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A Strategy for the Construction of Controlled, Three-Dimensional, Multilayered, Tissue-Like Structures

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Differentiated cells make up tissues and organs, and communicate within a complex, three dimensional (3D) environment. The spatial arrangement of cellular interactions is difficult to recapitulate in vitro. Here, a simple and rapid method for stepwise formation of 2D multicellular structures through the biotin-streptavidin (SA) interaction and further construction of controlled, 3D, multilayered, tissue-like structures by using the stress-induced rolling membrane (SIRM) technique is reported. The biotinylated cells connect with the SA-coated adherent cells to form a bilayer. The bilayer of two types of cells on the SIRM is transformed into 3D tubes, in which two types of cells can directly interact and communicate with each other, mimicking the in vivo conditions of tubular structures such as blood vessel. This method has the potential to recapitulate functional tubular structures for tissue engineering.

1. Introduction

Differentiated cells make up tissues and organs, and communicate mutually within a complex 3D environment. The spatial orientation and distribution of extracellular matrix components directly influence the function of organs and tissues.[1] Cellular interactions with extracellular matrix molecules and other cells are important issues of fundamental cell biology, and play a crucial role in tissue engineering. Proper cell-cell communication through physical contact is crucial for a range of fundamental biological processes and the normal function of tissues. The spatial arrangements of these cellular interactions lead to higher-order function, which is difficult to recapitulate in vitro. The researchers have developed various models to investigate these complex interconnectivities,[2–4] the major challenges of which are the abilities to force contact between multiple cell types in 3D, and to control the spatial arrangement of cellular interactions. Several physical approaches have been integrated within the design of 3D scaffold to produce multicellular structures which are 2D cellular arrays of multiple cell types[5–7] or defined 3D cell aggregates of a single cell type.[8–10] However, these approaches may not be readily applicable to generating multicellular structures with controlled cell-cell contacts in 3D.

Cell-surface engineering methodologies are the other way for producing multicellular structures with controlled cell-cell contacts in 3D, and they can introduce different functional groups with mutual reactivity on cell surfaces. In recent studies, some cell-surface engineering methods have been used for the construction of 3D tissue-like structures, such as metabolic introduction of reactive functional groups,[11] hydrophobic insertion into the cell membrane,[12–15] and chemical modification.[16,17] The resulting multicellular structures include multicellular heterospheroids of different cell types,[13,15,17] layered heterotypic aggregates of two cell types,[12,14,16] or 3D cellular multilayers,[15,18,19] but they lack the control of the interconnectivity among cells at single cell level. The metabolic methods can precisely control the interconnectivity among cells in 3D,[11] but need to introduce exogenous substances into cells which may disturb the cellular physiology.

Chemical cell surface modification is the most straightforward for the cell surface remodeling. Biotinylation of cell surfaces is one of the most common methods for the cell surface modification through the direct chemical reaction of primary amine groups presented on the cell membrane with the amine-reactive biotin, such as *N*-hydroxy-succinimide biotin derivatives.[20,21] Cells modified with biotin can be readily functionalized with a wide range of biotinylated molecules through the biotin-SA interaction.[22] Here, we report a simple and rapid method for stepwise formation of controlled 3D multilayered tissue-like structures through the biotin-SA interaction and the stress-induced rolling membrane (SIRM) technique. This method can deform the 2D surface containing a bilayer of two types of cells, which connect with each other through the botin-SA interaction, into 3D multilayered tube, in which the two types of cells could directly form intima and tunica media-like structures mimicking tubular tissues, especially blood vessels. Furthermore, the interaction and communication between the cell-cell and cell layer-cell layer could be maintained in 3D spatial arrangement. These tubes could well mimic in vivo tubular tissues.

2. Results and Discussion

Our method for rapid and steady formation of controlled contacts between different cell types is based on the biotin-SA

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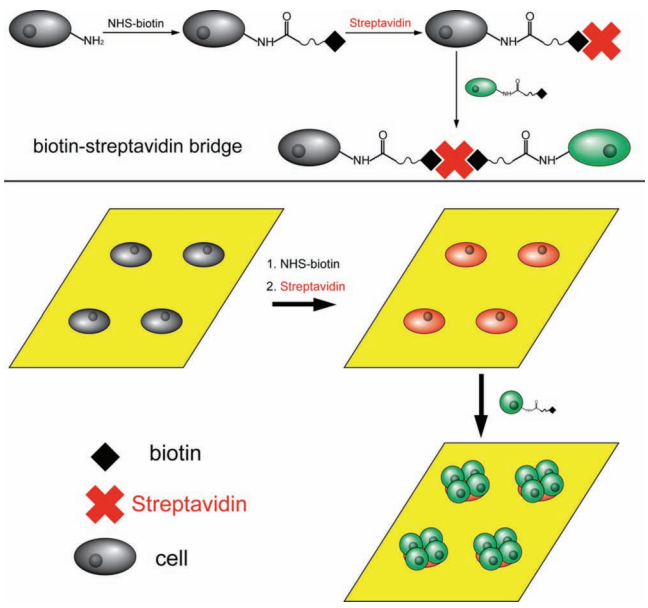

Figure 1. Schematic diagram of cell surface modification and stepwise formation of multicellular structures.

interaction. The cell surface modification procedure consists of two steps involving treating cell surface amines with sulphonated biotinyl-*N*-hydroxy-succinimide (NHS–biotin), followed by biotin-SA interaction. The adherent cells on the substrates were modified with NHS-biotin and SA successively. The population of another type of cells modified with NHS-biotin was added on the top of monolayer of the SA-coated adherent cells. So the biotinylated cells would contact with SA-coated cells through the biotin-SA interaction to form a bilayer (**Figure 1**). The structures can also be continuously coated with SA, followed by adding another population of biotinylated cells to from structures of multiple types of cells.

The primary amine groups present on the cell surface can react with the NHS-biotin to biotinylate the cell surface. After biotinylating the surface of the human umbilical vein endothelial cells (HUVECs), the cells were incubated with SA to form biotin-SA complexes on the cell surface. The red fluorescence of the cells indicates that they have been biotinylated successfully and coated with Cy3-conjugated SA (SA-Cy3), while the surface of the cells which are not treated with NHS-biotin cannot adsorb SA-Cy3 (**Figure 2**). The amount of the molecules of biotin or SA on the cell surface could be controlled by adjusting the concentration and reaction time of reagents at each step during modification. Short-term exposure to the NHS-biotin solution did not affect cell viability or the expression of phenotypic surface markers (e.g., CD144 for HUVEC) (Supporting Information, Figure S1–3).

We tested the contact efficiency of the surface-adherent cells with biotinylated suspended cells, which can not adhere to the substrates naturally. The NHS-biotin modified Jurkat cells were added and landed on the top of monolayer of the SA-coated HUVECs under gravity to contact with the adhered cell monolayer. Through the biotin-SA interaction, two types of cells combined with each other to form a cell bilayer (**Figure 3**a). Because the unmodified cells can not combine with the adhered cells

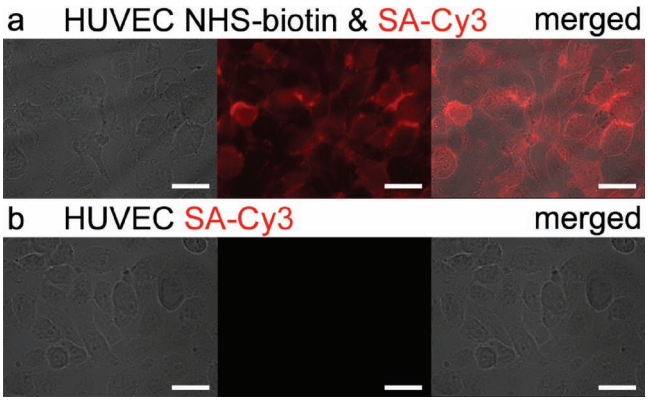

Figure 2. Phase contrast and fluorescent micrographs describe cell surface modification with NHS-biotin and SA-Cy3 (a), and only with SA-Cy3 (b). All the scale bars represent 100 μm.

which have been modified or not, through the biotin-SA interaction, they can be washed away from the surface of monolayer of the adherent cells (Figure 3b–d).

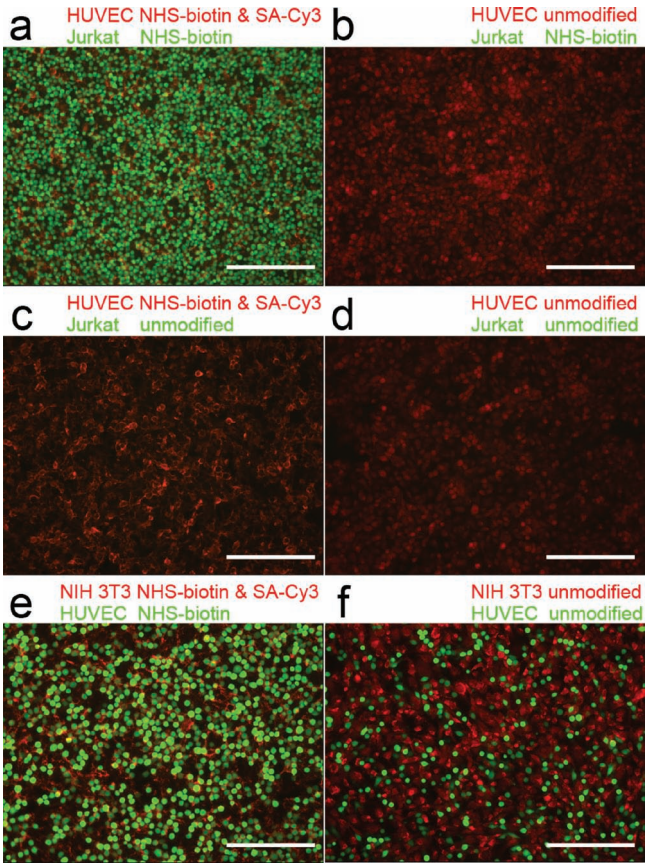

Figure 3. a) Fluorescent images representing the bilayer of HUVECs and Jurkat cells. The HUVECs were modified with NHS-biotin and SA-Cy3 (a,c) or stained with CellTracker Orange (red) without biotinylation (b and d). Jurkat cells stained with CellTracker Green (green) were biotinylated (a,b), or not (c,d). e) Fluorescent images represent the bilayer of HUVECs and NIH 3T3 cells. The NIH 3T3 cells were modified with NHS-biotin and SA-Cy3 (e), or stained with CellTracker Orange (red) without biotinylation (f). HUVECs stained with CellTracker Green (green) were biotinylated (e), or not (f). All the scale bars represent 250 μm.

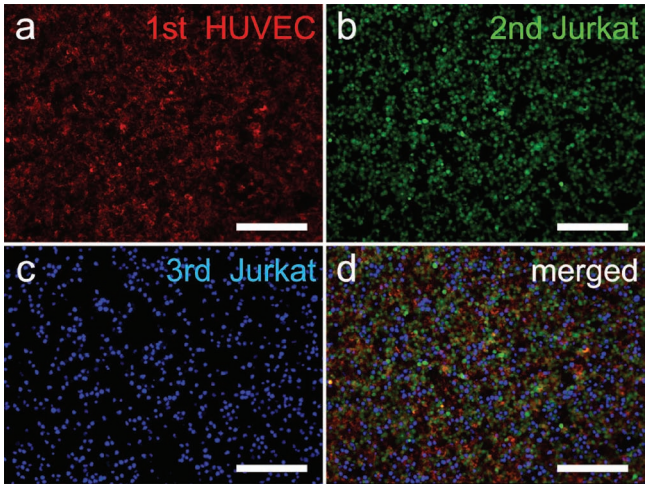

Figure 4. Fluorescent images of multilayered structures of Jurkat cells (c, blue) on the bilayer of HUVECs (a, red, first layer) and Jurkat cells (b, green, second layer). The HUVECs were modified with NHS-biotin and SA-Cy3. Jurkat cells were stained with CellTracker Green (b, green) or Hoechst 33258 (c, blue), followed by biotinylation. All the scale bars represent 200 μm.

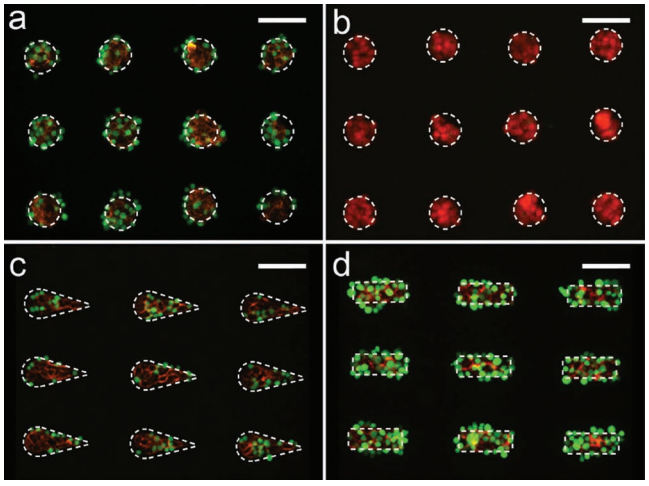

Figure 5. Fluorescent images representing the bilayer of Jurkat cells and HUVECs patterned in the agarose wells with different shapes: circle (a,b), teardrop (c), and rectangle (d). The HUVECs were modified with NHS-biotin and SA-Cy3 (a,c,d), or only stained with CellTracker Orange without biotinylation (b, red). Jurkat cells stained with CellTracker Green (green) were biotinylated (a,c,d), or not (b). All the scale bars represent 100 μm.

We also tested the contact efficiency of the method for assembling the bilayer of two adherent cells present in tubular structures in the body through the biotin-SA interaction using NIH 3T3 cells and HUVECs. We patterned SA-coated monolayer of biotinylated NIH 3T3 cells on glass surface, then added the suspension of the biotinylated HUVECs on the top of the SA-coated monolayer of NIH 3T3 cells, the biotinylated cells contacted with the SA-coated cells under gravity and formed the bilayer (Figure 3e). Although some unmodified HUVECs adhered to defects of the NIH 3T3 monolayer through nonspecific adhesion, the number of the unmodified HUVECs on the surface of the unmodified NIH 3T3 cells is obviously less than that of the biotinylated HUVECs on the surface of the SA-coated NIH 3T3 cells after the washing step (Figure 3f), indicating that the applicability of the biotin-SA interaction to adherent cells.

We added another population of the biotinylated Jurkat cells on the top of the bilayer of cells (**Figure 4**a,b), which have been previously coated with SA once more. The third population of the biotinylated Jurkat cells (Figure 4c) also can be connected to the top of the bilayer through the biotin-SA interaction to form multilayered structures (Figure 4d). This procedure could be repeated for several times.

We can also produce microscale patterns of multicellular structures by combination of biotin-SA interaction and soft lithographic technologies. Agarose gel is inert against protein adsorption and cell adhesion. We first patterned monolayer of HUVECs in the agarose wells fabricated by using polydimethylsiloxane (PDMS) stamps, and the cells were modified with NHS-biotin and SA successively. Then we added the biotinylated Jurkat cells on the monolayer. Due to the inert property of the surrounding areas of the agarose well, the biotinylated Jurkat cells can only connect with SA-coated monolayer of HUVECs to form a cell bilayer in the agarose wells (**Figure 5**a), and the unmodified Jurkat cells cannot adhere to the unmodified cell surfaces (Figure 5b). Other types of multilayers of cell patterns, for example, teardrop and rectangle, also have been fabricated (Figure 5c,d).

Tissues with tubular structures, such as the trachea, blood vessels, lymph vessels, and intestines are abundant in the bodies of higher animals. Tubular tissues have two distinguishing features: specific 3D shapes (tubular shapes), and different types of cells at specific locations (different parts of the tube wall are made up of different cells).[23] Mimicking both of these features is a prerequisite for fabricating functional tubular tissues in vitro, and the structural-tissue mimicry may have wide applications, but remains challenging.[24]

Our group has reported a general strategy to fabricate tubular structures with multiple types of cells as different layers of the tube walls.[25] This strategy provides a new perspective to precisely pattern different types of cells into 3D structures: first patterning different cells on a 2D surface, which could undergo changes in shape, and then deforming the 2D surface into a 3D structure (**Figure 6**a). However, in these tubes there is only monolayer of the same type of cells between the two layers of PDMS, and cells between different PDMS layers can't directly communicate with each other. In this study, the well-assembled cell layers can optimize the integrative structure and functionality of the tissues in 2D, and by using SIRM technique, we could further fabricate tubular structures containing multilayered cell architectures from 2D to 3D.

To get 2D cell bilayers on SIRM, we fabricated monolayer of NHS-biotin modified smooth muscle cells (SMCs) on the membrane, then modified the monolayer with SA, and added the suspension of the biotinylated HUVECs on the surface of the monolayer. HUVECs connected with the monolayer of SMCs to form the bilayer (Figure 6b). When we released one end of the SIRM, the cell-covered SIRM began to roll up into a tube due to internal stress, and the cell sheet was layered as the tubular wall. We fabricated "PDMS ambers"[25] to better characterize the 3D structure of the cell loading tubes using a confocal

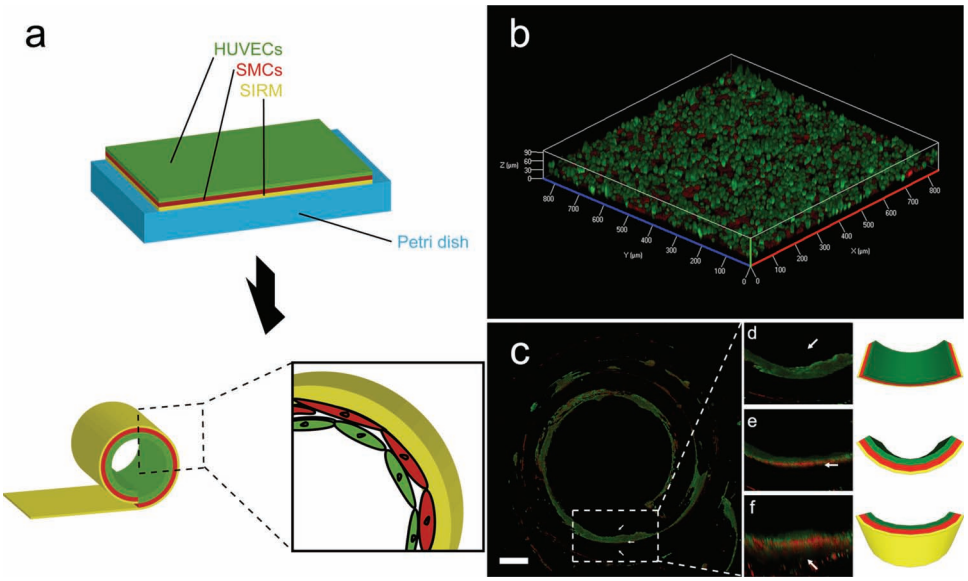

Figure 6. a) Schematic illustration of a stress-induced rolling membrane (SIRM) and tubes with multiple types of cells as the walls. b) Confocal image of a 3D reconstruction of the bilayer of two types of cells, SMCs (red) and HUVECs (green), on the SIRM before rolling. c) Confocal image of a 3D reconstruction of the bilayer on the SIRM after rolling. d–f) Magnified images of the side views of the portion of the tube in (c), indicated by the dashed line, respectively. The arrows indicate the direction of the observation, respectively. The scale bar represents 200 μm in (c).

microscope (Figure 6c). The structure of the tube is similar to the blood vessel: from inside to outside, the integrated bilayer of HUVECs (green) and SMCs (red) compose the structures mimicking intima and tunica part of the blood vessel wall.

3. Conclusions

We present a simple and rapid method for stepwise formation of 2D multilayered tissue-like structures through the biotin-SA interaction and further 3D tubular structures using SIRM technique. The biotin-SA interaction could be sequentially used to construct multilayered cell structures to mimick tissues in vivo. We believe that the method provides a useful tool for tissue engineering, and also a useful model for fundamental studies on cell-cell interactions. The 3D tubes containing bilayer of two types of cells is similar to blood vessels and would be potential for blood vessel repair.

4. Experimental Section

Cell Culture: Smooth muscle cells (SMCs), fibroblast cells (NIH 3T3), and HUVECs were cultured in Dulbecco's modified Eagle medium (DMEM) (Invirtogen, US) containing 10% fetal bovine serum (FBS) (Invitrogen, US), at 37 °C, 5% CO_2. Every passage of cells was conducted with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) (Invirtogen, US) every 3–4 days. Jurkat cells were cultured in RPMI 1640 medium (Invirtogen, US) containing 10% FBS, at 37 °C, 5% CO_2. Cell densities were maintained between 2×10^5 and 2×10^6 cells per mL. When cells grew into confluent or semiconfluent monolayers in the 60 mm petri dish, they would be trypsinized and collected for use.

Cell Surface Modification and Bilayer Formation: When HUVECs and NIH 3T3 cells grew into confluent, we collected them after trypsinization, and seeded them on the glass substrates which have been incubated with fibronectin. When growing into monolayer on the glass substrates, cells were incubated in NHS-biotin solution (1 mM in Hanks solution, 0.5 mL) for 1 h in the incubator (37 °C, 5% CO_2). After washing with Hanks solution, SA-Cy3 (Bioss, China) solution (50 mg/mL in DMEM without FBS, 0.5 mL) was then used to treat the cells for 1 h in the incubator (37 °C, 5% CO_2).

The suspended HUVECs and Jurkat cells were stained with dyes of different colours by Calcein-AM green (Invirtogen, US) or Hoechst 33258 (Invirtogen, US) before the surface modification. Briefly, we added 0.5% (V/V) of each dye in cell culture medium for 40 min for staining. Then cells were collected in a 15 mL centrifuge tube, washed with Hanks solution, and centrifuged for 3 min at 1000 rpm to increase cell density. Cells were dispersed in NHS-biotin solution (1 mM in Hanks solution, 1 mL), and the solution was allowed to incubate for 1 h in the incubator (37 °C, 5% CO_2). After washing with Hanks solution, cells were dispersed in DMEM without FBS.

The suspended HUVECs and Jurkat cells modified with NHS-biotin were add on the top of monolayer of surface-adherent cells on the glass substrates which have been treated with NHS-bioyin and SA-Cy3. After incubation for 1 h in the incubator (37 °C, 5% CO_2) and moving the unconnected cells, the cell bilayers were formed.

Fabrication of the Stress-Induced Rolling Membrane (SIRM) and 3D Tubular Structures: The method of the fabrication of SIRM has been described in the reference.[25] Briefly, when SMCs grew into confluent, we collected them after trypsinization, and seeded them on the SIRM which have been incubated with fibronectin. When growing into monolayer, the cells were stained with Cell Tracker Orange (Invirtogen, US). After washing with Hanks solution, NHS-biotin solution (1 mM in Hanks solution, 1.5 mL) was used to treat the cells for 1 h in the incubator (37 °C, 5% CO_2). After washing with Hanks solution, SA solution (50 mg/mL in DMEM without FBS, 0.5 mL) was also used to treat the cells for 1 h in the incubator (37 °C, 5% CO_2).

The suspended HUVECs after trypsinization were stained with green dyes by Calcein-AM green (Invirtogen, US) before the surface modification. Then cells were collected in a 15 mL centrifuge tube, washed with Hanks solution, and centrifuged for 3 min at 1000 rpm to increase cell density. Cells were dispersed in NHS-biotin solution (1 mM in Hanks solution, 1 mL), and the solution was allowed to incubate for 1 h in the incubator (37 °C, 5% CO_2). After washing with Hanks solution, cells were dispersed in DMEM without FBS.

The suspended HUVECs modified with NHS-biotin were added onto the top of monolayer of SMCs on the SIRM which have been treated with NHS-bioyin and SA. After incubation for 1 h in the incubator (37 °C, 5% CO_2) and moving the unconnected cells, the cell bilayers were formed. When one end of the SIRM was released, the cell-covered SIRM began to roll up into a tube due to internal stress, and 3D tubular structures were formed.

If the bulk PDMS tubes are directly observed using the confocal microscopy, the layers close to the lens may affect the fluorescence of the layers far from the lens, and the quality of the image was not good. For better observation, a "PDMS amber" was fabricated to show the 3D structures. The method of the fabrication of PDMS amber also has been described in the reference.[25] Briefly, the tube was immersed into liquid PDMS, and PBS and bubbles were removed from one end of the tubes with a pipette. After curing the liquid PDMS (2–3 h, 50 °C), the PDMS was cut into thin slices. The cutting direction should be vertical to the tube, and each slide was about 1–2 mm thick. The slices were put on a cover glass with liquid PDMS on it, and pressed slightly to remove the bubbles between the slices and glass, and then heated for curing (1 h, 50 °C). The surfaces of the slices were not flat or smooth after cut, so the light would be reflected, and the image of tubes became fragmentary if liquid PDMS was not cured between the slices and glass. The PDMS ambers could be preserved for several months, and faded very slowly.

Microscopy: The morphologies of bilayers were observed by the fluorescent microscopy (Leica DMI 6000B). The morphologies of tubes were observed by the confocal microscopy (Zeiss LSM 710).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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