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# Selective endothelial cells adhesion to Arg-Glu-Asp-Val peptide functionalized polysaccharide multilayer

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ABSTRACT

Thrombosis and in-stent restenosis are the main obstacles in the healing process after cardiovascular surgery. A promising way to achieve the healing process after percutaneous transluminal coronary angioplasty followed by stenting may rely on the rapid *in situ endothelialization* on the materials of implants. Several requirements are raised to achieve *in situ* endothelialization, of which the specifically endothelial cells (ECs) homing and the non-specific cells repulsion come first. In this work, heparin/chitosan multilayer was constructed with thromboresistant and non-specifically cell-resistant properties. The specific ECs adhesive peptide sequence Arg-Glu-Asp-Val (REDV) was then immobilized onto the pristine multilayer and the cell responses of ECs and smooth muscle cells (SMCs) were verified. It is interesting that ECs selective attachment was obtained on the REDV functionalized multilayer, whereas the multilayer maintains resisting to the SMCs. These results show that the REDV functionalized cell-resistant heparin/chitosan multilayer is a ECs selective surface, which may have great potential in cardiovascular biomaterials for *in situ* endothelialization.



## 1. Introduction

Coronary artery diseases (CAD) are the major causes of mortality in modern societies [1]. Percutaneous transluminal coronary angioplasty followed by stenting (PTCA/stenting) is one of the main options to cure CAD [2]. However, in-stent restenosis (ISR) may occur after PTCA/stenting and lead to failure of the implants [3]. The use of drug-eluting stents (DES) has significantly decreased the incidences of ISR. Nevertheless, the later stent thrombosis (LAST) and ISR remain in high risk after the drug elution [4]. It is indicated that the delayed/uncompleted endothelialization is the main cause of the LAST and ISR [5]. Recently, inspired from the blood vessel healing process, whose endothelium re-growth might be derived from the migration of neighboring ECs or the recruitment of circulating endothelial cells (CECs), a strategy has been developed: the *in vivo*/*in situ* endothelialization of materials after implantation [6]. CECs homing factors immobilized directly onto the stents can achieve rapid *in situ* endothelialization, whereas the clinical trials reveal that it did not effectively obviate ISR as expected [7]. The *in situ* endothelialization on the implant surface to prevent ISR is likely to require greater complexity than endothelial seeding alone [8].

It is reported that the migration, proliferation and extracellular matrix deposition of smooth muscle cells (SMCs) may be the leading causes of ISR [9]. As a result, the implantable coating should present rapid ECs adhesion, meanwhile be repulsive to SMCs. Designing an anticoagulant coating with chemical and physical cues to repulse the non-specific cells adhesion meanwhile specifically to capture ECs could significantly improve the *in situ* endothelialization.

Surface modification of biomaterials with polyelectrolyte multilayers (PEMs) to obtain biocompatible surfaces via layer-by-layer (LbL) technique has developed since 1990s [10,11]. PEMs could be deposited onto almost any kinds of materials, including intravascular stents [12–14] and grafts [15,16]. Many building blocks could be incorporated into PEMs, including polysaccharides, proteins, synthetic polyelectrolytes, and nanoparticles [17–20]. Through the adjustability of several parameters, such as the chemical components of polyelectrolyte [19,21], chemical crosslinking [22–26], pH [27,28], and ionic strength of the medium [29], properties of PEMs could be easily tuned. PEMs with antithrombogenic [30–32], antibacterial [33–35] and/or cytocompatible [16,36] properties have been developed for various biomedical applications.

We have previously described an anticoagulant and antibacterial multilayer constitutes of heparin (HEP) and chitosan (CHI) [27,34,35]. The cell-resistant properties of this multilayer and its conversion to ECs adhesive surface after chemical crosslinking have also been detected [23]. Arg-Glu-Asp-Val (REDV) peptide sequence is specifically recognized by the $\alpha_4\beta_1$ integrin that specifically expressed on ECs surface [37,38]. Synthetic surfaces grafted with REDV peptide are shown to induce selective ECs adhesion [39]. The aim of this work is to construct a surface coating with ECs selective adhesion property via immobilization of REDV peptide onto the surface of HEP/CHI multilayer. We synthesized REDV peptide coupled CHI (CHI-REDV). After buildup

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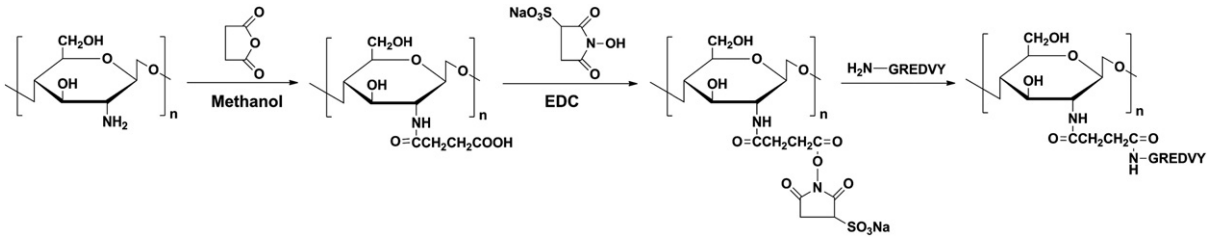


**Fig. 1.** The illustration of the procedure of the GREDVY peptide grafts onto chitosan chain.

of HEP/CHI multilayer, the CHI-REDV was then deposited as a terminating layer on the HEP/CHI multilayers. The multilayer growth and its structure were characterized. And its influence on adhesion of ECs and SMCs were also investigated.

## 2. Experimental details

### 2.1. Materials

HEP and CHI (Degrees of deacetylation > 92%, Mw ca. $3\times10^6$) were purchased from Shangdong Freda and Sanland Biochem Co., Ltd., respectively. polyethylenimine (PEI, Mw = 25,000), collagenase (type II) and fluorescein diacetate (FDA) were purchased from Sigma. The REDV contained peptide sequence Gly-Arg-Glu-Asp-Val-Tyr (GREDVY) and its scrambled sequence Gly-Arg-Glu-Val-Asp-Tyr (GREVDY) were supplied by Shanghai Science Peptide Biological Technology Co., Ltd. China. The EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and NHS (N-hydroxysulfosuccinimide) were purchased from Shanghai Medpep Co., Ltd. and were dissolved in 0.15 M NaCl (pH adjusted to 4.0) at 200 and 50 mM, respectively. All other reagents were local products of analytical grade.

### 2.2. Synthesis and characterization of the peptides coupled CHI

The synthesis of peptide coupled CHI was performed according to the protocol described in Ref. (Fig. 1) [39]. Briefly, CHI was dissolved in 2% (v/v) acetic acid solution. After stirring, the solution was filtered by a middle pore sand funnel to remove insoluble substances. The solution was then injected into a glass vessel. It was allowed to evaporate at 50 °C for 24 h to obtain a very thin CHI film. After that, the CHI thin film surface was treated with succinic anhydride to generate surfaces carboxyl group (denoted as SUC-CHI), which can react with the peptides. The –COOH groups on the SUC-CHI were used to immobilize the GREDVY peptide via EDC/NHS chemistry. The GREDVY and GREVDY peptides functionalized chitosan were denoted as CHI-REDV and CHI-REVD, respectively. UV–vis spectrophotometer (CARY 100 BIO, America) was used to identify the peptide grafting.

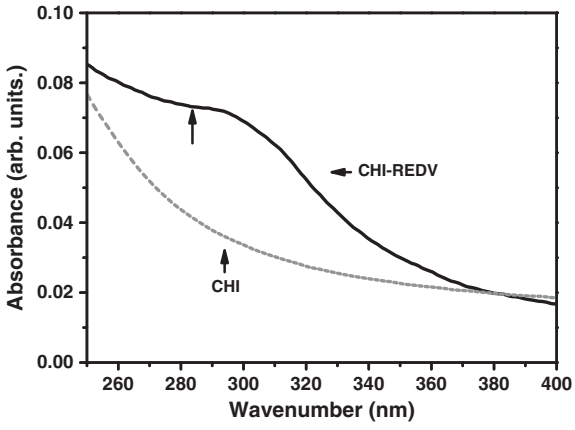


**Fig. 2.** UV–vis spectra of the chitosan (dash line) and GREDVY grafted chitosan (solid line).

### 2.3. PEMs fabrication and functionalization

The preparation of HEP and CHI solutions and the buildup of (HEP/CHI)n multilayer (n corresponds to the number of bilayers) were the same as the previous publications [23]. Briefly, HEP and CHI were dissolved at 1 mg/mL in acetic buffer solution (0.018 M NaAc, 0.082 M HAc, 0.14 M NaCl, pH = 4.0). The poly(ethylene terephalate) (PET) sheets were used as substrates to fabricate the HEP/CHI multilayer. Each experiment was initiated by PEI (3 mg/mL in Phosphate-Buffered Saline) solution adsorption for 1 h, which resulted in an aminolyzed positively charged surface [34]. The pre-coated substrates were dipped in HEP solution for 15 min and subsequently rinsed with acetic buffer solution for 1 min. The HEP adsorbed substrates were then dipped into CHI solution for 15 min and followed by same rinsing procedure. Alternate polyelectrolyte deposition cycles were continued until n bilayers of (HEP/CHI) multilayer was obtained. The multilayer crosslinking was performed with EDC/NHS conjugation reaction. The HEP/CHI multilayers coated PET sheets were incubated in an EDC/NHS solution (200/50 mM in 0.15 M NaCl, pH 4.0) for 12 h at room temperature, followed with NaCl solution rinsing. To get peptide functionalized multilayers, the CHI-REDV or CHI-REVD was deposited onto (HEP/CHI) multilayers (uncrosslinked one) and served as the outmost layer.

For the cell viability test, multilayers were directly fabricated onto 96-well plates. Briefly, 50 μL of HEP was introduced into wells for 15 min. The wells were then washed twice with the rinsing solution and 50 μL of CHI was introduced into wells for 15 min, and subsequently rinsing procedure. Alternate polyelectrolyte deposition cycles were continued until n bilayers of (HEP/CHI) multilayer was obtained.

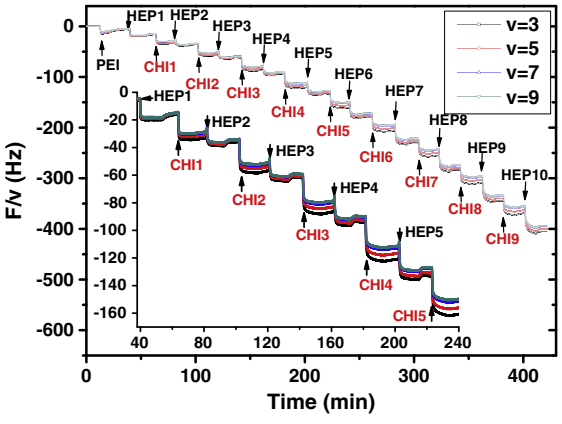


**Fig. 3.** QCM frequency shifts as a function of time during the fabrication of the heparin/chitosan multilayer at the 3rd (v = 3), 5th (v = 5), 7th (v = 7) and 9th (v = 9) overtone (15, 25, 35, and 45 MHz respectively). The arrows indicate the different polyelectrolytes injection. The insertion is a magnified view of the first five bilayers.