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Combined REDV Polypeptide and Heparin onto Titanium Surface for the Hemocompatibility and Selectively Endothelialization

Kun Zhang[1,2,3], **Jing-an Li**[3*], **Jian Wang**[3], **Tao Liu**[3], **Xue Wang**[3], **Jun-ying Chen**[3], **Nan Huang**[3] **and Fang-xia Guan**[1,2]

[1]*Center of Stem Cell and Regenerative Medicine, First Affiliated Hospital of Zhengzhou University, 40 University Road, Zhengzhou 450052, PR China*

[2]*School of Life Science, Zhengzhou University, 100 Science Road, Zhengzhou 450001, PR China*

[3]*Key Laboratory for Advanced Technologies of Materials, Ministry of Education, School of Material Science and Engineering, Southwest Jiaotong University, Chengdu 610031, PR China*

[*]**Corresponding author:** Jing-an Li, Key Laboratory for Advanced Technologies of Materials, Ministry of Education, School of Material Science and Engineering, Southwest Jiaotong University, Chengdu 610031, PR China, Tel: +86-13551284797; Fax: +86-28-87600625; E-mail: 4828713@qq.com

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Abstract

To enhance the blood compatibility and selective endothelialization of the cardiovascular implants simultaneously, in this study, the mixture of REDV polypeptide and heparin was used to modify titanium (Ti) surfaces in virtue of a polylysine layer. Blood compatibility tests revealed that the modified Ti prolonged blood coagulation time compared with Ti surface. Endothelial cells (ECs)/platelets and ECs/smooth muscle cells (SMCs) competed seeding results showed more attached ECs on the modified samples than that on Ti surfaces. Thus, *in vitro* evaluation indicates that the REDV polypeptide and heparin modified Ti surface kept excellent biocompatibility, which could improve the blood compatibility and selectively promotes the endothelialization simultaneously. We conceive that the cooperation of REDV polypeptide and heparin may provide a promising selection for biomaterials surface modification of vascular implants.

Keywords: Vascular stent; Surface modification; REDV polypeptide; Heparin

Introduction

Cardiovascular disease caused by atherosclerosis is a significant cause of death [1]. Percutaneous coronary intervention using stents is an effective clinical treatment [2]. Endothelialization would provide an anti-thrombogenic and anti-proliferative coating on the blood-contacting metal stent surface forever which mimics the normal lining of blood vessels [3].

The peptide sequence Arg-Glu-Asp-Val (REDV), which is present in the III-CS domain of human plasma fibronectin, is a special adhesion receptor on the human endothelial cell. After a synthetic peptide including this sequence was immobilized on otherwise cell non adhesive substrates to specifically attract endothelial cells attach and spread [4]. GREDVY polypeptide (Gly-Arg-Glu-Asp-Val-Tyr, shown in Figure 1) which has the REDV sequence also has the selective ability [5]. Heparin, as a common anticoagulant in clinical, can be coated on the stents simply [6,7] or combing with many drugs (such as abciximab [8]) or biomolecules (such as fibronectin [9], poly-l-lysine [10]) to improve the blood compatibility.

In the present work, GREDVY polypeptide and heparin were immobilized on Ti, in sights of the goal with hemocompatibility and selectively endothelialization. The construction and evaluation of the assembled GREDVY polypeptide/heparin layer on Ti plates were evaluated *in vitro*. We anticipate that this GREDVY polypeptide/heparin layer will be beneficial to selectively enhance the endothelial cell attachment on an artificial vascular graft.

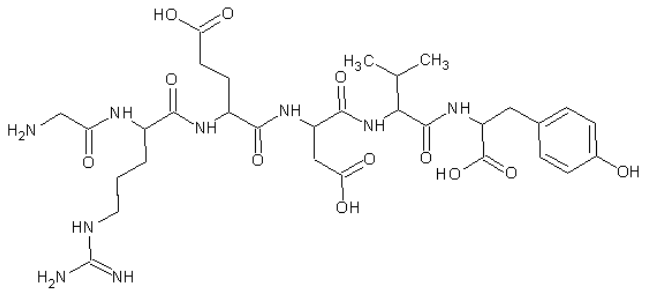

Figure 1: The molecular structure of GREDVY polypeptide

Material and Methods

Materials and reagents

Ti plate was cut into specifications of 10×10 mm (The purity was 99.5%, Baoji, China). Polylysine (30,000-70,000 kDa, Sigma–Aldrich, USA) was diluted to a concentration of 2.5 mg/ml. Heparin sodium (>160 IU/mg, Solarbio Corp., China) was dissolved to a concentration of 5 mg/ml. GREDVY polypeptide (Shanghai Science Peptide Biological Technology Co., Ltd, China) was diluted to 200 μg/ml [11]. All the solvent were phosphate buffer solution (PBS) at pH 7.4.

Activated partial thromboplastin time (APTT) kits for anticoagulation properties test were purchased from Sunbio, China. Alcian blue 8GX and Acridine orange (AO) were bought from Sigma–Aldrich. FITC-GREDVY polypeptide was synthesized by Shanghai

Science Peptide Biological Technology Co., Ltd, China. CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) and CellTracker™ Orange CMFDA were bought from Life Technologies Corporation, USA. All the antibodies used in the experiment were bought from Abcam Ltd, Hong Kong. Acetone, ethanol and other reagents were analytical of grade (purity >99.9%) and purchased from Sunbio, China.

Combined GREDVY polypeptide and heparin on Ti substrate

The schedule of pretreated Ti substrates was as the methods of the literature [12]. Figure 2 shows the fabrication process of GREDVY polypeptide/heparin layer assembled on Ti substrate. Ti was immersed into 2 M NaOH solution 24h at 80oC to obtain –OH groups on the surface which was named TiOH. 50 μl polylysine were dropped on TiOH to get TiOHP. A mixed solvent with 5 mg/ml heparin and 50 μg/ml GREDVY polypeptide was obtained, then 50 μl of the solution mixture were added onto TiOH for 2 h to obtain TiOHPHR, and 50 μl heparin were added onto TiOH for 2 h to obtain TiOHPH, each step was rinsed with deionized water to remove the unattached molecules (Table 1). All the Ti, TiOH, TiOHP, TiOHPH samples are contrast samples.

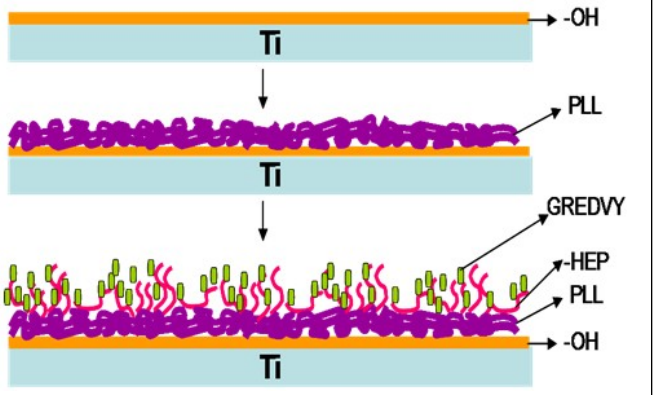

Figure 2: Sketch map of the preparation of GREDVY polypeptide / heparin film

FTIR

FTIR of Ti, TiOH, TiOHP, TiOHPH and TiOHPHR were detected in diffuse reflectance mode using a Fourier Transform infrared spectrometer (FTIR, NICOLET 5700, USA) with the Spectra range from 1000 to 4000 cm^{-1}.

Water contact angle measurement

All these dried samples were fixed to an object stage of the contact angle apparatus (JY-82, China), and then a droplet of dH_2O was dropped onto the surface to measure contact angle.

AFM

The surface roughness of the Ti, TiOH, TiOHP, TiOHPH and TiOHPHR samples were characterized by atomic force microscopy (AFM, JPK Instruments, Berlin, Germany) using tapping mode.

Lable	The process parameters
Ti	Pure titanium
TiOH	Pure titanium after activation in 2M NaOH solution
TiOHP	The TiOH surface assembled with polylysine
TiOHPH	TiOHP surface assembled with heparin
TiOHPHR	TiOHP surface assembled with the mixture of heparin and GREDVY polypeptide

Table 1: The label and the process parameters of the samples

Quatitative characterization of TiOHPHR

Heparin-Alcian Blue 8GX Staining: Alcian Blue staining method was used to view the assemblied heparin on the TiOHPHR surface as the methods of the literature [13]. After the staining, TiOHP and TiOHPHR samples were observed by optical microscopy (Leica, Germany).

FITC-GREDVY polypeptide Staining: The GREDVY polypeptide was replaced by FITC-GREDVY polypeptide to fabricate a FITC marked TiOHPHR. Then the samples were observed by fluorescence microscopy.

Biocompatibility of TiOHPHR

Hemocompatibility-APTT: The *in vitro* anticoagulation properties of TiOHPHR were evaluated by means of the intrinsic coagulation using a coagulation instrument (Hospitex Diagnostics, Italy), or researching the quantity, morphology and aggregation of the adherent platelets via platelet adhesion testing. Platelet-rich plasma (PRP) was acquired with anticoagulant citrate dextrose fresh human blood using centrifuge at 1500 rpm for 15 min. The samples were immersed in 350 μl PRP and incubated at 37 for 10 min, then 300 μl incubated untreated or treated PRP was moved to test the coagulation time.

Cell compatibility-Keen competition between ECs and platelets adhesion: ECs at the concentration of 5 X 10^3 cells/ml and PRP were mixed with the same volume and the Ti and TiOHPHR samples were immersed in 400 μl mixture. After the two samples were incubated at 37 for 45 minutes, parts of the samples were fixed with 2.5% glutaraldehyde solution and stained with Rhodamine and DAPI, finally ECs and platelets were examined from their different size by a fluorescence microscopy, and other samples were characterized by a scanning electron microscopy.

Cell compatibility-Keen competition between ECs and SMCs adhesion: Briefly, ECs marked with green tracker and SMCs marked with orange tracker [14] at the same concentration of 5 × 10^4 cells/ml were mixed with 2:1(v/v), and 600 μl/well of the mixture was added to the 24-well plates with the Ti and TiOHPHR samples. After the two samples were respectively taken out after 2 hours of incubation at 37°C the cells were observed by fluorescence microscope. Attached SMCs and ECs were counted from 6 random fields and averaged for multiple samples (n=3).

Data analysis

The results were reported as mean ± standard deviation (S.D.). The statistical analysis was performed via Student's paired t test. The

confidence range selected was 95% and the probabilities of $P<0.05$ indicated a significant difference. All experiments were repeated for three times.

Results and Discussion

FTIR

The DR-FTIR spectrum of different samples (Figure 3) indicates the presence of specific functional groups. The broad peak around 3370 cm^{-1} suggests that TiOH, TiOHP, TiOHPH and TiOHPHR are rich in hydroxy (–OH) groups. With the emergence of stretching vibration peak of C=O at 1672 cm^{-1} (amide I) and in-plane deformation vibration of NH at 1544 cm^{-1} (amide II), which indicated that polylysine description acid buffer layer was successfully constructed because a large number of amide bonds in the polylysine. Amide I and amide II also appear on TiOHPH and TiOHPHR, the reason is that the overlapping carboxyl stretching vibrations (1715~1650 cm^{-1}) on heparin belong to the broad peak around 1672 cm^{-1}, and GREDVY polypeptide also has many amide bonds [15]. So it might be speculated that heparin and GREDVY polypeptide are successfully assembled on the Ti surface.

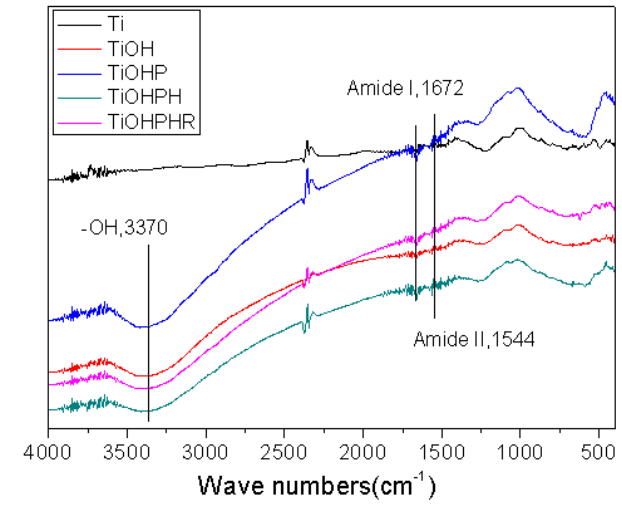

Figure 3: FTIR spectrum of Ti, TiOH, TiOHP , TiOHPH , Ti-R and TiOHPHR

AFM

Figure 4(a) shows the AFM images [16] of the Ti, TiOH, TiOHP, TiOHPH and TiOHPHR surfaces, respectively. It could be seen that the morphology of the Ti surface is smoother than that of TiOH which is flatter than that of TiOHP, due to activation by NaOH and assembled by polylysine. Polylysine is a long-chain molecule with a large molecular weight, while heparin has a low molecular weight and GREDVY polypeptide has the lowest molecular weight, so the small molecules can filled in large networks to make the surface be smoother. Then TiOHPH and TiOHPHR become smoother than TiOHP, which shows that heparin can be assembled successfully, but the molecular weight of GREDVY polypeptide is so low that it is difficult to distinguish the difference between TiOHPH and TiOHPHR.

Water contact angle

It can be seen from Figure 4(b) that the contact angle of water on the titanium surface is reduced to less than 10° after the alkali activation treatment, mainly for the hydrophilic group of -OH rich in TiOH surface; the water contact angle increased to 20~30° of TiOHP as hydrophobic group -NH2 of polylysine; Subsequently, the heparin or heparin/polypeptides mixture was fixed self-assembly on the surface, and the water contact angles of the two surfaces are decreased to 10~20° ($P<0.05$), because heparin is rich in acidic polysaccharides containing –COOH [17] which reduces the surface water contact angle values, GREDVY polypeptide containing both a free amino group and 3 free carboxyl groups, then the order of the water contact angle is TiOHPH<TiOHPHR<TiOHP, but no significant difference between the three samples ($P>0.05$).

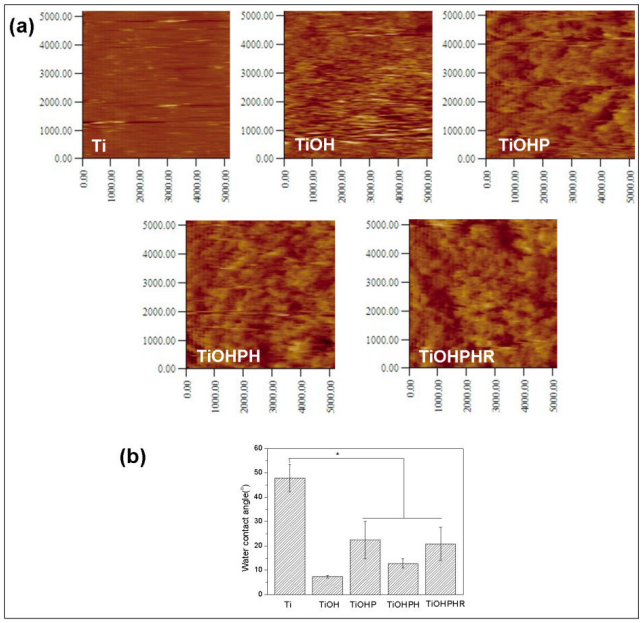

Figure 4: (a) AFM and (b) Water contact angle of Ti, TiOH, TiOHP, TiOHPH and TiOHPHR (*p<0.05, mean ± SD, N=3)

Alcian blue 8GX

The surface with heparin can be dyed blue with alcian blue staining by binding to the carboxyl group of heparin [12]. Figure 5(a) shows that the large areas of blue dots are scattered on the TiOHPHR surface, indicating that the heparin and polypeptide may be fixed on the surface successfully and covered the entire surface of the sample completely, due to both heparin and the polypeptide have the carboxyl groups.

GREDVY polypeptide qualitatively

The C-terminus labeled with FITC-GREDVY polypeptide was used to mix with heparin and to construct TiOHPHR. Figure 5(b) shows that the green fluorescence-labeled polypeptide assembled on the

TiOHPHR surface can be colored under a blue light excitation, while almost no green fluorescence shown on the TiOHP surface. The fluorescence intensity of TiOHPHR surface was stronger than that of TiOHP surface, it can be inferred that GREDVY polypeptide can be successfully secured to the TiOHP surface.

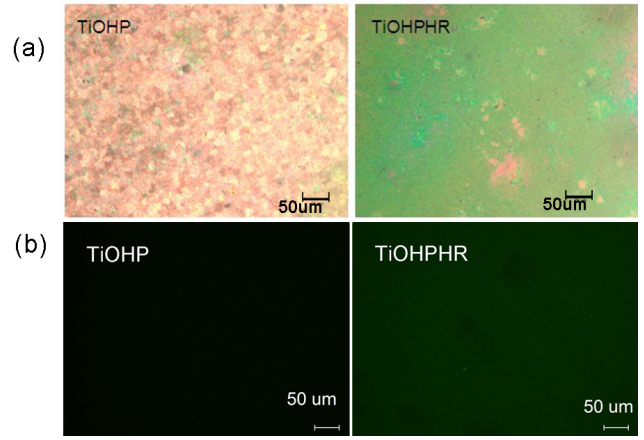

Figure 5: (a) The light microscopic images and (b) Immunofluorescence assay of TiOHP and TiOHPHR

Hemocompatibility-APTT

Activated partial thromboplastin times of different samples were tested using automatic coagulation analyzer and APTT testing kits, the results shown in Figure 6. The assembled heparin from TiOHP and TiOHPHR prolonged APTT over 10 s, and the APTT of TiOHPHR was prolonged approximately 20 s which has a certain clinical significance [18] Heparin can be combined with AT III which play a role in the intrinsic coagulation pathway [12]. Thus, heparin and GREDVY polypeptide make Ti based metal material with a certain anti-clotting function.

Platelets/ECs selectively adhesion

Platelets and Endothelial cells are two of the most important cells in the hemocompatibility evaluation and cell compatibility evaluation [19,20] It is necessary to examine the competitive adhesion of ECs and platelets onto the surface, the morphology and number of observations results of competitive adhesion were shown in Figure 7. From the different size of ECs and platelets [21], it can be seen that the quantity of platelets on TiOHPHR is far less than that on Ti, while the number of endothelial cells on TiOHPHR is a little more than that on Ti. And the statistical results of several different horizons counting fluorescence images were shown in Table 2. In the conditions of platelet and endothelial cell adhesion simultaneously, the number of platelets adhesion around each EC on Ti is much more than that on TiOHPHR, thus it can be inferred that the TiOHPHR surface has the ECs selective function and shows better hemocompatibility than Ti.

Samples	Ti	TiOHPHR
The number of platelet adhesion around each EC	126 ± 10.5	31.7 ± 1.78

Table 2: The statistical results of ECs/platelets competitive adhesion

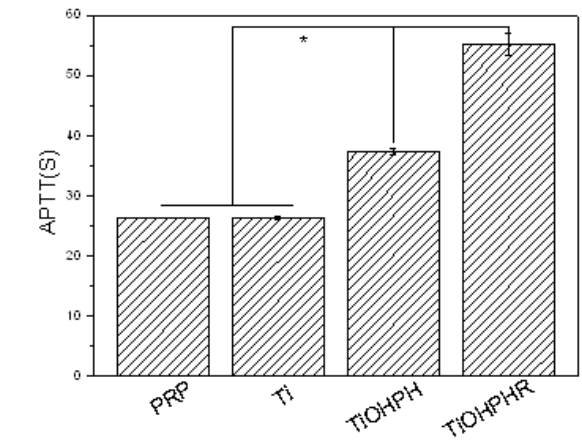

Figure 6: APTT values of PRP, Ti, TiOHPH and TiOHPHR (*p < 0.05, mean ± SD, N=3)

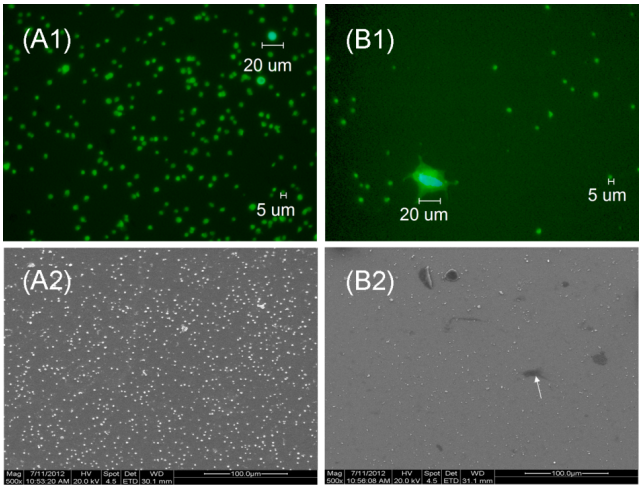

Figure 7: Immunofluorescence staining pictures (1) and SEM (2) of ECs/platelets competitive adhesion on Ti (A) and TiOHPHR (B) for 45 minutes

Heparin is a potent anticoagulant that is extensively used in the prevention and treatment of cardiovascular disorders [22]. Understanding the interaction between heparin and platelets will help in the development of safer anticoagulants. A significant advantage of heparin as a surface modifier is its specific interaction with AT III through the pentasaccharide structure, triggering the conformation change, which enhanced the inactivation rate of AT III to those coagulation proteases [23].

The peptide sequence Arg-Glu-Asp-Val (GREDVY) is specifically recognized by the integrin α4β1 which is abundant on ECs and scarce on platelets and SMCs [24]. Thus, it has been considered that the GREDVY immobilized on the surface can selectively promote ECs adhesion [24,25].

SMCs/ECs selectively adhesion

The ECs marked with CellTracker™ Green CMFDA and SMCs marked with CellTracker™ Orange CMFDA were mixed and planted on Ti and TiOHPHR surfaces for 2 h, the results of these immunofluorescence staining images are shown in Figure 8. There is more ECs (green) adhesion on TiOHPHR than that on Ti surface, while the quantity of adhered SMCs (orange) is similar. This is because the sequence of REDV in GREDVY polypeptide has a specific selective ECs adhesion function [26]. Liu et al. also reported that the heparin could significantly inhibit SMCs attachment to the surface and excessive proliferation [10], while the study of Ji et al. proved that GREDVY polypeptide had no effects on SMCs adhesion and proliferation [27]. Therefore, it can be concluded that the TiOHPHR surface has better ECs selective function than Ti.

Conclusion

Heparin and GREDVY polypeptide could be readily fixed self-assembly on Ti surface retaining their bioactivities, the combination of heparin and GREDVY polypeptide shows the selective endothelial cell compatibility, which may give both antithrombotic property and the property of anti-smooth muscle cell proliferation. This method can be extended to other different functional molecules assembled on kinds of biomaterials. While, based on the advantage of GREDVY polypeptide and heparin, there will be a potential improvement for implanted cardiovascular devices.

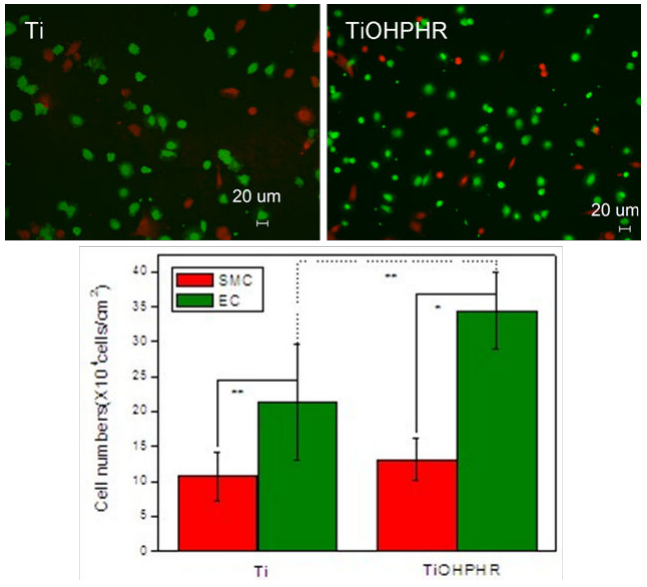

Figure 8: Immunofluorescence staining Images of ECs / SMCs competitive adhesion for 2 h

Acknowledgment

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