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Combinatorial coating of adhesive polypeptide and anti-CD34 antibody for improved endothelial cell adhesion and proliferation

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Abstract Improved attachment, adhesion and proliferation of the surrounding mature endothelial cells (ECs) and circulating endothelial progenitor cells (EPCs) is of primary importance to realize the in situ rapid re-endothelialization of cardiovascular stents. To achieve this, a combinatorial coating of synthesized mussel adhesive polypeptide mimics as well as anti-CD34 antibody was constructed onto the devices through a novel adsorption method in this study. To immobilize the polypeptide and target antibody effectively, polycaprolactone (PCL) was first spin-coated onto the substrate as intermediate. The immobilization of polypeptide and antibody was confirmed by the changes of water contact angles and the attachment, growth of ECs and EPCs on the substrates, respectively. The results showed that after adhesive polypeptide or/and antibody immobilization, the hydrophilicity of coated PCL substrate (PCLS) was obviously improved. The amount of the immobilized antibody, determined by enzymelinked immunoassay (ELISA) method, was enhanced with the increase of antibody concentrations in the range from 5 to 25 μg/ml. The coatings after BSA blocking prevented the unspecific protein adsorption as monitored by fluorescent microscopy. The results of in vitro cell culture showed that compared with the PCLS, polypeptide/anti-CD34 antibody coating could effectively enhance the attachment, growth and adhesion of ECs and EPCs, in particular EPCs. A platelet adhesion experiment revealed that the blood compatibility of the PCLS after polypeptide/anti-CD34 antibody coating was also obviously improved. The results showed that the surface modification with adhesive polypeptide and anti-CD34 antibody will be a promising coating technique for the surface modification of the intravascular prostheses for rapid re-endothelialization.

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1 Introduction

Restenosis is the main limitation after coronary artery stents implantation, which is mainly caused by thrombosis, abnormal hyperplasia and migration of smooth muscle cells (SMCs) in response to the mechanical injury within the vascular wall induced by overstretched stents [1]. It is generally accepted that the monolayer of endothelial cells (ECs), which provides an inherent nonthrombogenic potential and interrupts cytokine-driven activation of SMCs in vascular medial tissues, play an integral part in maintaining vascular homeostasis and normal functions. However, the mechanical injury during stent implantation inevitably exposed the thrombogenic arterial wall, along with stent to blood, directly triggered the formation of thrombus, and stimulated the proliferation of SMCs and finally resulted in the restenosis. Previous studies have shown that the lack of endothelialization is known to be one of the main reasons leading to the development of thromboses and/or intimal hyperplasia [2]. Although drug-eluting stents have achieved some promising results in reducing the proliferation of SMCs in recent years, it's not effective to recover the injured ECs at the site since the loaded anti-proliferation component, such as taxol, is non specific and is effective to both SMCs and ECs [3], and thereby the re-endothelialization was retarded. So a rapid in situ re-endothelialization of stent was indispensable.

Michael et al. [4] for example, reported that the stent thrombosis and neointimal hyperplasia would be simultaneously inhibited effectively if rapid re-endothelialization is achieved.

Due to ECs' ability in preventing thrombosis and restenosis, researchers have focused their attentions on seeding ECs on stent prior to implantation to realize the in situ re-endothelialization of stent [5–7]. The results, however, were inconsistent since the adherence efficacy of the seeded ECs to the stent has been revealed to be unpredictable, ECs showed little adhesion and no proliferation on currently available stent materials [8]. Allowing endothelial cells to attach and grow in situ to the stents under pulsatile flow might be an alternative strategy to solve this problem [9]. Re-endothelialization of the prosthetic surface can be achieved by the migration, attraction and adhesion of surrounding mature ECs and circulating EPCs to the injured region, which then differentiate into endothelial-like cells. To achieve this, many bioactive molecules such as polypeptide or antibody have been used to modulate the cell attachment and proliferation on the cardiovascular devices [10–15]. When substrates were pre-coated with bioactive proteins or polypeptide, such as gelatin, collagen, and RGD as well, the ECs attachment, growth and retention would be greatly improved [13, 16–19]. For example, Feugier et al. [9] investigated the attachment, morphology and adherence of human endothelial cells to vascular prostheses materials under the action of shear stress. They found that the collagen coating improved cellular adhesion and coverage significantly. Anti-CD34 antibody can specifically target $CD34^{+}$cells (endothelial progenitor cells are CD34 positive) in the vascular circulation. Kutryk and his coworkers revealed that once ECs or EPCs in blood flows contact with anti-CD34 antibody that immobilized onto the stent, the cells are then captured to the surface of the cardiovascular prostheses, and the growth and differentiation of the attached cells are simultaneously mediated. Consequently, the in situ re-endothelialization of implanted stent is accelerated [4].

Herein, we attempt to realize the rapid re-endothelialization of cardiovascular stents *via* the synergistic effect of polypeptide and anti-CD34 antibody. 316L stainless steel (316LSS), which is commonly used as vascular grafts, was used as substrate in the experiment. The polypeptide, which mimics the adhesive molecule of the mussel adhesive proteins, was composed of dihydroxyphenylalanine and L-lysine [20]. To effectively immobilize the polypeptide and anti-CD34 antibody, polycaprolactone, a biodegradable aliphatic polyester, which was widely used as stent stuff or coating material [19], was first spin-coated onto 316LSS as intermediate coating. The effects of adhesive polypeptide and anti-CD34 antibody on the attachment and growth, along with cell viabilities of ECs and EPCs on the surfaces were evaluated and compared, respectively.

2 Materials and methods

2.1 Preparation of PCL coating on 316L stainless steel

Biomedical 316LSS slides (Shanghai Biomedical Metal Company, China), 316LSS stents (DeviceTeck Co, USA) were employed as substrates. The biomedical 316LSS slides (1×1 cm^2) were polished with Buehler Phoenix 4000 to mirror surfaces. The biomedical 316LSS slides and stents were ultrasonically cleaned with acetone and isopropanol for 10 min to remove the surface containments.

PCL pellets (Mn = 80000, Sigma-Aldrich Company) were dissolved in dimethylsulphoxide/dimethyl acetamide (50% w/w) and spin-coated onto the 316LSS slides. The solvent was removed by evaporation under vacuum at 60°C, yielding a smooth and dense coating. To eliminate the remnant solvent completely, the PCL-coated substrates (PCLS) were further dried in vacuum at room temperature for 24 h. Before adhesive polypeptide and antibody immobilization, the PCLS were carefully washed with ethanol and PBS.

2.2 Immobilization of adhesive polypeptide and anti-CD34 antibody onto PCLS

The adhesive polypeptide was synthesized as reference [20]. The lyophilized polypeptide and antibody (monoclonal mouse anti-human CD34 antibody, Gene Tech Co.Ltd, China) were reconstituted in deionized water and further diluted with 10 mM/l Tris-buffered saline to various concentrations, respectively.

Firstly, PCLS were immersed in the polypeptide solution at 37°C. After 4 h, PCLS was thoroughly rinsed with distilled water to remove the unbound polypeptide and blown dry. Then, the polypeptide-coated substrates were incubated with anti-CD34 antibody at 4°C overnight and washed with PBS twice. To prevent the non-specific cells binding, all substrates were then blocked with 3% heat-denatured bovine serum albumin (BSA, Sijiqing Biotech. Co., China) at room temperature for 2 h.

In order to prepare the substrates for cell culture, PCLS were exposed to UV irradiation for 10 min under a 20 W mercury-vapor UV lamp (ZWS-UV 20 W, 50 Hz, Huanghai Lighting Co. China) integrated in the laminar air flow hood with a distance of 75 cm between samples and lamp. Prior to use, the polypeptide and antibody solution were sterilized by filtration (0.22 μm ultramicro-membrane). And the experiments were all carried out in sterilized room.

2.3 Surface characterization

The water contact angles of the substrates were measured using the sessile drop method with deionized water at room temperature (POWER EACH JC2000A, China). Each sample was repeated at more than three sites, the average values were obtained.

2.4 Quantitation of the immobilized anti-CD34 antibody

The antibody immobilized onto the substrates was quantitatively assayed with ELISA method [21]. The specimens were blocked with 3% BSA at room temperature for 2 h, after washing with PBS (0.01 M, pH = 7.3) for 3 times, the substrates were put into 24-well plates. 100 μl of 1 μg/ml horseradish peroxidase-conjugated goat anti-mouse antibodies (Pierce Biotechnology Inc., USA) was added to each specimen for 30 min at 37°C, and then washed with PBS solution. An aliquot of100 μl of super signal ELISA femto maximum sensitivity substrate (Pierce Biotechnology Inc., USA) was added to react for 30 min at room temperature; the amount of substrate converted by enzyme produced a fluorescent product related to the amount of bound antibody, the mean fluorescence intensity was measured using biochip reader (HD-2001A, matching protein chip system, Shukang Instruments, China).

2.5 Protein adsorption

The protein adsorptions on PCLS, polypeptide-coated PCLS and polypeptide/anti-CD34 antibody-coated PCLS before and after BSA blocking were assessed and compared. PCLS membranes were prepared by solvent evaporation method. For comparison, two set experiments were carried out. In the first set experiment, the membranes were modified by the adhesive polypeptide and anti-CD34 antibody according to the method given in Sect. 2.2. In the second set experiment, the membranes were also modified by the same methods as the first, but after modifications, the samples were subsequently blocked with BSA solution for 1 h at room temperature.

To investigate the protein adsorption, the PCLS-based samples were incubated with 1 mg/ml rhodamine-streptavidin solution overnight at 4°C. The streptavidin was labeled with rhodamine according to the protocol of EZ-Label^{TM} rhodamine Protein Labeling Kit [22]. After that, the samples were then washed twice with 1 × PBS buffer to remove excess rhodamine- streptavidin solution and analyzed for fluorescent activity. Fluorescent microscopy (Nikon Eclipse TE2000-U) was used to detect the intensity of protein binding. The fluorescent intensity was analyzed using the Fryer Metamorph Image Analysis System.

2.6 In vitro ECs culture

The human umbilical cord veins endothelial cells (HUVEC) were obtained from China Centre for Type Culture Collection and incubated in a culture medium consisting of 20% (v/v) fetal calf serum (FCS, Sijiqing Biotech. Co., China) and 80% RPMI 1640 (Gibco Co., USA) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin in humidified air containing 5% CO_2 at 37°C. The cells were cultured until they reached confluence in tissue culture flasks. Prior to cultivation with the substrates modified with different methods, the cells were trypsinized and resuspended.

To investigate the cells attachment on the substrates in the initial period, the substrates were kept in 24-well plates. ECs were trypsinized and washed with serum-free media, finally re-suspended in the media, and then seeded at an initial cell density of 5×10^4 cells per well. The cells were incubated for 1.5 h with the substrates. The non-adherent cells were eliminated by washing with PBS and the adherent cells were removed by digestion with 0.25% trypsin solution. The number of cells attached on different substrates was counted with haemacytometer. The cells attachment was expressed as a percentage of the number of cells attached on uncoated PCL surface.

For cells growth studies, the substrates were kept in 24-well plates and the cells were seeded at a density of 2.5×10^4 cells per well. The culture media was replenished every second day. The randomly selected wells were washed twice with PBS and the cells grown on the substrates were digested with 0.25% trypsin solution. The number of cells was determined by hemocytometer counting.

In order to evaluate the cells retention on different surfaces, the cells were exposed to an enzymatic treatment according to the reference [23]. The substrates were kept in 24-well plates and the ECs suspended in culture media were seeded at an initial cell density of 2.5×10^4 cells per well. After 48 h of incubation, the substrates were removed and the cells were washed with PBS and exposed to 400 μl of 0.0025% trypsin-EDTA solution in PBS for 5 min. The enzymatic digestion was stopped by adding of culture medium. The control was the same substrate without enzymatic treatment. The adherent percentage was calculated by the relation: (number of cells adherent on the treated substrates after enzymatic treatment/number of cells on the corresponding substrates without enzymatic treatment) × 100%.

Cell metabolic viability was measured by MTT (dimethylthiazol diphenyltetrazolium bromide) assay method. ECs were seeded at 1.3×10^4 cells each well. After a 48 h of culture, 20 μl MTT dissolved in PBS at 5 mg/ml was added to each well to continue incubation at 37°C for 4 h. And then, 200 μl of DMSO was added to

each well to dissolve the dark blue crystals. The optical densities of the centrifugal solutions were measured at 490 nm wavelength (Multiscan MK3, Thermo Electron Corp., USA). The cell viability was calculated by the relation: optical density value of the solution from the well/the number of cells grown in the well.

Cells were observed with optical microscope at different intervals. The cells were fixed with cold methanol/formaldehyde for 15 min and stained with fluorescein isothiocyanate isomer (FITC) for 5 min at 4°C, then washed with PBS, the cells images were taken with an inverted fluorescent microscope (TE2000-U, Nikon Corp., Japan)

2.7 In vitro isolation and culture of EPCs

Peripheral blood mononuclear cells were separated by density gradient centrifugation from porcine blood. The isolated cells were plated on culture dishes coated with fibronectin (Roche, Germany) in M199 (JRH,USA) supplemented with 20% fetal bovine serum (Hyclone, Australia). After 4 days, non-adherent cells were removed by washing with PBS. The adherent cells were trypsinized and washed with serum-free media, finally re-suspended in the media, and then seeded in 24-well plates, where the substrates were kept prior to cell culture. After 1.5, 24 and 48 h of culture, the non-adherent cells were eliminated by washing with PBS and the adherent cells were removed by digestion with 0.25% trypsin solution. The number of cells attached on different substrates was counted with haemacytometer. The cells attachment was expressed as a percentage of the number of cells attached on uncoated PCL surface.

2.8 In vitro platelet studies

For platelet adhesion studies, fresh human blood anticoagulated with acid citrate dextrose was centrifuged at 1000 rpm for 10 min to obtain platelet-rich plasma (PRP). The PCLS substrates with different modified methods were incubated for 1 h in PBS at 37°C in an incubator. After washing gently with PBS to remove the non-adhering platelets, fixing was done with 2.5% buffered glutaraldehyde solution for 1 h at 37°C. Specimens were then dehydrated with 50, 70, 95 and 100 dilutions of ethanol and water and dried. All the samples were coated with a gold layer and observed with SEM (JSM-6360LV, JEOL, Japan).

2.9 Statistical analysis

At least three independent experiments were performed for the assays described above. The results were expressed as a mean ± SD. One-Way ANOVA was used to analyze the date. P value smaller than 0.05 or 0.01 was considered as significant difference.

3 Results

3.1 Characterization of the PCLS before and after modification

Surfaces of the PCLS before and after polypeptide and antibody modification were all smooth and dense, with no obvious difference (data not shown). This will, to a certain extent, assure of the mechanical requirement when fabricated onto cardiovascular stents; on the other hand, the dense property of the coatings should be in favor of the sustained release of the drugs, if needed.

Table 1 presents the changes of the water contact angles of PCLS. As shown in the table, the original water contact angle of PCL film was 78.5°. After polypeptide or antibody immobilization, the contact angle decreased to about 54.2° or 71.5°, respectively. When PCL was sequentially coated with polypeptide and antibody, the water contact angle decreased to 51.4°. These results confirmed the occurrence of immobilization of polypeptide or/and antibody on the PCL surface.

The unspecific interaction of proteins is one of the major problems for biomaterials used for tissue regeneration in vitro or in vivo. Surface modifications that prevent unspecific protein adsorption and further allow immobilization of a specific biological function on the surface are necessary [24]. So, in this study, BSA was used to prevent the unspecific protein adsorption after surface modification and the protein adsorption on the modified PCLS before and after BSA blocking was evaluated. The representative fluorescence intensities are presented in Fig. 1. As shown in Fig. 1, high intensities were observed on the samples before BSA blocking, whereas very small fluorescence intensities on the samples after BSA blocking indicated very little of adsorbed proteins. That is to say, BSA can effectively prevent the nonspecific adsorption of protein.

3.2 Quantitative assay of anti-CD34 antibody immobilized on PCLS

Fluorescence intensities on specimens with different modifications were examined to confirm the amounts of

Table 1 Water contact angles on PCLS with different modifications

Samples	PCLS	Peptide–PCLS	Antibody-PCLS	Peptide-antibody-PCLS
Contact angle	78.5° ± 1.5	54.2° ± 2.3	71.5° ± 1.8	51.4° ± 1.6

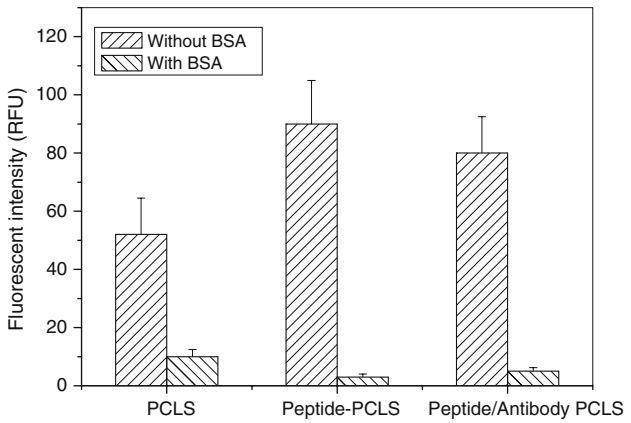

Fig. 1 Comparison of fluorescent intensities of rhodamine-labeled streptavidin adsorbed on the PCLS before and after BSA blocking

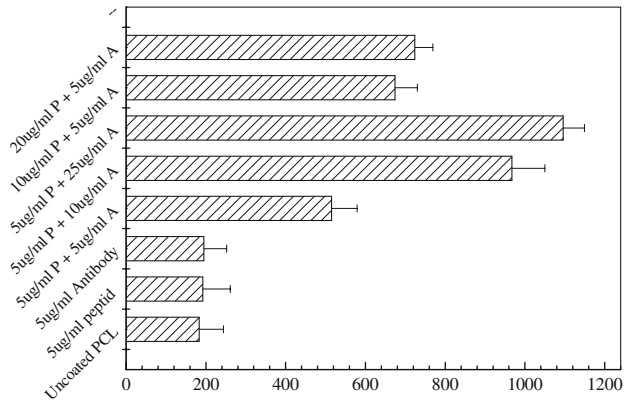

Fig. 2 Fluorescent intensities on PCLS with different modifications, where "P" represents the polypeptide and "A" represents anti-CD34 antibody

antibodies immobilized and the results are shown in Fig. 2. It can be seen that PCLS and polypeptide-coated PCLS sent faint fluorescence. The fluorescence intensity of the antibody-coated PCLS was almost equivalent to that on PCLS, indicating that antibody was hardly directly immobilized onto PCLS or may be easily washed out by this directly adsorption method. While the fluorescent intensities on the polypeptide/anti-CD34 antibody-coated specimens were much higher than that on the antibody-coated PCLS, suggesting that the immobilization efficiency of antibodies onto PCLS was enhanced with the aid of adhesive polypeptide. Furthermore, the fluorescence intensities slightly rose with the increasing of the polypeptide concentration. But at a fixed polypeptide concentration, the fluorescence densities were greatly improved when the concentration of antibody was increased from 5 to 25 μg/ml, a typical concentration-dependent manner.

3.3 Attachment of ECs on modified PCLS

To achieve optimal attachment of ECs, the concentrations of polypeptide and anti-CD34 antibody were first optimized according to the attachment of ECs to the PCLS. Figure 3 shows the effect of the concentrations of polypeptide at the fixed anti-CD34 antibody concentration. It can be seen that compared with the untreated control, the cells attached on the modified substrates increased approximately 14, 30 and 35% when the concentration of polypeptide was 1, 5 and 10 μg/ml, respectively. Further increasing of the polypeptide concentration to more than 10 μg/ml caused obvious decrease of the cells attachment.

Figure 4 shows the dependence of cells attachment on the anti-CD34 antibody concentrations. It can be seen that the cells attachment increased with the increasing of anti-CD34 antibody concentrations up to 10 μg/ml, and then slightly enhanced or remained constant afterwards. These results led to the selection of a polypeptide concentration 10 μg/ml and anti-CD34 antibody concentration of 10 μg/ml for all subsequent experiments unless otherwise noticed.

The attachments of ECs on PCLS modified at optimal conditions were evaluated. For comparison, PCLS were also coated with dBSA only. Cells were cultured for 1.5 h and the bound cells were counted with haemacytometer. The results showed that the number of cells attached to dBSA- or anti-CD34 antibody-coated PCLS were almost equivalent to that on PCLS (Fig. 5). It is obvious that when PCLS was modified with the adhesive polypeptide, the cells attachment significantly increased. It is also clear that compared with the cells attached on PCLS modified with polypeptide or antibody coating alone, a further enhanced attachment was noticed on PCLS sequentially coated with polypeptide and anti-CD34 antibody.

The morphologies of ECs after 1.5 h incubation on PCLS before or after modification were visualized (Fig. 6).

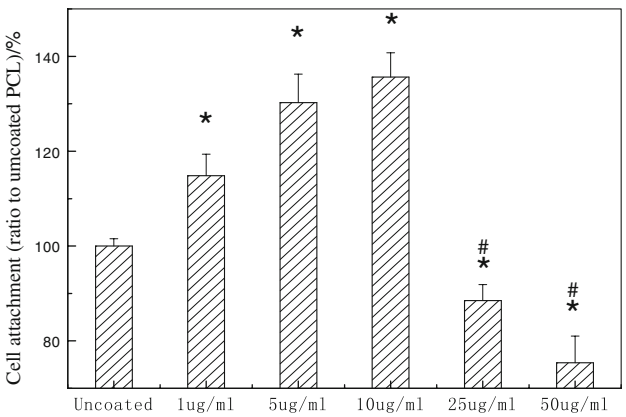

Fig. 3 Effect of polypeptide concentrations on the attachment of ECs. The concentration of anti-CD34 antibody was 5 μg/ml ($^{*}P < 0.01$ compared with uncoated PCL and $^{\#}P < 0.01$ compared with uncoated PCLS)

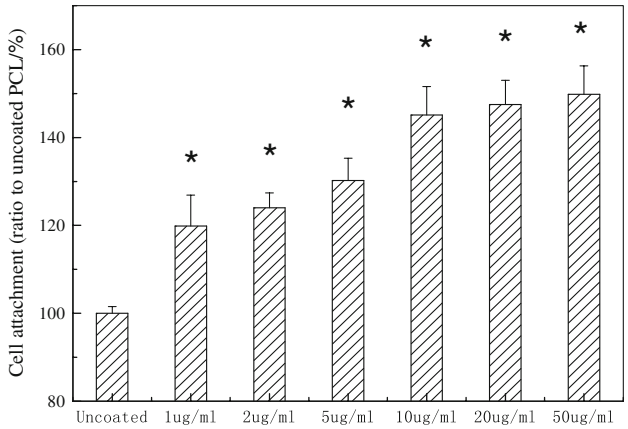

Fig. 4 Effect of antibody concentrations on the attachment of ECs. The concentration of polypeptide was 5 μg/ml (*$P < 0.01$ compared with PCLS)

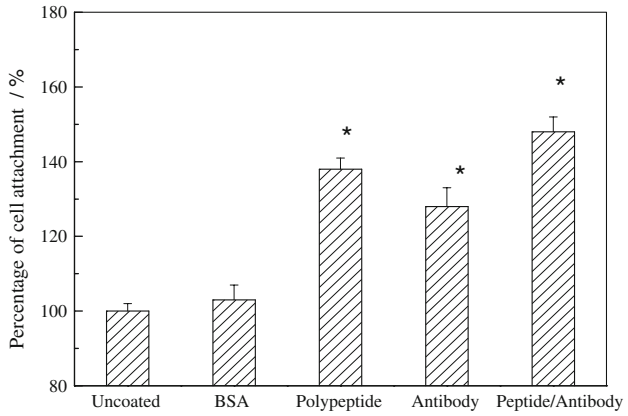

Fig. 5 ECs attachment on PCLS with different modification methods. The concentrations of polypeptide and antibody were all fixed at 10 μg/ml. (*$P < 0.01$ compared with the PCLS)

As we can see, few cells were attached to the surface of PCLS (Fig. 6a). After modified with polypeptide- or/and anti-CD34 antibody, the number of the cells on PCLS was increased (Fig. 6b, c). However, ECs could not spread well following a round-shaped morphology on the PCLS and the polypeptide-coated PCLS (Fig. 6a, b). In contrast, on the anti-CD34 containing PCLS, in particular on the PCLS simultaneously coated by polypeptide and anti-CD34 antibody, the ECs showed the typical cobblestone-like appearance. These results showed that polypeptide was able to improve the attachment of cells but not in favor of the spreading of cells.

3.4 The growth of ECs on the PCLS

The growth of ECs on the modified PCLS was further investigated. As shown in Fig. 7, compared with that on the corresponding control groups, the cells on the polypeptide-coated PCLS were not changed obviously, and the cells grown on PCLS modified with anti-CD34 antibody and polypeptide/anti-CD34 antibody were all significantly increased after 1-, 2- and 3-day culture. In particular when the PCLS was modified with polypeptide and anti-CD34 antibody simultaneously, 1.28-, 1.72- and 2.0-fold increases were observed after 1-, 2- and 3-day incubation, respectively. This showed that the antibody along with the polypeptide coating can greatly accelerate the growth of ECs.

The viabilities measurement of ECs grown on PCLS showed that the viability of ECs grown on antibody-coated surface was almost equivalent to that on uncoated PCL (Fig. 8). The cell viability on polypeptide and sequential polypeptide/anti-CD34 antibody coated PCLS was slightly improved. This result demonstrated that the surface modification methods developed here would not depress the metabolic viabilities of ECs.

3.5 Attachment and growth of EPCs on the PCLS modified under optimal conditions

Figures 9 and 10 presented the attachment and growth of the EPCs on the PCLS, polypeptide coated PCLS, anti-CD34 antibody coated PCLS and polypeptide/anti-CD34 antibody coated PCLS, respectively. It can be seen from Fig. 9 that, after 1.5 h culture, compared with the untreated PCLS, the attachment of EPCs on the polypeptide-coated, anti-CD34 antibody coated and polypeptide/anti-CD34 antibody coated PCLS all significantly increased, especially in the case of anti-CD34 antibody coated PCLS and polypeptide/anti-CD34 antibody coated PCLS.

As shown in Fig. 10, there were no obvious differences between the EPCs growth on the control and polypeptide-coated PCLS both at 1 day and at 2 days. But for the antibody-coated and peptide/antibody-coated PCLS, the cell growths were significantly enhanced, and the increasing extents were almost equivalent.

3.6 Retention of ECs and EPCs on the modified PCLS

After 0.0025% trypsin-EDTA treatment, the adhesion rates of ECs and EPCs on the specimens were evaluated. As shown in Fig. 11, in contrast with the control group, the percentage of the cells adhesion of ECs and EPCs were all significantly increased when PCLS was modified with polypeptide, anti-CD34 antibody and polypeptide/anti-CD34 antibody.

3.7 Platelet adhesion on the modified PCLS

Figure 12 shows the platelet adhesion on PCLS and PCLS modified with polypeptide and antibody contacting with PRP for 60 min. It can be found that the platelets were

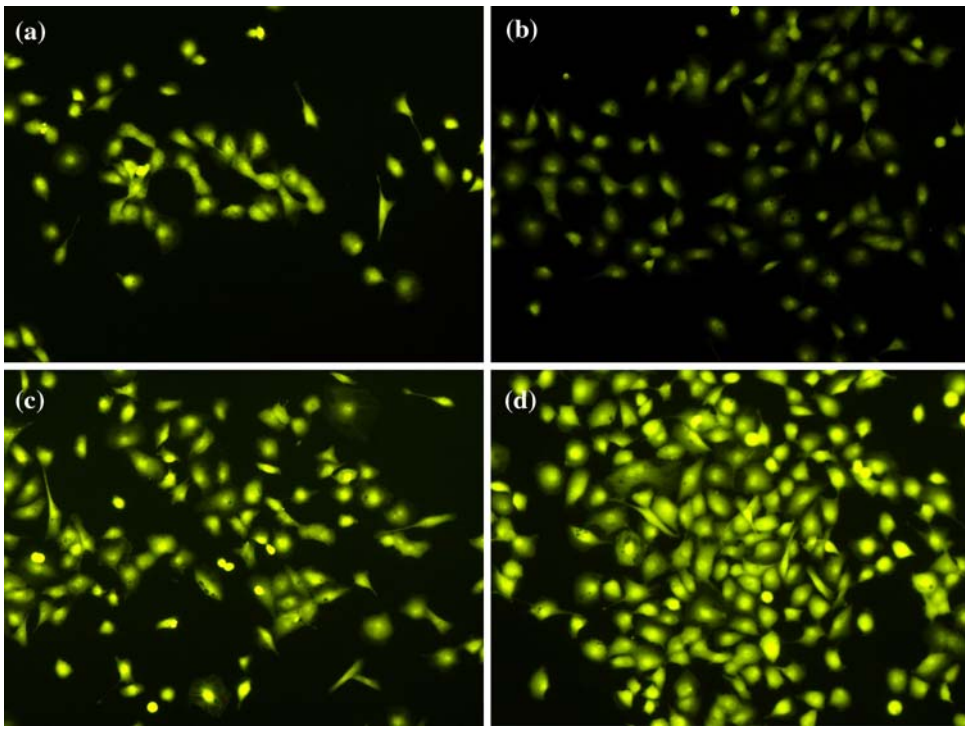

Fig. 6 Cells morphologies (×150) after 1.5 h of culture on PCLS coated with **a** uncoated **b** peptide-coated PCLS **c** anti-CD34-coated PCLS **d** peptide/anti-CD34-coated PCLS

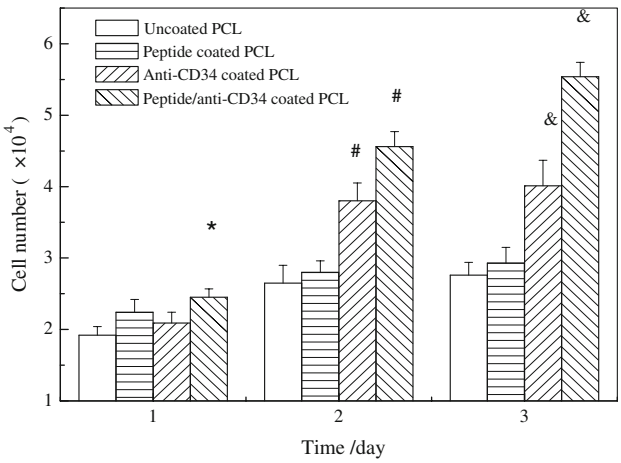

Fig. 7 Growth of ECs on PCL coated with polypeptide or/and antibody. * $P < 0.05$, significant difference compared with the control after 1 day incubation # $P < 0.05$, significant difference compared with the control after 2 day incubation & $P < 0.01$ significant difference compared with the control after 3 day incubation

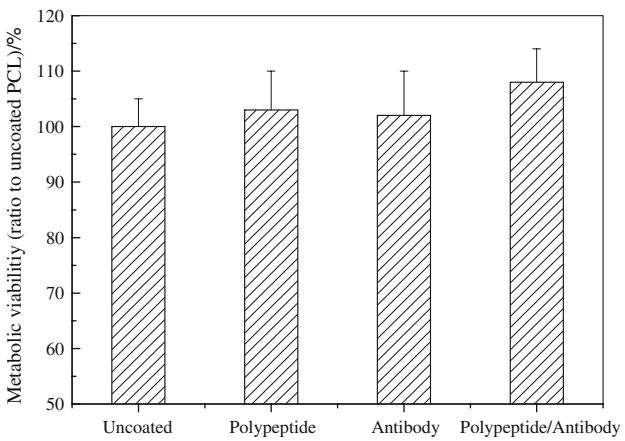

Fig. 8 Metabolic viability of EC grown on PCLS coated with polypeptide or/and anti-CD34 antibody

aggregated on the unmodified PCLS,while the platelets decreased on the PCLS treated by polypeptide/antibody and the platelets were not aggregated. Platelets play a major role in thrombus formation. Activated platelets would release several mitogens and chemotactic and stimulate SMCs migration and proliferation to the injury site and finally lead to the in-stent restenosis. By surface modification of PCLS with polypeptide and anti-CD34 antibody, the thrombogenicity was declined and the blood compatibility was improved.

4 Discussion

In situ rapid re-endothelialization, re-endothelium-paving or relining at denudated regions after stents implantation has been proposed and attempted by using a number of techniques in recent years [6, 7]. These strategies are all based on the coating of stent surface with a ECs-compatible bioactive protein layer, which can stimulate the attachment and proliferation of the surrounding mature ECs and the circulating EPCs to stents' surface, and thus accelerate the formation of an active ECs layer after prostheses implantation. So, the key of rapid re-endothelialization of stents is to make it suitable

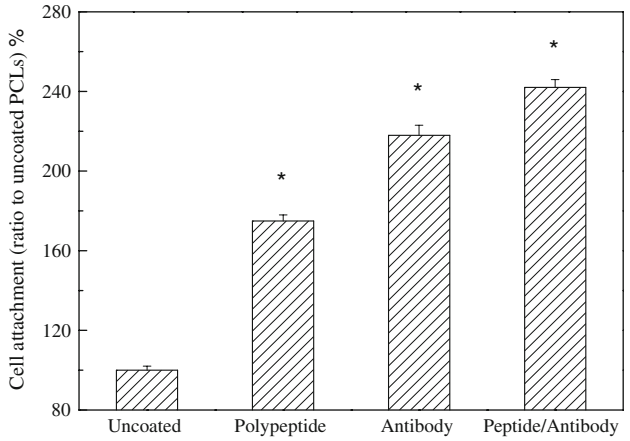

Fig. 9 EPCs attachment on PCLS with different modification methods after 1.5 h incubation. The concentrations of polypeptide and antibody were all fixed at 10 μg/ml. (* $P < 0.01$ compared with the PCL-coated substrate)

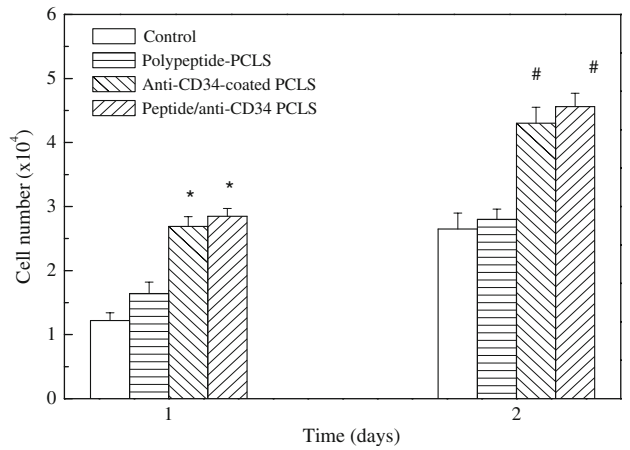

Fig. 10 EPCs growth on PCLS coated with polypeptide or/and antibody. * $P < 0.05$, significant difference compared with the control after 1 day incubation # $P < 0.05$, significant difference compared with the control after 2 day incubation

for the attachment and proliferation of ECs and EPCs by surface modification.

In this study, a combinatorial coating of polypeptide and anti-CD34 antibody was constructed onto cardiovascular devices to realize the re-endothelialization. The polypeptide containing dihydroxyphenylalanine and L-lysine was synthesized by open-opening polymerization in our lab. Dihydroxyphenylalanine, as the only essential amino acid participating in the adhesion of marine organisms, was chosen as the backbone of the polypeptide. L-lysine with the positive charges, which can not only advantageously attract and adsorb the cells that carried negative charge, but also can promote the cells growth [25], was also introduced into the polypeptide. To effectively immobilize polypeptide and anti-CD34 antibody, PCL was first spin-coated onto the 316LSS as an intermediate.

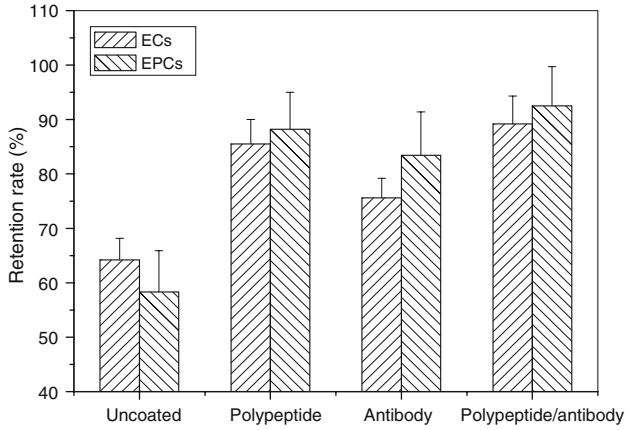

Fig. 11 Adhesion rates of ECs and EPCs on PCLS with polypeptide or/and antibody. (* $P < 0.01$ compared with the ECs adhesion rate on control, # $P < 0.01$ compared with the EPCs adhesion rate on control)

Initially, we have tried to develop the polypeptide- or anti-CD34 antibody-coated PCLS. But on the one hand, the directly adsorbed antibody layers on the surface were easily removed, especially by high shear forces once modified stent was contacted with blood flow, since the surface of PCLS is hydrophobic and does not have any physiological activities. From Fig. 2 we can see that hardly any antibodies were immobilized onto PCLS when the antibody was coated alone with this adsorption method. On the other hand, the spreading of cells was inhibited (Fig. 6b) on the polypeptide-coated PCLS, even at low concentration (5 μg/ml). We hypothesized that two reasons may restrict the spreading of ECs when coated with polypeptide alone: the first is that the polypeptide is too viscous and the attached cells were difficult to expand on it. The second reason maybe that the synthesized polypeptide contains the remnants of excessive HBr. So, in this study, we attempted to develop the polypeptide/anti-CD34 antibody-coated PCLS to accelerate the re-endothelialization of stents via the combinatory effects.

ELISA assay results demonstrated that compared with the polypeptide- or anti-CD34 antibody- coated PCLS, the fluorescence intensities on the polypeptide/anti-CD34 antibody-coated PCLS surface were greatly increased, implying that more antibodies were immobilized onto PCLS. The variation of polypeptide concentrations did not contribute to the immobilization of antibody significantly. We think that since the polypeptide has strong adhesive properties, it was easily reached adsorption saturation, even at a relative lower concentration in a short time. If only the concentration of antibody was assured, the amount of deposited antibody to the substrates will not change obviously. But for the anti-CD34 antibody, the fluorescence intensities were greatly improved with the increase of antibody concentrations in the range from 5 to 25 μg/ml.

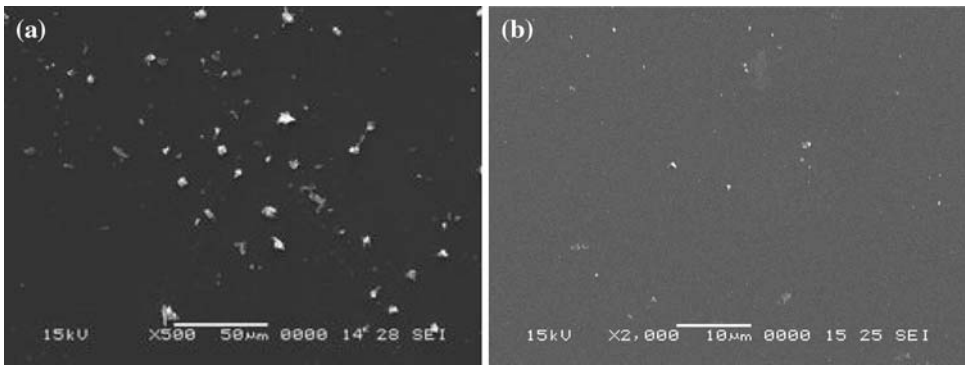

Fig. 12 SEM images of the coating surfaces contacting with PRP for 60 min **a** PCLS, **b** polypeptide-antibody-PCLS

As an ideal vascular prosthesis suitable for in vivo endothelialization, the materials should prevent unspecific proteins adsorption effectively. So, in this study, the unspecific proteins adsorption was assessed with the rhodamine-labeled streptavidin as model. The results indicated that the commonly used BSA blocking could inhibit the nonspecific protein adsorption.

To obtain the optimal cell attachment, we first investigated the effect of the concentrations of peptide and anti-CD34 antibody on the ECs attachment. The results demonstrated the polypeptide/anti-CD34 antibody-coated PCLS significantly enhanced the attachment of ECs within the polypeptide concentrations of <10 μg/ml. This result is similar to Sawyer's [26], where they evaluated the attachment and spreading of mesenchymal stem cells on the hydroxyapatite coated with RGD peptide and serum proteins, and found that RGD/FBS coatings stimulated cells attachment and spreading compared to either coating alone, but only at low RGD coating concentrations. In this study, we think that excessive adhesive polypeptide may limit the activities of antibody for bounding too tightly; on the other hand, excessive polypeptide may introduce more remnants of HBr, which, in turn, may restrain the attachment of ECs. The antibody, which consists of two F_{ab} (antigen binding) and one F_c (constant region) fragments, should be immobilized by the F_c fragment, allowing the Fab fragments to be sterically unrestricted and hence to available for antigen binding. So, how to achieve maximum immobilization amounts while avoid losing of bioactivities is the key for the antibodies immobilization [27]. ELISA assay confirmed that more antibodies were immobilized onto PCLS with the increase of antibody concentrations as discussed. However, the ECs attachment is not necessarily "proportional" to the amount of adsorbed antibody. The antibody may be bound too tightly to the surface with a higher molecular density and lost activities. So, as the antibody concentration further increased to 10 μg/ml, the attachment of cells became almost equivalent, as shown in Fig. 4.

After the optimal conditions for peptide and anti-CD34 antibody immobilization was determined, the cells attachment and cells growth of the ECs and EPCs on the PCLS with different modification methods were compared and found that the cells attachment, growth as well as cells retention on the PCLS modified by peptide/anti-CD34 antibody was greatly enhanced, especially for the EPCs cells. The noticeable differences with regard to cells attachment and cells growth on the PCLS modified by peptide or anti-CD34 antibody alone between ECs and EPCs arouse our attentions. Figures 5 and 7 indicated that the improvement of ECs attachment can be attributed to polypeptide coating and the improvement of the ECs growth was mainly the contribution of the anti-CD34 antibody coating. But for the EPCs (Figs. 9 and 10), both the polypeptide coating and anti-CD34 antibody coating are all responsible for the improved cell attachment. That is to say, the anti-CD34 antibody coating can contribute to the attachment of EPCs, but not to ECs. We believe that these phenomena might be associated with the characterizations of ECs and EPCs. It is well-accepted that EPCs are CD34 positive in nature [28]. From this viewpoint, the anti-CD34 antibody immobilized on the PCLS can specifically target the EPCs, but not the ECs. On the other hand, EPCs are proved to have much higher proliferative potential than mature ECs [29]. So, the polypeptide/anti-CD34 antibody combinatorial coating is more suitable for the enhanced attachment, growth and adhesion of EPCs.

As a blood-contacting biomedical device, the stent surface should prevent the thrombus formation. In general, when materials contact with blood, proteins are first adsorbed instantaneously onto surfaces and deformed, then platelets are adsorbed, activated and aggregated, so platelets play a major role in the initial thrombus formation. Therefore, a study on platelet adhesion is the first step to evaluate the blood compatibility of materials. Bare 316LSS is thrombogenic and is easy to initiate platelet adhesion and activation. The platelet adhesion, however, after polypeptide and anti-CD34 antibody modification, was greatly reduced, indicative of the improvement of blood compatibility.

Taken together, a novel combinatorial coating of mussel adhesive polypeptide and anti-CD34 antibody was

developed on the vascular stent to accelerate the re-endothelialization. Polypeptide and anti-CD34 antibody, although, have been used to modify the vascular stent. The combination coating of this mussel adhesive polypeptide and anti-CD34 antibody has never been reported for the rapid re-endothelialization. The results showed that the mussel adhesive polypeptide coating can significantly enhance the attachment of ECs and EPCs. This method developed here is simple but very useful. Bioactive molecules such as antibodies are able to be immobilized by this adsorption method, which achieves goodish results while keep the bulk properties of the substrates. No matter what the really mechanisms between the immobilized antibody and ECs and EPCs are, selective binding with ECs antigens or other mechanisms, though the investigation at the molecular and cellular levels are under taken, the attachment and growth of ECs and EPCs were greatly accelerated. The method could thus be used as a way to modify the surfaces of vascular stents or be used in tissue engineering applications.

5 Conclusion

To accelerate the re-endothelializaiton of cardiovascular stent, synthesized mussel adhesive polypeptide mimics and anti-CD34 antibody were coated using a novel adsorption method with PCL as intermediate coating. As PCLS were pre-coated with polypeptide (<10 μg/ml) and over-coated with antibody, the attachment, growth and retention of ECs and EPCs on PCLS were both significantly improved, and the viabilities of ECs grown on the substrates were not affected by this modification method. The method could thus be used as a way to modify the surfaces of vascular stents.

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