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Protocols

# Stiffness of polyelectrolyte multilayer film influences endothelial function of endothelial cell monolayer

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ABSTRACT

Endothelialization has proved to be critical for maintaining long-term success of implantable vascular devices. The formation of monolayer of endothelial cells (ECs) on the implant surfaces is one of the most important factors for the endothelialization. However, endothelial function of regenerated EC monolayer, which plays a much more important role in preventing the complications of post-implantation, has not received enough attention. Here, a vascular endothelial growth factor (VEGF)-incorporated poly(L-lysine)/hyaluronan (PLL/HA) polyelectrolyte multilayer film was fabricated. Through varying the crosslinking degree, stiffness of the film was manipulated, offering either soft or stiff film. We demonstrated that ECs were able to adhere and proliferate on both soft and stiff films, subsequently forming an integrated EC monolayer. Furthermore, endothelial functions were evaluated by characterizing EC monolayer integrity, expression of genes correlated with the endothelial functions, and nitric oxide production. It demonstrated that EC monolayer on the soft film displayed higher endothelial function compared to that on the stiff film. Our study highlights the influence of substrate stiffness on endothelial function, which offers a new criterion for surface design of vascular implants.



## 1. Introduction

Cardiovascular disease is the leading cause of mortality worldwide [1]. Implantable vascular devices, including vessel bypass grafts, stents and heart valves, have been widely developed for treatment of cardiovascular diseases [2,3]. Though synthetic implants are able to mimic the structural integrity of the native tissues, metabolic and biochemical functions of native tissues are not easily reproduced without the involvement of cellular machinery. Endothelial cells (ECs), plays a vital role in maintaining healthy blood vessel conditions through its diverse physiological functions, such as anti-coagulation, anti-thrombosis, and regulating growth of other types of cells [4]. With the lack of ECs on the surface of implants, post-implantation complications, particularly intimal hyperplasia and thrombosis, are more prone to take place [5,6].

To avoid undesired post-implantation effects [4,7], many surface modification strategies have been developed to improve endothelialization and subsequently formation of EC monolayer on the surfaces of vascular implants [8–12]. However, it was found that monolayer of ECs can be obtained on non-treated bare steel stents after several weeks of implantation [13,14]. In addition, formation of monolayer ECs on implant surfaces does not imply a full restoration of endothelial function [15–17]. It has demonstrated that the endothelium on steel stent lost their original phenotype, and showing dysfunction compared to normal endothelium *in vivo* [13]. Although the CD34-antibody-immobilized stent was able to significantly improve endothelialization, endothelial function remained impaired, resulting in limited effect against restenosis [18]. Therefore, improvement of endothelial function of regenerated endothelium is more critical in preventing more severe complications, such as late-thrombosis and neoatherosclerosis, after vascular device implantation [15,19].

Stiffness, which is one of the most important physical properties of materials, is known as a powerful stimulus capable of modulating cellular processes [20,21]. Being anchorage-dependent, the

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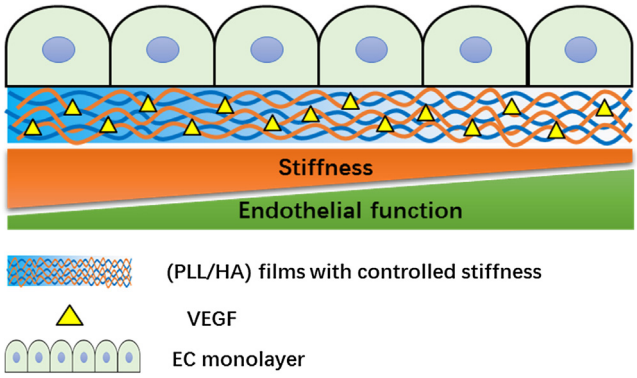


**Scheme 1.** Illustration of influence of stiffness of (PLL/HA) polyelectrolyte multilayer films with substrate-bound VEGF on endothelial function of regenerated EC monolayer. Reducing substrate stiffness favors improvement of endothelial function.

behaviors of ECs are greatly influence by substrate stiffness. For instance, adhesion and proliferation of ECs were showed to be inhibited on soft substrates, while promoted on higher stiffness substrates [8,22,23]. Our group previously has also demonstrated that substrate stiffness significantly influenced EC phenotype and low substrate stiffness showed to be able to retain endothelial marker (CD31) of ECs [24]. Other studies revealed that high stiffness substrate stimulated ECs to produce functional pro-angiogenic and osteogenic factors [25].

Recently, our group has developed a soft multilayer film through alternative deposition of poly(L-lysine) (PLL) and hyaluronan (HA) [26]. By incorporating matrix-bound vascular endothelial growth factor (VEGF) at an optimal density in the film, the film with matrix-bound VEGF was able to specifically promote EC adhesion rather than smooth muscle cells, and it further significantly improved endothelial function compared to traditional materials (including glass, stainless steel, and tissue culture polystyrene) [26]. However, elastic modulus of these traditional stiff materials is usually at GPa level, which is much higher than the elastic modulus of (PLL/HA) films and the stiffness level that cells can distinguish. How stiffness at kPa level influences endothelial function still remains unclear.

With the purpose of gaining this missing piece of information, the VEGF-bound (PLL/HA) films with two stiffness (soft and stiff) were applied here. First, EC adhesion and proliferation were characterized to investigate the process of formation of EC monolayer. Further, endothelial functions of as-formed EC monolayer were investigated by evaluating monolayer integrity, expression of genes correlated with the endothelial functions, and nitric oxide (NO) production. We demonstrated that the difference of substrate stiffness in kPa levels had significantly influence on endothelial function, which gives insight into the importance of surface stiffness of vascular implants.

## 2. Materials and methods

### 2.1. Materials

Poly(L-lysine) (PLL, Mw 30,000–70,000), polyethylenimine (PEI, branched, Mw 25,000), Triton X-100 and 4,6-diamidina-2-phenylin (DAPI) were purchased from Sigma-Aldrich (USA). Hyaluronate acid (HA, Mw 351–600 kDa) was purchased from Lifecore Biomedical (USA). N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (Hepes, free acid, high purity grade), phosphate buffered saline (PBS), bovine serum albumin (BSA) and tris-buffered saline (TBS) were obtained from Sangon Biotech (Shanghai, China). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N—Hydroxysulfosuccinimide sodium salt (sulfo-NHS) were purchased from Aladdin (Shanghai, China). Human recombinant vascular endothelial growth factor 165 (VEGF) was purchased from PeproTech (USA). Endothelial Cell Medium was purchased from ScienCell Research Laboratories (USA). All solutions were prepared using deionized water (18 MΩ cm, Milli-Q Ultrapure Water System, Millipore).

### 2.2. Fabrication and crosslinking of (PLL/HA) multilayer film

PLL (0.5 mg/mL) and HA (1 mg/mL) were dissolved in a Hepes-NaCl buffer (20 mM Hepes at pH 7.4150 mM NaCl). $(PLL/HA)_{12}$ films (where subscript 12 indicates for the number of layer pairs) were prepared as previously described [26,27]. Briefly, glass coverslips (diameter is 14 mm) were dipped in the PLL solution for 8 min. After being rinsed three times with NaCl solution (150 mM), the coverslips were dipped in the HA solution for 8 min. The coverslips were then rinsed again. This sequence was repeated 12 times. For 6-well plates, films were fabricated starting with a first layer of PEI (3 mg/mL in Hepes-NaCl buffer). The $(PLL/HA)_{12}$ films were crosslinked for 18 h at 4 °C using a crosslinking solution containing EDC (30 and 100 mg/mL) and sulfo-NHS (11 mg/mL) in NaCl (150 mM, pH = 5.5). After crosslinking, the films were rinsed with Hepes-NaCl buffer at least 8 times (30 min each time).

### 2.3. VEGF incorporation

VEGF was reconstituted to 1 mg/mL in Mili-Q water according to provider protocol. The loading process of VEGF in film was carried out according to previous reported method [26]. Briefly, VEGF was diluted to 20 μg/mL by HCl solution (1 mM, pH = 3.0). Film was pre-equilibrated for 30 min in the HCl solution. A volume of 50 μL for 14 mm glass coverslips or 0.6 mL for 6-well plates of VEGF at desired concentration was deposited onto film and left to adsorb overnight at 4 °C. Hepes-NaCl buffer was added onto film and left at room temperature for 15 min and this washing process was repeated 7 times in order to keep only substrate-bound VEGF. During each washing, the rinsing solution was collected for testing concentration of VEGF.

### 2.4. Measurement of amount of VEGF in films

In order to calculate amount of VEGF in films, free VEGF left in the supernatant during the washing steps was quantified using an enzyme-linked immunosorbent assay (ELISA) kit, as recommended by the manufacturer (Boster Bio-engineering, China). The density of substrate-bound VEGF in films was calculated from the difference between the initial and remaining amount of VEGF [28]. To investigate the loading stability of VEGF in films, the VEGF-bound films with controlled stiffness were incubated into PBS (pH = 7.4) at 37 °C. 100 μL solution was collected on day 1–3 for ELISA assay. The percentage of VEGF remaining in films was then calculated.

### 2.5. Young's modulus measurement

The Young's modulus of the soft and stiff films, with and without substrate-bound VEGF was characterized by Atomic Force Microscope (AFM, multimode 8, Bruker). The probe (SNL-10, Bruker) had a nominal spring constant of 0.06 N/m. The samples were immersed in a drop of HEPES-NaCl buffer, and the tests were performed in a liquid environment. For each sample, measurements were obtained at eight different sites. Data were analyzed by NanoScope Analysis software using the Sneddon model as modulus fit model. The fitting range was from 30 to 90%.