaxial stretching of a water-swollen and fixed cellulose gel, resulting in the orientation of cellulose molecular chains along the stretching axis. A unique characteristic of the prepared NOC film is its dominant noncrystallinity, despite the well-ordered molecular orientation [1]. In addition, oriented anhydroglucose units on an NOC surface are vertically pitched at ∼60° to the surface, which indicates that the neighboring anhydroglucose ring planes tend to face each other. Simultaneously, the hydrophilic hydroxyl groups in the pitched individual molecular chains are also aligned like tracks along the stretching axis and across the entire NOC surface. Between the hydrophilic molecular tracks of hydroxyl groups, the hydrophobic phase due to the anhydroglucose plane also appears, resulting in both hydrophilic and hydrophobic tracks next to each other alternating across the NOC surface. These amphiphilic molecular tracks enhance the unique surface properties of NOCs, which include serving as a template or scaffold for ordered deposition of various substances such as nanofibers or inorganics [2, 5].

The interfacial affinity between cells and culture plates is in general affected by differences in the plate surface properties because a relatively hydrophobic culture plate is believed to induce the proliferation of cells following cell adhesion and subsequent settlement [6]. Recently, some scaffolds for cell culture have been proposed to establish a technique for regeneration medicine. Among available scaffolds, collagen [7–11], hyaluronic acid [12–14], chitosan [15], silk fibroin and spider silk [16, 17] are major biopolymers playing a bioactive role in such medical purposes. Although cotton cellulose is not believed to be biocompatible, it was recently clarified that bacterial cellulose pellicle produced from *Gluconacetobacter xylinus* has biocompatibility [18] and could be used for medical materials and tissue engineering [18, 19]. The latest research

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reported that cell divisions were induced toward the direction of cellulose nanofiber as the scaffold [20, 21]. Here, we attempt to culture epidermal cells on the noncrystalline cellulose template (NOC) having ordered cellulose molecules, instead of crystalline cellulose nanofibrils.

In this study, the unique NOC surface described above was examined regarding its potential as a scaffold for two- and three-dimensional cell culture of epidermal keratinocytes. Initially, surface characterization of NOC templates was carried out by contact angle measurements and atomic force microscopy (AFM) analyses. Then, cell behaviors when cultured on NOC templates were observed using inversed phase contrast microscopy, followed by attempts to produce a three-dimensional (3D) culture of epidermal keratinocytes on a newly modified NOC system proposed in this study.

Materials and methods

Preparation of NOC templates

The NOC templates were prepared according to a previous procedure [1]. As the starting cellulose sample, cotton fibers with a degree of polymerization (DP) of 1300 were swollen sequentially in water, ethanol and *N*,*N*-dimethylacetamide (DMAc) using a solvent exchange method. Here, after samples were soaked in each solvent overnight and then squeezed to remove the solvent at least four times between each step, they were subjected to dissolution as follows. The swollen cellulose was put into the LiCl/DMAc (7% w/w) solution with constant stirring at room temperature for at most three weeks. The resulting solution was centrifuged and filtered to remove any insoluble material. The filtrate was then poured into a surface-cleaned glass Petri dish with a flat bottom and placed in a closed box containing saturated water vapor at room temperature. The sample was allowed to stand overnight for at most a few days until precipitation of a gel-like film appeared, at which time the film was washed with distilled water for several days to thoroughly remove the solvent, resulting in a water-swollen transparent gel-like film. Next, the cellulose film was cut into strips to be clamped in a manual stretching device and elongated uniaxially at a draw ratio of >2.0 at room temperature; such a draw ratio is enough to induce the orientation of cellulose molecular chains to form an NOC template [1]. Following air-drying, the drawn specimen was vacuum-dried while being kept stretched in the device at 25 °C for 24 h.

Analyses of the surface structure of NOC templates

Contact angle measurements of a water drop on the template were performed by a contact angle meter (DM300, Kyowa Interface Science Co., Ltd., Niiza, Japan) at 25 °C. Dried NOC template specimens were stored into a desiccator until measurement and the water drop volume set at 1 μl.

The surface structure of the templates was observed by an AFM system (SPM-9500J3, Shimadzu Corp., Kyoto, Japan). All AFM images were acquired in contact mode using silicon nitrate cantilevers at room temperature. The scanning area was from 1 × 1 to 10 × 10 μm^2. Prior to measurements, each dried sample was cut into a square and fixed on the sample stage for AFM observation. Scanning was carried out perpendicular to the drawing axis. For surface characterization, the width and height of the surface roughness were measured using a cross-sectional line profile analysis. The apparent width data were supposed to include geometrical enhancement (E) depending on the radius (R) of the AFM tip. In this observation, as the height of the aggregates was smaller than the radius of the tip, the following correction equation was employed [2]. The equation takes the tip cone angle (A) into account in the form,

$$E = 2 \times (RH - H^2)^{1/2}, \quad w = W - E,$$

in which E is the geometrical enhancement on the real width value (w), W is the apparent width observed in AFM, H is the height of the object observed in AFM and R is the AFM tip radius. In this study, an R-value of 20 nm in radius was employed.

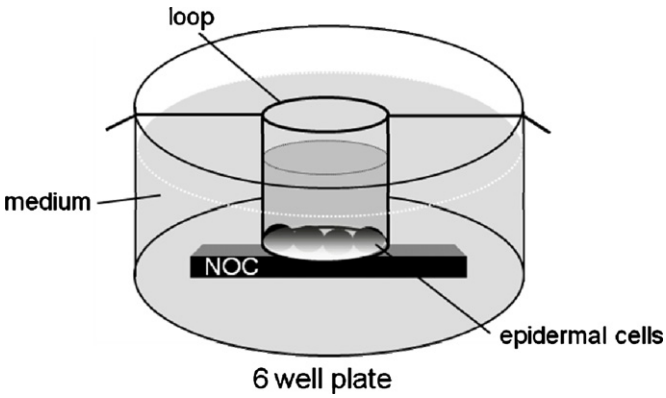

Figure 1. Schematic image of the three-dimensional cell culture system.

Cell culture

Normal human epidermal keratinocytes (NHEK(B), Kurabo Co. Ltd., Osaka, Japan) were cultured on normal plates (T-25 flask: BD Falcon™ cell culture flask, BD Japan Co. Ltd, Tokyo, Japan), NOC templates and the modified NOC system.

NOC templates were sterilized before cell cultivation. Briefly, the template was soaked in 70% aqueous ethanol solution, washed with sterilized water and then soaked in culture media, with the process repeated three times over 15 min for each cycle.

For cultivation and subculture of epidermal keratinocytes, cells cultured in HuMedia-KG2 (Kurabo Co. Ltd.) were detached from the culture flask surface by treatment with trypsin plus ethylenediamine tetra acetic acid (the concentration was as indicated by the provides), and then collected by centrifugation (1100g for 5 min). The resulting pelleted cells were then placed onto the desired culture plates, adjusted to 2500 cells cm^{-2} and cultured at 37 °C under 5% CO_2.

Three-dimensional cell culture

For 3D cell culture, a new culture plate system using the NOC film was fabricated (figure 1). In this system, the NOC film template was fixed to the bottom of a cultivating loop, placed in

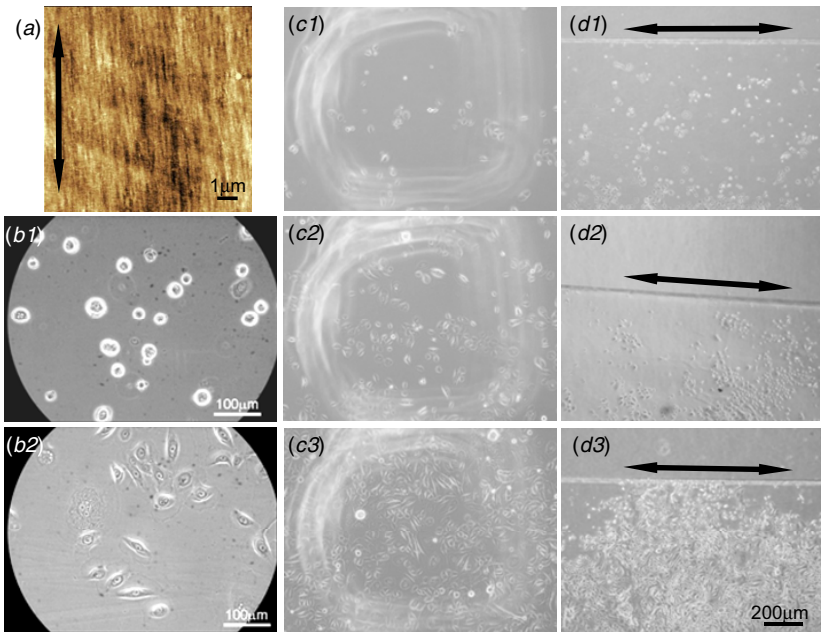

Figure 2. Culturing epidermal cells on NOC scaffolds. (*a*) AFM image of the NOC template; typical fixation behavior of epidermal cells before (*b*1) and after (*b*2) placement on the normal culture plate of the T-25 flask; (*c*) and (*d*) series indicate cell behavior on the T-25 flask and NOC templates, respectively; cultivation periods: 4 (*c*1, *d*1), 6 (*c*2, *d*2) and 8 (*c*3, *d*3) days; double arrow—stretching direction (*a*, *d*1–3); scanning area—10 × 10 μm (*a*).

a six-well plate, sterilized as previously described and medium poured both inside and outside of the loop. In this device, NOC templates served as a semipermeable membrane. Next, cells were placed onto the NOC template inside the loop and cultured at 37 °C under 5% CO_2. The medium outside the loop was exchanged daily to provide and preserve fresh culture conditions.

Observation and analysis of cellular behavior on culture plates

Cultured cells were observed using an inversed phase contrast microscope (CKX41, Olympus Corp, Tokyo, Japan) at two-day intervals of 2, 4, 6 and 8 days after introduction onto the medium.

To observe cell surfaces, cells retrieved from culture plates were rapidly frozen in liquid nitrogen, after which the frozen samples were fixed with 2.5% cooled glutaraldehyde solution for 48 h and washed with cooled commercially available phosphate buffer saline (PBS) at pH 7.2 (Sigma-Aldrich, Inc., St Louis, MO, USA). After washing, samples soaked in PBS solution for a few minutes were then dehydrated in an ethanol series of 30, 50, 70, 80, 90, 99.5 and 100% (v/v), and finally exchanged with *t*-butyl alcohol for lyophilization. Prepared samples were observed by AFM to measure the thickness of the cell layers.

Results and discussion

Characterization of NOC templates for culturing epidermal cells

The possibility of cell fixation onto a culture template depends on appropriate surface hydrophobicity. A contact angle range of 60–80° has been proposed as the most suitable for cell fixation and proliferation [6]. Here, the contact angle of the NOC template was indeed 71.9 ± 1.3°. This value is in the middle of the 60–80° range and indicates suitability for cell culture. A uniaxial orientation in NOC was observed on the template surfaces, confirming it to be similar to previous reports [1, 3]. The periodic distance appeared to be 107 ± 28 nm (figure 2(*a*)).

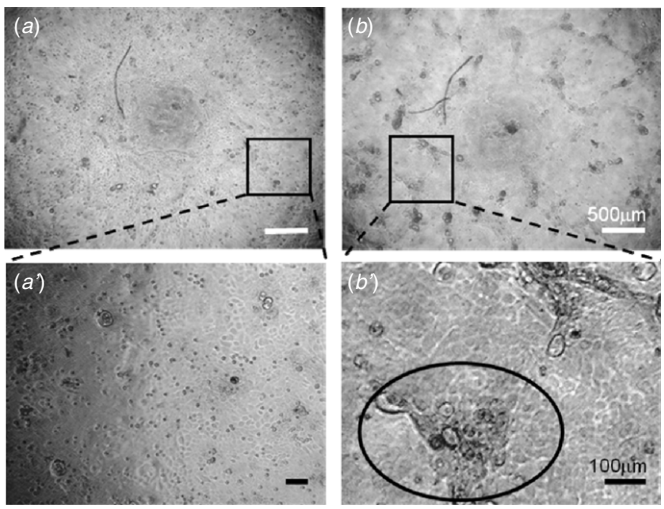

Figure 3. Cell proliferation on NOC templates provided medium from the loop bottom to the top through the semipermeable NOC template. Culture times: (*a*, *a*′) 3 days' and (*b*, *b*′) 5 days' cultivation; circle—colony.

Epidermal cells were then cultured on both the T-25 flask and the NOC template and, after attachment, observed by inversed phase contrast microscopy. As described above, the contact angle for NOC was 71.9° ± 1.3°, whereas the T-25 flask exhibited 69.4° ± 11.1°. The surface of the T-25

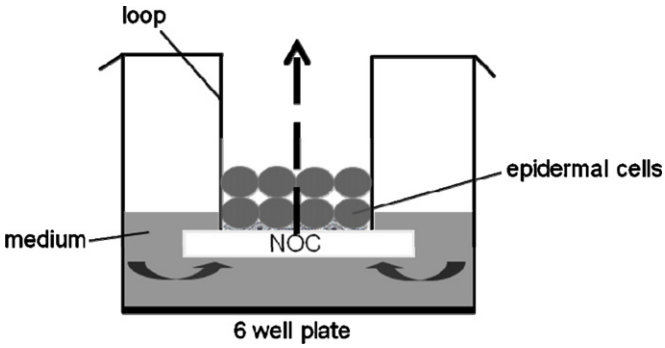

Figure 4. Schematic image of three-dimensional cultivation on the semipermeable NOC template. Broken arrow—possible proliferation direction of epidermal cells; arrows—flowing tide direction of medium into and through NOC template.

flask is in general modified to be suitable for cell fixation and proliferation, although the contact angle had a larger standard deviation. Both templates were assumed suitable for cultivation for the epidermal cells, judging by the contact angles. In fact, cells were fixed and proliferated well on both the NOC template and the T-25 flask. The cell shapes were observed to change from spherical (figure 2(*b*1)) to spreading (figure 2(*b*2)) several hours after introduction to both the T-25 flask and NOC templates. Spreading shapes indicated that cells were successfully attaching onto both culture plates and such a behavior of cells also indicated preference of the cells for particular plates. As control tests, a non-stretched gel cellulose (prior to the stretching for NOC) and cellophane were employed as non-ordered cellulose template. On both of the templates, fixation and proliferation of cells were not well performed, presumably because of their higher hydrophilicity.

Following adherence to the template or culture flask surfaces, cell division began. It should be noted that T-25 flask surfaces are usually modified by negative electric charges to induce stable cell attachment. On NOC templates, it was observed that cells successfully attached and proliferated on both these templates and T-25 flasks; the calculated growth ratio showed similar tendencies to proliferate (figures 2(*c*1)–(*c*3)). At the beginning of the culture periods, cells were scattered across the NOC template (figure 2(*d*1)). After 8 d of cultivation, cells completed covered the surface and were considered confluent (figure 2(*d*3)) and stably attached. Taken together, these behaviors indicated that the NOC template surface was capable of supporting these cells. Growth observation results also verified the relationship between cell attachment and the contact angle value of 71.9 $\pm$ 1.3°.

On the other hand, cells appeared to be randomly distributed after proliferation on the NOC surface (figures 2(*d*1) and (*d*2)). This may have indicated that the cellulose molecular orientation on the NOC surface was not effective in controlling the direction of cell growth. In this case, it appeared more important for cell attachment and proliferation to have surface hydrophobicity because of the cellulose molecule orientation.

Three-dimensional cell culture using a modified NOC culturing system

This study also attempted to establish a 3D cell culture using a NOC template by confirming it to be a suitable culture template, as in the previous section. A 3D formation of epidermal cells is important as a bio-mimicking sample for medical and cosmetic analyses without involving living organisms. The stability of cell adherence and the continuous introduction of nutrients are required for 3D cell culture. As NOC templates were swollen in the medium, forming a semipermeable membrane because of its dominant noncrystalline domains, they served as a means for constantly providing fresh nutrient for growing cells (figure 1). Using the new system in 3 days' cultures, cells proliferated and formed one layer over the entire NOC template surface (figures 3(*a*) and (*a*′)). Furthermore, in 5 days' cultures, some colonies having 36 μm differences in height were observed above the previously formed cell layer (figure 3(*b*′)). This indicated that cell division was induced upward, perpendicular to the NOC template bottom. Specifically, this effect indicated that the film played a role as a semipermeable membrane in providing and maintaining fresh culture conditions (figure 4). To support the

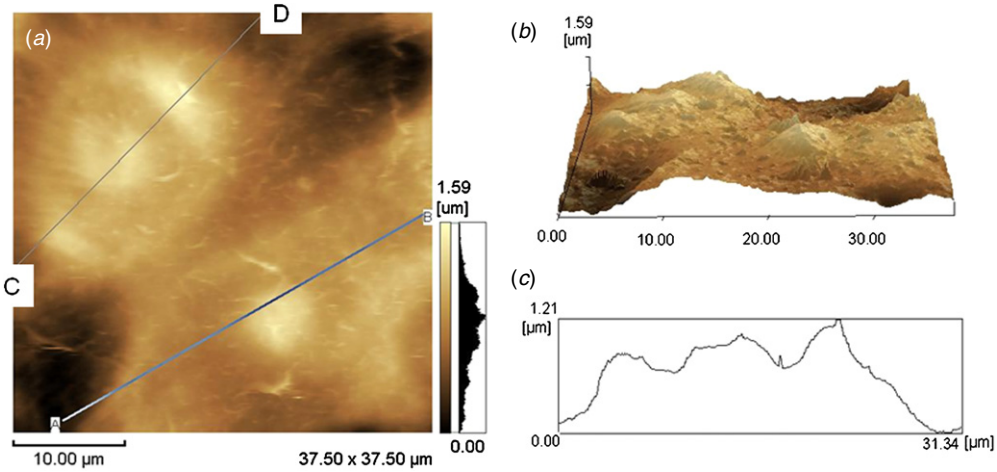

Figure 5. AFM image of a single layer of epidermal cells cultured on templates. (*a*) Height image of cells; (*b*) three-dimensional image and (*c*) cross-sectional image along line C–D in (*a*).

3D features mentioned above, the height of a single cell was measured by AFM in a cultivation that was allowed to build only a single cell layer on the NOC surface in the loop bottom (figure 4). An AFM image of the single layer of fixed and proliferated cells on the NOC is shown in figure 5. The cross-sectional image along the line C–D in this figure was assumed to indicate the height of a single fixed cell, at 1.42 $\pm$ 0.13 μm. Although measurement of the height of some layered cells, as a colony, was attempted (figure 3), it was too high to detect by AFM. Alternatively, the height of these layers was measured at $\sim$36 μm via a through-focus series of images using light microscopy. The colony, as shown in figure 3, was concluded to consist of $\sim$30 layered cells.

These results also suggested that the very bottom layer of cells did not detach from the NOC template during culture; therefore, the formerly established layered cells were considered to fulfil the role of scaffold for the next newly formed cell layer.

Conclusion

This study served to establish that NOC, as a cellulose film having a unique surface structure and properties, could be employed as a three-dimensional (3D) culture template for epidermal cells. Initial light microscopic observations of cell attachment and growth (or proliferation) on NOC templates indicated that the template was suitable as a cultivating scaffold for animal cells. It also indicated that the template was suitable for inducing construction processes leading to the formation of a 3D cell accumulation because the NOC structure played roles as both a scaffold and semipermeable membrane. The functions of the scaffold in supporting cell adherence and proliferation were enhanced by the amphiphilic molecular tracks on the unique NOC surface. However, the semipermeable membrane function was attributed to the dominant noncrystalline domains of the NOC film, providing fresh nutrient for cells through the bottom to the top, similar to the process of human skin formation.

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