Special Issue: *Analytical Biomaterials*

Analysis of Thickness and Roughness Effects of Artificial Basement Membranes on Endothelial Cell Functions

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Abstract

Various cells and tissues are highly organized in vivo by basement membranes (BMs) and thus promising artificial BMs (A-BMs) constructed by electrospinning and layer-by-layer (LbL) assembly have recently attracted much attention in tissue engineering field. However, control of cell adhesion, morphology, and migration of the attached cells on the A-BMs was not reported yet. In this study, we investigated both thickness and roughness-dependent effects of A-BMs on the functions of endothelial cells (ECs), which resulted from different assembly concentrations. The results indicated that the roughness of A-BMs increased gradually with the increase of nanofilm thickness. ECs adhesion, spreading and proliferation were inhibited on thicker A-BMs surface with larger roughness, while interendothelial junctions and barrier effect of confluent ECs monolayer on thicker A-BMs surface were compensated by increasing seeding cell number and expanding culture time. Our study highlights the influence of LbL assembly conditions on endothelial functions, which offers a new criterion for the design of A-BMs in well-organized 3D tissues.

Keywords: Artificial basement membranes; Thickness; Endothelial cell function
Introduction

Endothelial cells (ECs) align on the interior surface of microvasculature and their barrier functions play important roles in maintaining the integrity of vessels and regulating the selective mass transport between blood vessels and surrounding tissues.\(^{[1,2]}\) The rapid endothelialization of ECs on implant-blood contacting surfaces has been recognized as essential for improving the long-term biocompatibility of implanted cardiovascular materials, thus avoiding severe complications.\(^{[3,4]}\) Hence, understanding the impacts of biomaterials on EC behaviors, will contribute to the development of scaffolds for vascular tissue engineering.

Basement membranes (BMs) are specialized sheet-like types of extracellular matrix (ECM), which lie beneath the endothelium and are surrounded with vascular smooth muscle cells. BMs provide not only mechanical support and compartmentalized organization but also biological signals to adjacent cells.\(^{[5]}\) Col-IV and LM, serving as the main components of BMs, play critical roles in the biophysical and biochemical characteristics of BMs to maintain cell phenotype and guide cell functions.\(^{[6]}\) For example, the assembly and degradation of Col-IV are associated with ECs adhesion, migration, and angiogenesis\(^{[7]}\) and LM can promote the differentiation of ECs in vitro and angiogenesis.\(^{[7,8]}\) Due to the sophisticated functions of BMs,\(^{[9]}\) the construction of artificial basement membranes (A-BMs) in vitro to mimic the structure and functions of native BMs will contribute to developments of tissue engineering. To this end, a better understanding of the interactions between ECs and A-BMs becomes particularly important.

Inspired by the ultra-thin, flexible, permeable, and fibrous structure of BMs, several attempts have been reported for patterned cell co-culture, including simple polymer membranes\(^{[10,11]}\) and artificial porous nanofilms.\(^{[12–15]}\) Suzuki\(^{[10]}\) et al.
developed a free-standing porous nanosheet based on biodegradable poly(D, L-lactic acid), to mechanically support cell monolayers and assist the assembly of layered structure in 3D tissues. Meanwhile, due to the controllable fibrillar microstructure and high porosity, electrospun scaffolds have attracted great attention for the construction of BM-like membranes.\cite{14-16} Although these porous ultra-thin membranes and nanofiber meshes showed good barrier properties and permeability, the effects of physicochemical properties of A-BMs on EC functions were not investigated in detail. Furthermore, synthetic materials are limited in their ability to display the complicated bio-functions in vitro of natural BMs. Moreover, the interactions between cell functions and A-BMs still require further investigations.

In contrast to electrostatic spinning and porous membranes, the layer-by-layer (LbL) assembly technique is a widely used method for ultrathin film construction, which allows the use of multiple components, including synthetic polymers, nanostructures, polysaccharides, and proteins.\cite{17} Ji et al. reported a polyelectrolyte film with controllable stiffness through LbL assembly of poly(L-lysine) and hyaluronic acid.\cite{3,18} They demonstrated that an ECs monolayer on the soft film displayed higher endothelial function compared to that on the stiff film,\cite{18} and that ECs tended to undergo endothelial-to-mesenchymal transition, losing their endothelial phenotype with increasing substrate stiffness.\cite{3} However, adjustments in the assembly parameters, such as components and conditions can also affect the physicochemical properties of LbL films. To the best of our knowledge, there has been no relevant research concerning the regulation of LbL thickness on ECs. Moreover, the application of LbL nanofilms as A-BMs appears to warrant further investigation.

All tissues are surrounded by ECM that not only provides physical and mechanical support to cells but also mediates cell behaviors by biochemical signals.\cite{19} To mimic
the complicated properties and structures of ECM in the human body, ECM proteins suggested that they would be the best candidates to effectively support cell survival in vitro.\cite{20,21} Our group has recently developed a multilayered nanofilm using the main components of BMs, Col-IV and LM, to mimic the structure and bio-functions of BMs in vitro.\cite{22,23} Benefiting from a fibrous, porous sheet-like structure, the Col-IV/LM nanofilm used as an A-BM showed a helpful cell compartment effect for organized 3D tissue construction and a size-dependent permeability for successful cell-cell crosstalk. However, evaluations of the relationships between Col-IV/LM LbL assembly conditions and cell behaviors are still lacking. This study aims to explore the effects of thickness and surface morphology of Col-IV/LM LbL nanofilms on the functions of ECs.

**Results and Discussion**

*Characterization of the (Col-IV/LM)₅ nanofilms with different thicknesses*

Studies on biologically specific recognition between Col-IV and LM have reported that LM binds preferentially to Col-IV over other collagens,\cite{22,24,25} suggesting they would be the promising candidates for the LbL assembly technique. To investigate the effects of the (Col-IV/LM)₅ nanofilms thickness and roughness on ECs, we prepared a series of nanofilms with various thicknesses which were checked by quartz crystal microbalance (QCM), as shown in Figure 1. Using the simple alternate deposition process of Col-IV and LM, the thickness of the (Col-IV/LM)₅ nanofilms can be easily controlled at 5.9, 25.6, 26.1, 36.0, and 136.7 nm by changing the assembly concentrations, 4, 40, 200, 400, and 1000 μg/mL, respectively, which were named as nanofilms (NFs)-4, 40, 200, 400, and 1000.

AFM images further evaluated the surface morphology of the (Col-IV/LM)₅ nanofilms with the increase of assembly concentrations (Figure 2a). Compared with the
control sample (tissue-culture treated plate (TC-PS)), the gradually increased roughness demonstrated the successful assembly of nanofilms, even for low concentrations at 4 and 40 μg/mL. At the higher concentrations of 200 and 400 μg/mL, the assembled nanofilms exhibited a uniform surface morphology and fibrous structure. Due to the specific interaction and self-assembly property of Col-IV and LM in vitro,[24,25] the LbL assembly process endows a fibrous but dense microstructure to the (Col-IV/LM)$_5$ nanofilms.[22] At 400 and 1000 μg/mL in particular, the height difference of the nanofilm surfaces reached 81 and 194 nm, in some way, revealing the variation in thickness. However, the surface morphology observed by AFM was quite different from that found by phase contrast microscopy (Figure S1). As shown in Figure S1, particle-like aggregates were observed on the surfaces of NFs-400 and 1000, which probably occurred because of the self-assembly of LM at high concentrations.[26–28] Unlike AFM that is affected by the AFM tips, color 3D laser scanning microscopy performs a variety of non-contact 3D measurements using the laser, which includes surface profile, roughness, 3D, and comparative measurements. Further evaluation of surface scanning of the (Col-IV/LM)$_5$ nanofilms was performed using color 3D laser scanning microscopy, as shown in Figure 2b. As with the results from AFM, (Col-IV/LM)$_5$ nanofilm obtained from low concentration had a smooth surface, with lower roughness value (<100 nm, Figure 2c). However, the nanofilm surface became increasingly rough with higher assembly concentrations. The roughness of NFs-400 ranged from 100 nm to 1.0 μm, while the value for the NFs-1000 surface was above 17.0 μm. Aggregates were clearly observed on NFs-1000, where the height difference even reached 117 μm that is much larger than single-cell size. These results differed from the nanofilm thicknesses seen in Figure 1 where QCM was used to check the assembly results, probably because of the different substrates and the presence of drying
steps using N₂ between each step.

In conclusion, the thickness of the \((\text{Col-IV/LM})_5\) nanofilms can be controlled in the range of 5 ~ 137 nm on QCM chips, and the surface morphology of the nanofilms was greatly affected by the thickness change, whose roughness increased gradually with the increase of assembly concentrations.

**HUVECs adhesion on \((\text{Col-IV/LM})_5\) nanofilms**

It has been reported that chemical and topographical factors have a considerable impact on ECs adhesion and phenotype.\(^{[29]}\) Owing to the specific recognition of Col-IV and LM with cells, \((\text{Col-IV/LM})_5\) nanofilms were shown to be helpful for fibroblasts and endothelial cells adhesion in our previous study.\(^{[22]}\) However, surface morphology of multilayered nanofilms greatly varied with the increase of nanofilms thickness. Thus, HUVECs adhesion on the \((\text{Col-IV/LM})_5\) nanofilms obtained from different assembly concentrations was evaluated, and the quantitative results are shown in Figure 3. Taking the 2 hours-culture results as an example, although no significant difference was observed from the quantitative results of adhered cell density as shown in Figure 3b and the adhered cell number on NFs-1000 was even higher than that on NFs-40, cells spreading morphology in each group became markedly different. HUVECs adhered to TC-PS, NFs-4 and 40 already started to spread on the substrates after a short culture time (2 hours), while cells on thicker nanofilms maintained their un-spread state (bright dots in Figure 3a). In Figure 3c, the spreading area of each HUVEC on NFs-200, 400, and 1000 after 2 hours was clearly lower than other groups because of the un-spread cell morphology. Moreover, after 2 days culture, HUVECs adhered well to TC-PS, NFs-4, 40, and 200, but still did not adhere well on either NFs-400 or 1000, especially for NFs-1000, showing an elongated shape. Cell density and spreading area on NFs-1000
were also lower than other groups (Figure 3b, c).

To provide further insights into the relationship between thickness and roughness of the (Col-IV/LM)$_3$ nanofilms and cell adhesion state, the aspect ratio (L/D) of each cell was calculated and then all of the cells were classified into different shapes (Figure 3d, e). An aspect ratio of 1.0 represents a round shape, indicating poor cell spreading morphology, while L/D ratios over 2.0 suggest a good cell adhesion state. Even after just a short incubation time (2 hours), HUVECs started to attach and spread on the substrates. The average of the aspect ratio of cells on NFs-4, 40 was around 2.0, and fewer than 40 % of cells were observed to have an aspect ratio of 1.0 (Figure S2), while the aspect ratio of all of the cells on NFs-400, 1000 were still 1.0, corresponding well with the cell morphology shown in Figure 3a. Meanwhile, when the incubation time was extended to 2 days, the increasing aspect ratio on NFs-4, 40, and 200 indicated the spreading cell shape and good adhesion state. However, there were still some un-spread cells (aspect ratio 1.0) on NFs-400 and 1000 after 2 days culture. Although most of the cells showed an elongated shape in these two samples (Figure 3a, d, e), the morphology of HUVECs was quite different from those on TC-PS. In general, the poor cell adhesion state on NFs-400 and 1000 may relate to the surface morphology and mechanical properties of the (Col-IV/LM)$_3$ nanofilms. LM was demonstrated to show a tendency of self-assembly in vitro, especially at high concentrations (over 100 μg/mL), which also enhanced the assembly of Col-IV.$^{[30,31]}$ After the assembly of 5 bilayers at 37 °C, aggregation of LM at the nanofilm surfaces was clearly observed on NFs-400 and 1000. Even though ECs exhibited a better morphology onto the substrate with submicron-scale roughness than a smooth surface,$^{[32]}$ it’s still difficult for ECs (~20 μm) to adhere and spread through the oversized barriers. Based on the above results, we conclude that the adhesion and spreading morphology of HUVECs were affected by the
roughness of (Col-IV/LM)$_5$ nanofilms, as well as nanofilms thickness.

**HUVECs proliferation onto (Col-IV/LM)$_5$ nanofilms**

Since the thickness and roughness of (Col-IV/LM)$_5$ nanofilms have a great impact on HUVECs adhesion, proliferation was also investigated as it is another one important cell behavior (Figure 4). The proliferation results of HUVECs on NFs-4, 40, 200, and 400 indicated a similar growth ratio with TC-PS (without nanofilms). The proliferated cell number on (Col-IV/LM)$_5$ nanofilms was higher than the control sample, and the cell number also increased gradually with the increase of nanofilm thickness, suggesting an enhanced effect of ECM proteins on cell proliferation. The results on NFs-400 did not agree well with the adhered cell number shown in Figure 3, probably because of the variation of LbL assembly results. However, the proliferated cell number on NFs-1000 was somewhat lower than other substrates due to the larger roughness which is not ideal for cell adhesion and spreading. In conclusion, the thickness and surface morphology of the (Col-IV/LM)$_5$ nanofilms affected by the assembly concentrations account for the different behaviors of HUVECs, including cell adhesion, spreading, and proliferation.

**Barrier effect of HUVECs monolayer on (Col-IV/LM)$_5$ nanofilms**

Alignment of vascular endothelial cells lining the inner surface of blood vessels contributes to their barrier effects, controlling the substance exchange between blood and interstitial compartments. This intact ECs layer attaches tightly to BMs which play an essential role in regulating EC functions. Therefore, the barrier effect of the HUVECs monolayer on A-BMs was evaluated including interendothelial junction protein expression and trans-endothelial electrical resistance (TEER) value.

Endothelial junctions composed of adherens junctions and tight junctions create
adhesive structures between adjacent cells, accounting for a unique ECs barrier on the vessel walls.\textsuperscript{[33–35]} As shown in Figure 5a, interendothelial adhesive proteins, including CD31 and VE-cadherin were clearly observed to be distributed on the margins of cells, demonstrating the development of cell connections. Similar results were also observed for tight junction proteins expression, including ZO-1 and claudin 5. CD31 and ZO-1 were only located on the edge of cell-cell joints, meanwhile VE-cadherin and claudin 5 tended to move to the edge of the cellular surface from the cytoskeleton. It has been demonstrated that uniform and continuous signals of junction proteins on the edge of cell-cell joints suggests an abundance of junction proteins and strong barrier integrity of an endothelial monolayer.\textsuperscript{[34]} Comparing the morphology of intercellular junctions in each sample, no significant differences were founded. Owing to the promotion of ECM proteins to the differentiation of ECs, the distribution and margin of junction proteins on NFs-1000 were even clearer than those on transwell membranes (Figure 5a), even though a large fluctuation of HUVECs monolayer was observed on NFs-1000 due to the larger roughness (Figure S3). Moreover, by quantitative analysis, the mean interendothelial junction area of each cell and percentage of HUVECs monolayer coverage showed no statistical differences on different substrates (Figure 5b, c). However, the confluent ECs monolayer formation on NFs-1000 showed different proliferation results to those shown in Figure 4 where the proliferated cell number on NFs-1000 was lower than the other groups after 7 days culture. Even though there were no significant differences of interendothelial junction contact area of HUVEC on different substrates, the spreading area of each HUVEC on NFs-1000 was slightly higher than that on the transwell membrane and NFs-40 (Figure S4). Thus, the smaller amount of HUVECs with a larger spreading size were enough to cover the NFs-1000 surface and form a confluent ECs monolayer with larger fluctuation (Figure S3).
Intercellular junction proteins expression results indicated that although HUVECs cannot adhere well to thicker (Col-IV/LM)$_3$ nanofilms, the thickness or roughness would not affect the barrier functions of confluent HUVECs.

Unlike the cell adhesion and proliferation on thicker nanofilms, there was no statistically significance difference of intercellular junction protein expression after 5 days culture between NFs-40 and 1000. The barrier effects of confluent HUVECs on the (Col-IV/LM)$_3$ nanofilms with different thickness were further investigated. Integrating the (Col-IV/LM)$_3$ nanofilms into a 24-well transwell insert (0.4 μm pores), the underlying porous insert membrane allowed the measurement of TEER value of confluent HUVECs. As shown in Figure 6, the TEER values increased gradually over the period of a 5-days culture, suggesting the growing barrier effects of the HUVECs monolayer. Meanwhile, the TEER values of confluent HUVECs cultured on the (Col-IV/LM)$_3$ nanofilms (e.g. 4.0 μg/mL: 39.2 Ω·cm) were remarkably higher than those on the transwell membrane (28.7 Ω·cm) after 3 days culture. This improved ECs barrier function on A-BMs compared with a commercial porous membrane benefits from the specific recognition between (Col-IV/LM)$_3$ nanofilms with cells by integrins.$^{[36,37]}$ However, due to the limited adhesion and proliferation speed on thicker (Col-IV/LM)$_3$ nanofilms, the TEER values on NFs-1000 were much lower than other samples up to 5 days. Beyond 5 days culture however, no significant differences were found between different nanofilms. Even though HUVECs showed a less tendency to adhere and proliferate on the thicker (Col-IV/LM)$_3$ nanofilms, which would affect the formation of the ECs monolayer, an increased seeding cell number and extended culture time narrowed this difference (Figure 5). To investigate the relationship between seeding cell number and the formation of confluent monolayers, the VE-cadherin secretion of HUVECs after 2 days and 5 days culture on NFs-200 and 1000 were
compared as shown in Figure S5 where the initial seeding cell number was $2.0 \times 10^5$ in a 24-well insert and around 60 times higher than that shown in Figure 3. As shown in Figure S5, although the confluent ECs monolayer did not form at day 2, there were no differences in adhered cell number (Figure S5a) and the secretion of VE-cadherin on NFs-1000 was even higher than that on NFs-200 (Figure S5b). After 5 days culture, VE-cadherin tended to distribute at the joints of adjacent cells, forming the confluent monolayers. The margin of VE-cadherin was clearer and the joints was tighter on NF-200 than that on NFs-1000, but no significant difference of intercellular contact area was observed on different nanofilms (Figure S5b). Thus, owing to the higher seeding cell number, the adhered cells were sufficient to form confluent HUVECs monolayers, and then improve their barrier functions.

**Conclusions**

This study, based on the previous discovery that Col-IV/LM LbL nanofilms can be used as A-BMs to maintain the organized cell co-culture structure, further investigated the effects of nanofilm thickness and roughness on the functions of ECs. In summary, the thickness of (Col-IV/LM)$_5$ nanofilms can be controlled in the range of 5-137 nm by changing the assembly concentrations. The surface morphology of nanofilms showed increasing roughness with the increase of thickness. HUVECs behaviors, including cell adhesion and proliferation, varied with the different thickness of nanofilms, e.g. HUVECs could not adhere well to the thicker nanofilms obtained from 1000 μg/mL in a short time (less than 2 days) because of the rough surface (Ra value > 17.0 μm). Fortunately, these limitations were overcome by increasing seeding cell number and extending the cell culture time. There were no statistically significant differences in ECs barrier effects on (Col-IV/LM)$_5$ nanofilms with different roughness. However, in our
previous study, thicker (Col-IV/LM)₅ nanofilms contributed to a stronger barrier effect to maintain cell compartmentalization in 3D tissues.¹²² In order to perform the given functions related to the BM in different tissues, a trade-off has to be made between the cell functions and barrier effects when selecting appropriate assembly concentrations. This research lays a theoretical foundation for the application of Col-IV/LM LbL nanofilms used as A-BMs in regulating the cell fate of endothelial cells and provides a promising design criterion for surface modification of vascular implants.

Supporting Information

The detailed experimental process and additional figures were shown in Supporting Information.

This material is available free of charge on the web at http://www.jsac.or.jp/analsci/.

Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research (A) (20H00665), and Bilateral Joint Research Projects of the JSPS (20199946), as well as AMED-MPS (19be0304207h003).

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Figure 1. Formation of Col-IV/LM multilayered nanofilms on QCM chips. (a) Frequency shift and thickness increase of Col-IV (○) and LM (●) assembly for 5 bilayers were measured using QCM in 50 mM Tris-HCl buffer solution (pH=7.4) at 37 °C. The concentrations of assembly components, namely Col-IV and LM, were controlled in the range of 4-1000 μg/mL. n=3. (b) Thickness of Col-IV/LM nanofilms assembled from different concentrations of Col-IV and LM solutions for 5 bilayers. n=3. *p<0.05, **p<0.01, ***p<0.001. Reproduced with permission from ref. 22. Copyright 2020 WILEY-VCH.
Figure 2. (a) AFM images and (b) color 3D laser scanning microscopy images of (Col-IV/LM)s nanofilms obtained from various assembly solutions with different concentrations. A tissue culture treated plate composed of polystyrene (TC-PS) served as the control sample. (c) The arithmetic averages of the roughness profile (Ra) of each nanofilm, which were measured by color 3D laser scanning microscope.
Figure 3. Endothelial cells adhesion on (Col-IV/LM)$_5$ nanofilms. HUVECs cultured on a TC-PS substrate served as the control sample. Cell seeding density was $1.0 \times 10^4$ cells/cm$^2$. (a) Morphology of HUVECs adhered on (Col-IV/LM)$_5$ nanofilms with various thicknesses after 2 hours and 2 days culture. Scale bar is 100 μm. (b) Adhered cell density of HUVECs and (c) spreading area of each HUVEC cultured on (Col-IV/LM)$_5$ nanofilms with various thicknesses after 2 hours and 2 days culture. n=3. HUVECs morphology aspect ratio distribution after (d) 2 hours and (e) 2 days culture was plotted, HUVECs cultured on a TC-PS substrate served as the control sample. Representative images for adhered cell shape are shown at the top of each graph. For 2 hours culture, $n_{\text{Control}} = 158$; $n_4 = 128$; $n_{40} = 132$; $n_{200} = 167$; $n_{400} = 146$; $n_{1000} = 190$. For
2 days culture, \( n_{\text{Control}} = 115; n_4 = 100; n_{40} = 96; n_{200} = 84; n_{400} = 44; n_{1000} = 34 \). *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
Figure 4. Proliferation of HUVECs (3.0×10^4 cells/cm^2) on (Col-IV/LM)_5 nanofilms with various thicknesses after 1, 3, and 7 days culture. The cell number is normalized as a percentage of cells adhered to TC-PS at Day 1. *p<0.05, **p<0.01, ***p<0.001.
Figure 5. Characterization of intercellular junction proteins and cell morphology of the confluent HUVECs after 5 days culture. (a) Immunofluorescence images of confluent HUVECs on (Col-IV/LM)$_3$ nanofilms with various thicknesses. Cells were labeled for CD31 and VE-cadherin to show the location of adherens junction proteins, and ZO-1, claudin 5 to show the location of tight junction proteins. Nuclei were stained with Hoechst 33342 and are shown in blue. HUVECs monolayer cultured on a naked 24-well insert served as the control sample. The scale bar is 50 μm. Quantitative analysis of (b) intercellular junction coverage area of each cell and (c) percentage coverage area of confluent HUVECs monolayer by analyzing the immunofluorescence intensity in Figure 5a. n=4.
**Figure 6.** The electrical resistance through HUVECs monolayers cultured on (Col-IV/LM)$_8$ nanofilms with various thicknesses was measured over 7 days culture in the 24-well transwell. HUVECs monolayer cultured in a naked 24-well insert served as the control sample. n=3. **p<0.01.**
Graphical Index