Preparation of gelatin density gradient on poly(ε-caprolactone) membrane and its influence on adhesion and migration of endothelial cells

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Abstract

Directional migration of endothelial cells (ECs) can be achieved by gradient cues in vitro, which mimics the corresponding biological events in vivo. Currently, most of the gradients have been prepared on model surfaces which are too simple compared to real degradable biomaterials. In this study, the amino group density gradient was prepared on poly(ε-caprolactone) (PCL) membrane surface by a gradient aminolysis method, which was transferred into gelatin density gradient by covalent linking with glutaraldehyde. The resulted gelatin density gradient ranged from 0.49 to 1.57 g/cm² on the PCL membrane. The adhesion, orientation and migration of ECs on the PCL membrane with the gelatin density gradient were studied. The ECs showed preferred orientation and directional migration toward the gradient direction with enhanced gelatin density at proper position (gelatin density), forwarding a new step toward the preparation of applicable gradient biomaterials in tissue regeneration.

1. Introduction

The endothelium is a confluent monolayer of thin, flattened, rhomboid-shaped cells lining at the intimal surface of all blood vessels and, thus, is situated at the vital interface between circulating blood and body’s tissues [1]. In the last decade, the importance of the endothelium in cardiovascular physiology and homeostasis has been well recognized. The endothelium plays a significant role on maintaining vessel integrity with dynamic mechanisms that prevent thrombosis [2,3]. The study of in vitro endothelialization with cultured endothelial cells (ECs) prior to
implantation proves that a confluent endothelium has the ability of preventing thrombogenic complications and improving long-term patency [4,5]. For example, Gaudio et al. found that revascularization was always supported by penetration of endothelial cells inside the transplanted islets from outside [6]. Therefore, in cardiovascular tissue engineering, a directional and speedy migration of ECs is desired to favor fast endothelialization, which is critical to lower the risk of thrombogenesis and restenosis, etc.

The cells in vivo can migrate in response to gradients of stimuli including both biochemical and biophysical signals such as soluble chemoattractants (chemotaxis), surface-immobilized or attached molecules (haptotaxis) and biophysical contact cues ( durotaxis or mechanotaxis) [7,8]. Chemical signal gradients drive embryonic development, whereas gradients in cellular-extracellular architecture exist throughout the human body, within tissues and at tissue interfaces, to satisfy spatially diverse functional needs [9]. Plenty of studies demonstrate that the signal gradients generated on model materials, such as a density gradient of stiffness [10], bioactive molecules [11,12] or small molecules [13], and even complementary gradient of hydrophobic polymers and cell signaling peptides [14], also can dominate the cell migration behaviors.

Many physical and chemical signals have been implemented to prepare functional biomaterials to modulate the mobility of ECs. Smith et al. prepared a surface-bound fibronectin (Fn) linear density gradient to promote the directional migration of bovine aortic endothelial cells (BAECs). Compared to a uniform Fn surface, the frequency of discrete cellular motion in the gradient direction increased with gradient slope [15,16]. Liu et al. built a vascular endothelial growth factor (VEGF) gradient and compared to the Fn gradient, and found that the directional cell migration was increased by about 2-fold on the VEGF gradient, and was further increased by another 2-fold on the combined gradients of both proteins compared to the VEGF gradient alone [17]. Sundararaghavan et al. fabricated an electrospun fiber substrate with VEGF gradients, and found that the mobility of ECs could be enhanced due to the synergistic effect of chemical gradients and oriented electrospun fibers [18].

However, so far most of the signal gradients have been fabricated on model materials such as glass slides and silicon wafers, which are simple and easy for characterization. However, they are too simple compared to those real biomaterials with special bioactivity and biodegradability. Poly(e-caprolactone) (PCL) is one of the most widely used polyesters in biomedical field due to its good mechanical property and biocompatibility [19]. PCL is always in the rubbery state and has high material permeability under physiological conditions. PCL can be degraded by microorganism, hydrolytic, enzymatic, or intracellular mechanisms under physiological conditions at a relatively slow rate [20]. However, its relative inert surface with poor hydrophilicity cannot provide signals to guide efficient cell migration [21]. Gelatin is a mixture of peptides and proteins produced by partial hydrolysis of collagen, with the ability to induce cell adhesion and proliferation [22]. Ai et al. fabricated a gelatin coating on a silicone surface, which enhanced endothelial cell attachment and growth [23]. Shin et al. built a gelatin-immobilized poly(L-lactide-co-epsilon-caprolactone) (PLCL) substrate, which could promote the spreading, proliferation, and differentiation of human mesenchymal stem cells (hMSCs) [24].

In this work, an amino group density gradient is fabricated on the PCL membrane surface by a gradient aminolysis method, which is transferred into a gelatin density gradient via covalent immobilization with glutaraldehyde. The adhesion, orientation and migration of ECs on the PCL membrane with the gelatin gradient are studied.

2. Experiments section

2.1. Materials

Polycaprolactone (PCL, Mn~80 kDa) and gelatin Type B were purchased from Sigma-Aldrich, USA. Glutaraldehyde, isopropyl alcohol, 1,6-hexanediamine and 1,4-dioxane were purchased from Sinopharm Chemical Reagent Co., Ltd., China. All the chemicals were of analytical grade and were used without further treatment if not specially mentioned. The water used throughout the experiments was purified by a Milli-Q water system (Millipore, USA).

2.2. Preparation of aminolyzed PCL membrane

The PCL membrane with a thickness of 200 μm was prepared by casting 10% (w/v) of PCL/1,4-dioxane solution into a glass Petri dish, allowing solvent evaporation at 37 °C for 24 h. The membrane was cut into pieces with an accurate size of 10 × 22 mm for the following procedures. The membranes were aminolyzed in 0.43 mol/L 1,6-hexanediamine/isopropanol solution at 30 °C for 5 min, 15 min and 25 min, respectively, according to the procedures reported previously [25]. To generate the –NH2 density gradient, the PCL membrane was placed vertically in a centrifuge tube, into which 1,6-hexanediamine/isopropanol solution was slowly injected by a micro-infusion pump (WZS-50F2, Zhejiang University Medical Instrument, China) at 30 °C. The injection rate was precisely controlled at the longest reaction time of 25 min. After aminolysis, the PCL membrane was rinsed with large amount of water at room temperature to remove free 1,6-hexanediamine, and then dried under a nitrogen gas flow.

2.3. Preparation of gelatin density gradient

The aminolyzed PCL membrane was immersed in 2% (v/v) glutaraldehyde (GA) solution at room temperature for 4 h, and then washed 5 times with water. The PCL membrane functionalized with aldehyde groups was reacted with 1% gelatin solution at 37 °C for 4 h to covalently immobilize gelatin. After washed with water 5 times, the gelatin immobilized PCL membrane was dried under a nitrogen gas flow.

2.4. Characterization of the gradient

Quantitative determination of the –NH2 groups on the aminated PCL membrane was conducted by a ninhydrin assay [25]. In brief, the membrane was immersed in a glass tube containing 0.1 mol/L ninhydrin/ethanol solution, and then was treated at 80 °C for 15 min to expedite the reaction between ninhydrin and amino groups on the membrane. Subsequently, 8 mL 1,4-dioxane was added to the solution to dissolve the PCL membrane. The absorbance at 538 nm was recorded on a UV-vis spectrophotometer (Shimadzu UV-2550, Japan). The density of –NH2 groups was calculated by referring to a calibration curve constructed with known concentration of 1,6-hexanediamine at the same conditions.

The densities of gelatin and adsorbed serum proteins were assessed using a standard BCA assay kit (Generay Biotech Co., Ltd., China) following the user’s manual. Briefly, the PCL membranes (both the uniform and the gradient) were sliced into a pre-calculated size fitting for 96-well plates. Total mass of the proteins before and after 12 h incubation in phosphate buffered saline (PBS, pH = 7.4) containing 10% fetal bovine serum (FBS, Sijiqing Inc., Hangzhou, China) at 37 °C on the membranes was calculated according to a standard curve constructed at the same conditions.
The static water contact angles of PCL membranes were measured by a sessile-drop method on a DSA 100 contact angle measuring system (Krüss, Germany). The volume of each droplet was 2 µL. The results were averaged from 5 independent measurements.

The surface zeta potential of PCL membranes was measured in 10 mM NaCl solution using the Delsa Nano Series zeta potential/submicrometer particle size analyzers (Beckman Coulter, USA) via electrophoresis technology.

2.5. Cell culture

Human vascular endotheliocytes (ECs) were purchased from the Cell Bank of Typical Culture Collection of Chinese Academy of Sciences (Shanghai, China). The ECs were cultured with Roswell Park Memorial Institute-1640 medium (RPMI-1640) supplemented with 10% FBS, containing 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified incubator with 95% air and 5% CO₂ at 37 °C.

2.6. Cell migration

All samples were sterilized in 75% ethanol for at least 0.5 h, and then washed in sterilized PBS for 6 times. Three positions were selected on the gradient membrane and their respective uniform surfaces with the same aminolysis time and thereby the same gelatin densities were used as controls. Single-cell model was applied to avoid cell-cell interaction, and the ECs were seeded at a density of 5 × 10⁴/cm². 12 h later the cell migration traces were recorded in situ for 12 h under a time-lapse phase-contrast microscope (DMI6000B, Leica) equipped with an incubation chamber (37 °C and 5% CO₂ humidified atmosphere).

NIH ImageJ software was employed to trace each individual cell in situ, and thus exported a variety of (x, y) position coordinates over the period of observation time with the manual tracking plugins. The ECs trajectories were reconstructed from the center positions of individual cell, and the initial position of each single cell was automatically defined as the original position (0, 0). The cell migration distance S was then calculated by Chemotaxis Tool (Ibidi, Germany) with a 15 min time intervals according to the following equations.

\[ S = \sum_{i=1}^{n} \sqrt{(x_i - x_{i-1})^2 + (y_i - y_{i-1})^2} \]  (1)

At least 30 points were calculated for each position. Mitotic and spherical dead cells were excluded from the analyses. The cell migration rate is obtained from the formula \( v = S/t \) [26]. The data were finally analyzed by Rayleigh test with a pre-set significant level of \( p < 0.05 \), which suggests a statistically significant asymmetric distribution of the end points [27].

2.7. Interaction between cells and substrate

The relative cell adhesion force of ECs on the membranes was measured according to the method described in Reyes’s work [28]. After cell seeding for 12 h, the membranes were gently washed 5 times to remove floating cells. The number of ECs still adhering on the substrate was counted under a microscope. Simultaneously another two membranes were placed vertically into the centrifuge tubes incubated with PBS, and then the fraction of adhesion cells was calculated after being centrifuged at 900 rpm or 1500 rpm for 5 min, respectively. The adhesion force of ECs was evaluated according to the equations reported previously [26].

Cell nucleus and F-actin organization were imaged through fluorescent staining. In brief, ECs cultured on the substrates were fixed with 4% paraformaldehyde at 37 °C for 30 min, followed by 3 washes in PBS. The cells were then permeabilized using 0.5% Triton X-100 in PBS for 10 min. After 3 washes with PBS, the membranes were incubated in 1% bovine serum albumin (BSA)/PBS for 30 min at 37 °C to block nonspecific interactions. After washed twice in 1% BSA/PBS, the cells were counter-stained with rhodamine phalloidin (Invitrogen), 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) at room temperature for 1 h, followed by 3 washes in PBS. The cells mounted on microscope slides for examination under confocal laser scanning microscopy (Zeiss, LSM-510, Germany), and the relative fluorescent intensity was analyzed. Angles of cell alignment to the X axis were analyzed by using ImageJ software.

2.8. Statistical tests

All experiments were repeated three or more times with triplicate samples. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a Tukey posthoc method, and a significant level of \( p < 0.05 \) was chosen for all the tests. A few comparisons were also made between individual groups with t-test.

3. Results and discussion

3.1. Preparation and characterization of gelatin gradient on PCL membrane

The aminolysis is an effective and facile method to introduce primary amine groups on the surface of polyester materials and thereby endow the surface with reactivity for subsequent functionalization [29]. The 1,6-hexanediol molecules break the ester bonds and introduce aminoethyl amides (–CONH(CH₃)NH₂) and –OH groups. In this study, the 1,6-hexanediol concentration was fixed at 0.43 mol/L since higher concentration resulted in a decrease of –NH₂ density due to the dissolution or dissociation of the oligomeric fragments of smaller size (data now shown). By combining the micro-infusion method, the –NH₂ density gradient with a length of 20 mm can be fabricated on the PCL membrane. The 0 position was defined as the upper end with the smallest –NH₂ density. As shown in Fig. 1a, the density of amino groups almost linearly increased with the aminolysis time, suggesting the feasibility of gradient formation. The conjugation of gelatin molecules onto the amino gradient on PCL membrane was realized by glutaraldehyde cross-linking, transferring the amino gradient into a gelatin density gradient. As shown in Fig. 1b, the surface gelatin density increased almost linearly along with the aminolysis time or gradient position, and reached to 0.49, 0.73, 1.00, 1.25 and 1.57 µg/cm² on the gradient surfaces with a position of 4, 8, 12, 16, and 20 mm, respectively. Due to the presence of relatively hydrophilic gelatin molecules, the water contact angle of the gradient surface decreased slightly along with the position (Fig. 1c). It is worth noting that the water droplet moved toward the direction with larger gelatin density on the gradient surface (Fig. 1d), demonstrating the existence of hydrophilicity gradient. All the results suggest the successful preparation of a linear gelatin density gradient on the PCL membrane. Besides, the preparation processes are thermodynamically controlled except of the micro-infusion step, leading to the precise control of the quality over a micron scale and in different batches.

It is widely acknowledged that surface hydrophilicity and surface charge have significant impact on cell behaviors such as adhesion, migration and proliferation. Hladys et al. [30] and Ruardy et al. [31] pioneered the study in this field, and demonstrated that cells prefer to attach on the surfaces with intermediate hydrophilicity.
However, in our study the surfaces in general are hydrophobic with water contact angles ranging from 71° to 78°. We believe such a small variation of hydrophobicity of the surfaces will not lead to significant impact on the cell behaviors.

Surface charge is another governing factor, which has been widely acknowledged, for cell adhesion and migration [30,32]. The zeta potentials of the uniform surfaces with different gelatin densities were measured. As shown in Fig. 1e, the PCL-NH₂ surface was positively charged due to the presence of amino groups. After immobilization of gelatin, which is a negatively charged protein in physiological environment, the surfaces became negatively charged (<15 mV), even on the surface with the lowest gelatin density (0.40 µg/cm²). Further enhancement of the gelatin density (0.82 µg/cm²) resulted in slightly increasing of negative charge on the surfaces, and finally reached the highest value (<36 mV) on the surface with the highest gelatin density (1.44 µg/cm²). Since the gelatin-modified surfaces are all negatively charged within a limited variation range, we assume the surface charge does not significantly influence the cell behaviors in our study.

It is worth mentioning that protein adsorption is not significant on the gelatin-covered surfaces (Fig. 1f). This is understandable because the adsorbed protein amount is very small (60–70 ng/cm²) [33,34] compared to that of the immobilized gelatin. Besides, most of the adsorbed proteins should be the relatively inert albumin, and only a small portion (about 10%) will be the cell-anchoring proteins such as fibronectin, laminin, vitronectin and so on [35]. Therefore, the impact of adsorbed serum proteins on cell behaviors can be safely ruled out in current study.

3.2. Cell adhesion

Previous studies reveal that cell-substrate interactions have an obvious impact on the cell behaviors including the cell migration [36]. Therefore, cell number, adhesion force and cell morphology on the uniform gelatin and the gradient gelatin surfaces with variable gelatin densities were firstly studied. As shown in Fig. 2a, the densities of adherent ECs 24 h post cell seeding on the gelatin-immobilized surfaces were significantly higher than those on the pristine PCL membrane regardless of the uniform or gradient distribution of gelatin, which is consistent with the feature of good cell anchoring ability of gelatin. The cell density slightly increased on the surfaces with higher gelatin densities (1.00 and 1.57 µg/cm²). However, the absolute cell densities are very close to each other, suggesting that 0.49 µg/cm² gelatin is high enough to accelerate adhesion of ECs.

The interaction between cells and substrates still can be different although all the surfaces can well support the cell adhesion. Fig. 2b shows that the cell adhesion force was enhanced obviously along with the gelatin density, i.e. from 92 pN/cm² on the pristine PCL membrane to 279 pN/cm² on the gelatin-immobilized membrane with the highest gelatin density. The differences of cell adhesion force on the uniform and gradient gelatin surfaces were very small, even though the absolute values on the uniform gelatin surface were statistically larger than those of the gradient at the same positions. Therefore, the gelatin density takes a critical role on cell adhesion rather than the distribution manner of gelatin molecules.

The cytoskeleton organizations of ECs on the uniform and the gradient gelatin surfaces with the same gelatin density were further studied. As shown in Fig. 3, compared to those on the pristine PCL membrane, the cells cultured on the gelatin-immobilized surfaces showed more stressed actin fibers, suggesting better cell adhesion which is consistent with the results in Fig. 2a. The ECs on the gradient surfaces showed some tendency to align to the gradient direction (Fig. 3a–c), especially on the surface of the lowest gelatin density (Fig. 3a). By contrast, the ECs on the uniform surfaces showed a random organization of cell cytoskeleton without a preferential direction (Fig. 3d–g).
To mimic the vascular vessel structure, it is important to consider the alignment and orientation of the cells [37]. The cell orientation was further quantitatively analyzed (Fig. 4). By contrast to the random orientation of ECs on the pristine PCL and uniform gelatin surfaces, 52% of the ECs orientated in ±30° to the X-direction on the gelatin gradient surface with a gelatin density of 0.49 μg/cm² (Fig. 4a). Although these ratios decreased to 47% and 39% on the surfaces with gelatin densities of 1.00 μg/cm² (12 mm) and 1.57 μg/cm² (20 mm), respectively (Fig. 4b and c), they were still significantly higher than that of the cells on the uniform surfaces (22–28%). The results suggest that ECs prefer to orientate to the gradient direction on the gradient surfaces, and their degree can be mediated by the gelatin density.

### 3.3. Cell migration

Vascular diseases usually start with ECs dysfunction, which is followed by the proliferation and migration of smooth muscle cells (SMCs) toward the blood vessel lumen [38]. Therefore, it is of paramount importance to promote the migration performance of ECs including both directionality and cell mobility by tissue regenerative biomaterials.

The migration behaviors of ECs on the biodegradable PCL membranes immobilized with gelatin gradient were monitored and statistically analyzed. The cells were seeded at a low density for the sake of avoiding cell-cell interactions. Under this condition, the mobility of cells can be dominated merely by the cell-substrate interactions. The ECs were monitored continuously to acquire the migration trajectories. As shown in Fig. 5a, 90% of ECs migrated toward the +X direction of the gradient at the 4 mm position with a gelatin density of 0.49 μg/cm². On the gradient positions of 12 mm and 20 mm with gelatin densities of 1.00 and 1.57 μg/cm², respectively (Fig. 5b and c), the ratios of ECs moving directionally toward the +X direction of the gradient decreased to 73% and 65%, respectively. By contrast, the ECs moved randomly without a preferential direction on the pristine (Fig. 5d) and the uniform gelatin surfaces (Fig. 5e–f). The directional migration of ECs is attributed to the directional polarization of ECs under the guidance of gelatin density gradient (Fig. 4), and the less oriented cells on the positions of higher gelatin densities should be responsible for the weakened directionality.

As shown in Fig. 6, the ECs had the highest migration rate (12.4 μm/h) on the gelatin gradient at 4 mm position (0.49 μg/cm²). At this position the ECs also showed significantly higher mobility on the gelatin gradient than that on the uniform gelatin.
surface (10.9 μm/h). Both values were significantly larger than that on the pristine PCL membrane (7.8 μm/h), suggesting that the gelatin can not only enhance cell adhesion but also promote cell mobility. The cell migration rate slightly decreased along with the increase of gelatin density on both the uniform and gradient surfaces, with values of 10.5 μm/h and 8.6 μm/h on the surfaces with gelatin densities of 1.00 μg/cm² and 1.57 μg/cm², respectively (see Scheme 1).

Gelatin is a natural protein with good cytocompatibility which can support cell adhesion, spreading and migration. Rosenquist et al. found a 2.5-fold increase of infiltrating endothelial cells into the gelatin-modified scaffolds compared to that of the control scaffold [39]. In this work, a gelatin density gradient was generated on the PCL membrane, and three representative positions with different gelatin densities were chosen to study the cell responses including adhesion, spreading and migration. The surfaces with the gelatin densities from 0.49 to 1.57 μg/cm² had a similar ability to support the adhesion of ECs, resulting in a larger adherent cell number compared to that on the pristine PCL membrane. However, the cell adhesion force increased along with the increase of gelatin density, suggesting stronger interaction between the cells and substrates with a higher gelatin density.

On the gelatin density gradient, ECs were found to move toward the direction with a higher gelatin density at all three tested positions, although the ratio of directionally migrated ECs decreased at the higher gelatin density. By contrast, ECs showed random migration on the uniform surfaces. One possible reason for the directional migration of ECs is a result of the preferential orientation

![Distribution of the angles between ECs and +X direction of the gradient after ECs were cultured for 12 h on (d) pristine PCL membrane, and PCL membranes immobilized with (a–c) gelatin density gradient and (e–g) uniform gelatin molecules with a density of 0.49, 1.00, and 1.57 μg/cm², respectively. At least 100 cells were analyzed for each sample.](image)

![Migration traces of ECs on (d) pristine PCL membrane, and PCL membranes immobilized with (a–c) gelatin density gradient and (e–g) uniform gelatin molecules with a density of 0.49, 1.00, and 1.57 μg/cm², respectively. At least 30 cells were tracked for each sample. The number on the bottom left side on each figure indicates the percentage of cells moving to the +X direction. The mobility of ECs was continuously tracked every 15 min for 12 h after being seeded and cultured for 12 h.](image)
of cells, which is induced by the gradient cues. Another possible mechanism is the general random movement of ECs, which is captured by the regions of a higher gelatin density due to the stronger cell–material interaction.

The ECs showed the highest migration rate of 12.4 μm/h at 4 mm position on the gelatin gradient (gelatin density 0.49 g/cm²), which was significantly higher than that on the uniform surface with the same gelatin density (10.9 g/cm²) and the pristine PCL membrane (7.8 g/cm²). However, the mobility of ECs also decreased along with the increase of gelatin density. The reduced cell mobility can be partially due to the significant increase of cell adhesion force with a higher gelatin density, indicating that the gelatin density plays a more important role than its distribution in tuning the mobility of ECs.

Recently, Wu et al. fabricated a VEGF density gradient on silicon wafers and studied its influence on ECs’ migration [40]. They found that at the best case 88.9% of ECs moved directionally with the highest speed around 6 μm/h. Our results showed that a more robust gelatin density gradient on biodegradable PCL membrane can induce directional migration of ECs with a relatively higher migration rate, indicating the potential for fast endothelialization in blood vessel formation or regeneration.

4. Conclusion

A gelatin density gradient was fabricated via glutaraldehyde linking on the PCL membrane with an amino gradient which was created by gradient aminolysis. The adhesion of ECs was improved on the gelatin-immobilized surface regardless of the distribution manner (uniform or gradient) of gelatin. The ECs showed highly directional orientation and migration with enhanced mobility on the gelatin gradient with a proper gelatin density. This study demonstrates the manipulation of migration behaviors of ECs by a gelatin gradient on real degradable biomaterials, highlighting the possibility to prepare gradient biomaterials and to realize their applications in tissue regeneration.

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References