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Abstract

Efficient immobilization of the antibody to the substrate is of crucial importance in the development of anti-CD34-based endothelial progenitor cells capturing cardiovascular devices. This should go along with precise control of the antibody orientation by appropriate immobilization technology for retaining antibody activity, like in immunosensors. Recently, great attention was paid to immobilization of anti-CD34 antibody onto substrates by covalent binding, but at random orientation. Here, to investigate the biological effect of antibody orientation, we have prepared two kinds of anti-CD34 antibody coated surfaces, with random immobilization and oriented immobilization. The immunological binding activity (IBA) of the antibody at oriented immobilization was 3.48 times higher than at random immobilization, indicating that the two different surfaces were successfully prepared. The endothelial progenitor cell-capturing capability of oriented antibody-immobilized surface was 1.35 and 1.64 times higher than for the random immobilized surface after seeding for 2 and 12 h under flow condition, respectively. The endothelial progenitor cell-capturing efficiency per antibody by oriented immobilization was 5.16 and 6.26 times higher than for the random after seeding for 2 and 12 h under flow condition, respectively. In addition, the oriented antibody-immobilized surface possessed better blood-compatibility. These results clearly revealed the significance of antibody orientation which could retain its biological effect and may revolutionize the antibody-immobilization protocols used in cardiovascular and other blood-contacting biomedical devices.

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Keywords

Endothelial progenitor cells, anti-CD34 antibody, random orientation, oriented immobilization, cardiovascular device

Introduction

Many cardiovascular and other blood-contacting biomedical devices have been widely applied, such as vascular stents, vascular grafts, artificial heart valves, and blood pumps,¹ and contribute significantly to the quality and effectiveness of the healthcare system. However, in the environment of blood, complications such as blood clotting and thrombus formation or, in the case of vascular stents, proliferation of smooth muscle cells (SMCs) and restenosis frequently lead to the failure of the device.² In vivo/in situ endothelialization soon after device implantation has been shown to be among the main factors in the prevention of restenosis and stent thrombosis.³

Since Asahara et al. first discovered endothelial progenitor cells (EPCs) in 1997, revolutionary new perspectives for rapid in vivo/in situ endothelialization by capturing endogenous circulating EPCs from the blood have been investigated.^{4–11} For example, capturing EPCs in situ via coating of anti-CD34 antibodies on vascular grafts or stents have been reported with promising results to demonstrate the efficacy of an EPC-capturing strategy.

Anti-CD34 antibodies have been immobilized on the surface of biomedical materials in random orientation mainly by nonspecific physical adsorption⁴ or by formation of covalent bonds between functional groups on protein molecules (e.g. $-\text{NH}_2$) and complementary coupling groups (e.g. aldehydes or $-\text{COOH}$) introduced on the solid surfaces.^{6–10} These methods, however, require chemical modification of the surface, and it is not possible to control the molecular orientation of the immobilized antibody, so the EPC-capturing capacity (EPC-CC) of the surface, determined by the number of adherent EPC, did not raise significantly in some studies.^{6,9}

When antibodies are immobilized on a solid surface, their IBA, which is the affinity of the antigen-binding site (F(ab') fragments) of an antibody for its binding partner (antigen),¹² usually decreases. The study of immunological binding analysis revealed that oriented immobilization of antibodies had antigen–antibody reaction equilibrium dissociation constants (Kd) as low as 8.6×10^{-10} mol/L, whereas randomly immobilized antibodies showed Kd values of 2.0×10^{-7} mol/L. Strict control of antibody orientation not only formed in average an approximately 100-fold stronger antigen–antibody complex than the random immobilization but also sustained the native antibody Kd (10^{-10} – 10^{-9} mol/L). These findings support the significance of antibody orientation, because controlling the orientation results in high IBA.¹² The reduced IBA for the randomly oriented antibody is ascribed to steric hindrance of the antibody on the solid phase.¹² Another plausible reason is partial denaturation of the antibody molecules by interaction with the solid surface.¹³ Therefore, it is very important to elaborate immobilization protocols to control the antibody orientation.

Staphylococcal protein A (SPA) is a 42-kDa cell wall protein of the bacterium *Staphylococcus aureus* that binds specifically to many mammalian immunoglobulins, most notably immunoglobulin G (IgG), via the Fc fragment.¹² The IBA of antibody linking with SPA is not lowered because the antigen-binding sites of antibody are located on the distal ends of the F(ab') variable regions.¹⁴ Thus, SPA has been widely used for oriented antibody immobilization to retain their IBA in immunosensors.¹⁵ Recently, oriented immobilization of anti-CD34 antibody on biomaterials through SPA was described by our group and this coating had a good EPC-CC under flow conditions.¹⁶

Until now, anti-CD34 antibodies have been widely immobilized on biomedical material surfaces, but only EPC-CC of these CD34-modified surfaces was evaluated. Although the theory of

Figure 1. Ideal reaction scheme for random and oriented immobilization of anti-CD34 antibodies.

IBA of immobilized antibody depending on the antibody orientation is widely accepted in the field of immunosensors, it seldom has been transferred to biomedical materials.¹² Therefore, it is reasonable to study the relationship between antibody orientation (random immobilization versus oriented immobilization) and IBA of anti-CD34 antibody immobilized on the surfaces.

The EPC-CC of these CD34-modified surfaces in the so far reported studies was only evaluated in a static incubation system (cells settle on the substrates), which is different from the physiological condition. A flowing system, in which cells flow over the substrates, is closest to the physiological condition in the vasculature.¹⁷ This motivates to study the difference of EPC-CC between static system and flow system.

The aim of this study is to investigate the significance of antibody orientation by IBA and EPC-capturing efficiency (EPC-CE, which is EPC-CC per antibody) of immobilized antibodies in static system and flow system. Here, IBA, EPC-CC and EPC-CE were calculated with the following formulas. Assessing the significance of antibody orientation requires strict and elaborate methods of orientation control, so we used two methods to immobilize antibodies (Figure 1): (1) random immobilization (SS-DA-CD34): covalent immobilization of anti-CD34 antibody onto a dopamine (DA)-modified surface. DA contains high concentrations of catechol and amine functional groups, which are capable of mediating protein immobilization to most organic and inorganic surfaces.^{18–21} (2) Oriented immobilization (SS-DA-SPA-CD34): affinity immobilization of anti-CD34 antibody via SPA covalently conjugated onto the DA-modified surface according to a previously described method.¹⁶ The antibody orientation was assessed by the IBA of the immobilized antibodies, assuming that oriented antibodies possess higher binding capacity. Additionally, EPC-CC of the CD34-modified surfaces and EPC-CE of immobilized antibodies were estimated during static incubation and in a flow system. Anti-CD34 antibody with oriented immobilization exhibited much higher retention of IBA and EPC-CE than random immobilization. The advantage of oriented immobilized antibodies on EPC-CE was especially pronounced under flow condition compared to the static incubation. In addition, the surface with oriented antibody immobilization exhibited much better blood-compatibility than the random immobilization. These results clearly reveal the significance of antibody orientation and may revolutionize the antibody-immobilized protocols used in cardiovascular and other blood-contacting biomedical devices

$$\text{IBA} = \frac{\text{The amount of F(ab') fragment exposure on the surface}}{\text{The mass of antibody on the surface}} \quad (1)$$

$$\text{EPC-CC} = \frac{\text{The number of EPC adherent on the surface}}{\text{Surface area of the sample}} \quad (2)$$

$$\text{EPC-CE} = \frac{\text{EPC-CC}}{\text{The mass of antibody on the surface}} \quad (3)$$

Materials and methods

Materials

Dopamine hydrochloride, Tris–HCl buffer base, SPA, human fibrinogen, and rabbit anti-goat IgG F(ab')₂-peroxidase antibody were obtained from Sigma–Aldrich (St. Louis, MO, USA). Polyclonal goat anti-CD34 IgG (anti-CD34 antibody for detection of CD34 of mouse, rat, and human origin) and goat anti-human fibrinogen IgG horseradish peroxidase (HRP)-conjugated antibody were, respectively, purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Beijing Biosynthesis Biotechnology Co., Ltd (Beijing, China). Monoclonal mouse anti-human P-selectin antibody and monoclonal sheep anti-mouse IgG Cy3-conjugated antibody were purchased from BD Biosciences (San Jose, CA, USA). All the other reagents used in the experiments were of the highest analytical purity (>99.9%).

Method

Preparation and characterization of the modified surface

Preparation of anti-CD34 antibody–modified surfaces. 316L stainless steel (SS) disks were polished to a reflective mirror-like finish. The samples were ultrasonically cleaned sequentially in a detergent solution, acetone, ethanol, and finally in ultrapure water. After cleaning, the samples were immersed in a 2 mg/mL solution of DA (in 10 mM Tris buffer, pH 8.5) for about 24 h at room temperature (RT) in the dark. Then, the samples were sonicated for 10 min in ultrapure water (three times) to remove the nonattached DA; the samples are denoted as SS-DA. The DA-coated samples were then placed in a 24-well plate and incubated with a 300-μL aqueous solution of SPA (5 μg/mL) or anti-CD34 antibody (0.1 μg/mL) at 37°C overnight, respectively, and rinsed with ultrapure water; the samples are denoted as SS-DA-SPA and SS-DA-CD34 (random immobilization), respectively. The samples of SS-DA-SPA were then placed in a 24-well plate and incubated with a 300-μL aqueous solution of polyclonal goat anti-human CD34 IgG (0.1 μg/mL) at 37°C overnight, rinsed with ultrapure water; the samples are denoted as SS-DA-SPA-CD34 (oriented immobilization).

Characterization of anti-CD34 antibody–modified surfaces. The surface composition of the samples was analyzed by X-ray photoelectron spectroscopy (XPS; PerkinElmer, Norwalk, CT, USA) with an Al Kα X-ray source (1486.6 eV photons). A wide-scan survey spectrum over a binding energy (BE) range of 0–1400 eV was recorded at pass energy of 80 eV for estimation of the chemical elemental composition and 10 eV for high-resolution detailed scans. The system was calibrated using the C 1s peak at 284.8 eV. All spectra were recorded at a take-off angle of 15°. The maximum information depth of the XPS method is not more than 10 nm. In order to determine the quantitative surface composition from XPS data, spectrum background was subtracted according to the Shirley method. The parameters of the component peak fitting were their BE, height, full width at half maximum, and the Gaussian–Lorentzian ratio.

Quartz crystal microbalance-dissipation (QCM-D) is a useful technique for evaluation of surface-related processes in liquids, including protein adsorption. The mass (Δm) of the molecules adsorbed on the surface of the quartz crystal is measured via the changes in the resonance frequency (Δf). Here, the mass density of anti-CD34 antibody on the different surfaces was measured as follows: prior to the test, DA was coated on an Au-coated quartz crystal (diameter of the Au films: 10 mm) by immersing the quartz crystal into the DA solution (2 mg/mL) in Tris buffer (pH 8.5) for 24 h. Then, the obtained DA-coated quartz crystal was settled in the chamber of QCM-D (Q-Sense AB, Gothenburg, Sweden) and ultrapure water was injected continuously at a rate of 100 μ L/min until the QCM traces reached steady state. Subsequently, aqueous solutions of SPA (5 μ g/mL) and anti-CD34 antibody (0.1 μ g/mL) were, respectively, injected at 10 μ L/min until no variation appeared in the adsorption curves, and then rinsed with ultrapure water at 100 μ L/min. Aqueous solution of anti-CD34 antibody (0.1 μ g/mL) was injected at 10 μ L/min for building up the coating on the quartz crystal surface modified with SPA until no variation appeared in the adsorption curves, and then rinsed with ultrapure water at 100 μ L/min for 20 min.

For evaluating the stability, the coating was rinsed with ultrapure water at 500 μ L/min for 12 h. Measurements were all conducted at a temperature of 37°C. Q-Tools software (Q-Sense AB) was used to analyze the QCM data and to extract quantitative parameters of the adsorption of each component on the surface of the quartz crystal at fifth overtone of the fundamental resonant frequency.

The amount of F(ab') fragment but not Fc fragment exposure of anti-CD34 antibody on the surfaces was determined by enzyme-linked immunosorbent assay (ELISA) as follows: (1) samples were immersed in 1% sheep serum for 60 min to block non-specific adsorption, and then the solution was decanted; (2) 20 μ L of rabbit peroxidase-conjugated anti-goat IgG F(ab')₂ antibody (1:100) was added to the surface of the samples, incubated for 1 h at 37°C, and subsequently washed with phosphate buffered saline (PBS) for 5 min three times; (3) 140 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic solution was added into the well containing the samples and reacted for 10 min; subsequently, 200 μ L of 2M H₂SO₄ was added to stop the reaction; and (4) 150 μ L of the reacting solution was transferred into a 96-well plate and was measured at 452 nm.

Water contact angles (WCAs) were measured with DSA100 drop shape analysis system (DSA100; Krüss, Hamburg, Germany) using ultrapure water at RT. The droplet volume was kept at 0.5 μ L and each measurement was taken 20 s after dispensing. Five samples were measured of each group, and two separate measurements were made on each sample.

In vitro testing of blood-compatibility

Quantification of adsorbed fibrinogen on the material surface. Fibrinogen is one of the most abundant plasma proteins (4%) and plays an important role in thrombosis. Here, the exact mass of adsorbed fibrinogen on the material surface was measured by QCM-D as follows: after testing about the stability of the CD34-modified surfaces, aqueous solution of human fibrinogen (1.5 mg/mL) was injected at 10 μ L/min until no variation appeared in the adsorption curves, and then rinsed with ultrapure water at 500 μ L/min until no variation appeared. The mass density of adsorbed fibrinogen at fifth overtone of the fundamental resonant frequency was analyzed by Q-Tools software.

For imitating the physiological condition, the samples were incubated with platelet-poor plasma (PPP) for 30 min at 37°C, and then the relative amounts of adsorbed fibrinogen on the material surfaces were measured by direct immunochemistry using goat anti-human fibrinogen IgG HRP-conjugated antibody. The description of the procedure is presented in detail elsewhere.⁶ Data are normalized to the fibrinogen adsorption to bare 316L SS as 100%.

Platelet adhesion testing. Fifty microliters of fresh platelet-rich plasma (PRP) was placed onto the sample surface and kept in contact with the surface for 2 h at 37°C. After incubation, the samples were gently rinsed in PBS to remove loosely adherent platelets from the surface. Then the number of adherent platelets was determined by measuring the lactate dehydrogenase (LDH) activity of the cells lysed with Triton X-100. A linear relationship was then established between the LDH activity of the aliquots of the cell suspension thus obtained and the number of platelets was counted using a hemocytometer. The LDH activity was determined by measuring the initial rate of nicotinamide adenine dinucleotide (NADH) oxidation in the presence of pyruvate.²² The procedure was as follows: the adherent platelets were lysed by the addition of 40 μ L of 0.1% Triton X-100 to the sample. After 5 min of incubation at RT, 25 μ L of each lysate was collected and mixed with 200 μ L reagent solution containing 297 μ L of 10 mg/mL reduced NADH, 187 μ L of 10 mg/mL pyruvate, and 10 mL of tris(hydroxymethyl) aminomethane buffer, pH 7.2. The LDH activity in the lysate was then determined by recording the decrease in absorbance at 340 nm using a microplate photometer.

Platelet activation: P-selectin detection. Surface-induced platelet activation was measured by immunofluorescence for P-selectin expressing on the surface of activated platelets.²³ The procedure was as follows: (1) samples with adherent platelets were fixed with 2% glutaraldehyde in PBS for 1 h and subsequently washed with PBS for 5 min; (2) 1 mL of 1% sheep serum was added into each well and incubated for 30 min at 37°C to block nonspecific adsorption; (3) 30 μ L of monoclonal mouse anti-human P-selectin antibody (1:100) was added onto the sample surfaces, incubated for 2 h at 37°C, and subsequently washed three times with PBS for 5 min; (4) monoclonal sheep anti-mouse IgG Cy3-conjugated antibody(1:100) was added to the sample surfaces, incubated for 1 h at 37°C, and subsequently washed with PBS for 5 min three times; and (5) the samples were immediately examined with fluorescence microscope with the binning 4 $\times$ 4 and the exposure of 15 s (Leica DMRX Polarization microscope; Leica, Wetzlar, Germany).

In vitro testing of EPC-compatibility and SMC-compatibility in static and flow condition

Isolation and culture of EPCs and SMCs. As previously described, mononuclear cells were isolated by density gradient centrifugation (1800 r/min, 20 min) from the bone marrow of the femur of an SD rat. Mononuclear cells without further purification steps were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY, USA) culture medium containing 20 % newborn calf serum, 10 ng/mL vascular endothelial growth factor (VEGF), 10 ng/mL stem cell factor (SCF), penicillin (100 U/mL), and streptomycin sulfate (100 U/mL) at 37°C, 5% CO₂. Cells were fed with fresh medium every third day and subcultured regularly after the adherent cells reached about 80% confluence. After 2–3 weeks culturing under VEGF condition, primary mononuclear cells differentiate into EPCs.²⁴ Human umbilical artery SMCs were isolated from newborn umbilical cords as described previously.²⁵ Cells in passages 2–5 were used. The SMCs were cultured in DMEM culture medium containing 10% newborn calf serum, penicillin (100 U/mL), and streptomycin sulfate (100 U/mL) at 37°C under 5% CO₂. Cells were fed with fresh medium every third day, and subcultured regularly after the adherent cells reached about 80% confluence. EPC and SMC were fixed and double-stained for EPC markers with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 antibody; cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

Cell attachment of the modified surfaces in flow conditions in vitro. The flow chamber system consisted of four basic modules: a parallel plate flow chamber, a flow loop, a reservoir, and a roller pump. The loop linked the flow chamber and reservoir. The speed of the liquid was controlled by

the roller pump. Different samples were positioned at the bottom of a parallel plate flow chamber. EPCs or SMCs were re-suspended in DMEM medium containing 10% fetal calf serum and 50 mL of suspensions of EPCs or SMCs at a density of $1 \times 10^6/\text{mL}$ was perfused through the flow chamber at a shear rate of 1.0 Pa, similar to a small arterial flow. The parallel wall flow chamber was placed into a cell incubator (at 37°C and 5% CO₂). After 2 or 12 h incubation, the samples were gently rinsed in PBS to remove loosely adherent cells from the surface. Then, the attached cells onto the samples were determined by an MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and inspected after actin expression using immunofluorescence staining.¹⁶

Cell attachment of the modified surfaces in static conditions in vitro. Different samples were placed into the wells of a 24-well flat-bottom plate. EPCs or SMCs were re-suspended in DMEM medium containing 10% fetal calf serum. One milliliter of cell suspension at a density of $1 \times 10^4/\text{mL}$ was added into the well, and then the plate was put into a cell incubator (at 37°C and 5% CO₂). After 2 or 12 h incubation, the samples were gently rinsed in PBS to remove loosely adherent cells from the surface. Then, the attached cells on the samples were determined by an MTT assay and inspected after actin expression using immunofluorescence staining.¹⁶

Statistics. All experiments were performed at least three independent times. All data were compared with one-way analysis of variance (ANOVA) to evaluate statistical significance using SPSS software. Tukey multiple comparisons' test was performed as post hoc test to find significant differences between pairs. A probability value less than 0.05 was considered statistically significant. In the figures, statistically significant differences ($p < 0.05$) are denoted with *.

Ethics statement

Fresh human whole blood and newborn umbilical cords were obtained from healthy volunteers after informed consent. All animals or human subjects experiments used protocols that were approved by Anhui Medical University (Protocol Number: 20131412).

Results and discussion

The chemical composition of the surfaces at various stages was analyzed by XPS. In order to obtain the detailed chemical composition of each sample, here further investigations of C1s, N1s, and O1s peak fittings were performed and are presented in Figure 2. In the survey spectrum of the SS-DA, the presence of N, which did not exist on the surface of 316L SS (data not presented), indicates that DA was successfully immobilized onto the surface of 316L SS. In parallel, the Fe2p and Cr2p peaks disappeared (data not presented), indicating that the DA had covered the substrate materials completely.

The detailed high-resolution spectra of C1s presented broad peaks which could be curve fitted into four peak components (Figure 2), with BEs at 284.5 eV for the C-C/C-H species, 285.5 eV for the C-N species, 286.6 eV for the C-O species, and 288.1 eV for the C=O species.²⁶ By calculating peak areas, the molar concentration ratios of C=O/C for the SS-DA, SS-DA-SPA, SS-DA-SPA-CD34, and SS-DA-CD34 were 9.9%, 16.3%, 11.0%, and 12.1%, respectively. The O1s core-level spectrum could be resolved into two typical peaks, with BEs at 531.4 eV for the O=C species and 532.9 eV for O-C species (data not presented).²⁷ By calculating peak areas, the molar concentration ratios of O=C/O for the SS-DA, SS-DA-SPA, SS-DA-SPA-CD34, and SS-DA-CD34 were 38.1%, 49.5%, 44.9%, and 47.6%, respectively. The results of C1s and O1s showed that there were more C=O species on the SPA-modified surface (16.3% and 49.5%) than on other

Figure 2. XPS spectra for different surfaces.

surfaces. This analysis proved that more than 40% of the amino acid composition of SPA were Asx (asparagine and aspartate) and Glx (glutamine and glutamate) having two carbonyl groups ($\text{C}=\text{O}$);²⁸ therefore, the highest molar concentration ratios of $\text{C}=\text{O}/\text{C}$ and $\text{O}=\text{C}/\text{O}$ for SS-DA-SPA indicate that SPA was immobilized onto the DA-modified surface. Compared with the SPA-modified surface (16.3% and 49.5%), the molar concentration ratios of $\text{C}=\text{O}/\text{C}$ and $\text{O}=\text{C}/\text{O}$ decreased greatly in the SS-DA-SPA-CD34 (11.0% and 44.9%), confirming the successful immobilization of the antibody onto the SPA-modified surface. Compared with the DA-modified surface (9.9% and 38.1%), the molar concentration ratios of $\text{C}=\text{O}/\text{C}$ and $\text{O}=\text{C}/\text{O}$ increased greatly in the SS-DA-CD34 (12.1% and 47.6%), confirming the successful immobilization of the antibody onto the DA-modified surface. Deconvolution of the N1s core-level spectra was in three peaks at 399.2, 400.1, and 401.1 eV, which were respectively assigned to $\text{C}-\text{NH}_{x=1,2}$ (amine), $\text{N}-\text{C}=\text{O}$ (amide), and ammonium salt $\text{C}-\text{NH}_3^+$. The possible existence of $\text{C}-\text{NH}_3^+$ could be ascribed to a proton transfer from the acid (catechol, $\text{C}-\text{OH}$) to the amine ($\text{C}-\text{NH}_2$).²⁹ By calculating peak areas, the molar concentration ratios of $\text{C}-\text{NH}_x/\text{N}$ for the SS-DA, SS-DA-SPA, SS-DA-SPA-CD34,

Figure 3. Changes in mass density determined by QCM-D measurement.

and SS-DA-CD34 were 76.9%, 42.0%, 60.1%, and 53.3%, respectively. Compared with the SPA-modified surface (42.0%), the molar concentration ratios of $C-NH_x/N$ increased in the SS-DA-SPA-CD34 (60.1%), confirming the successful immobilization of the antibody onto the SPA-modified surface. Compared with the DA-modified surface (76.9%), the molar concentration ratios of $C-NH_x/N$ decreased in the SS-DA-CD34 (53.3%), confirming the successful immobilization of the antibody onto the DA-modified surface. Because $C-NH_{x=1,2}$ (amine) on the antibodies was used to react with phenolic hydroxyl/o-quinone on the DA, the molar concentration ratio of $C-NH_x/N$ on SS-DA-CD34 (53.3%) was lower on SS-DA-SPA-CD34 (60.1%). Thus, XPS analysis confirmed that anti-CD34 antibodies were immobilized onto the DA-modified surface and the SPA-modified surface, respectively.

QCM-D has been applied to monitor the assembly process of each component on the DA-modified quartz crystal surface in real time. The mass density versus time curves (Figure 3) clearly demonstrated the buildup process of the coating. The protein conjugation on the DA surface was based on a simple one-step Schiff base reaction between the quinone groups of DA and amine groups of the protein.²⁹ The mass densities of immobilized SPA and anti-CD34 antibody on the DA-modified surface were ~ 605 and ~ 428 ng/cm², respectively. The anti-CD34 antibody conjugation on the SPA surface was based on bio-specific affinity between Fc receptor on the SPA and the Fc fragment of the antibody.^{12,15} The mass density of immobilized anti-CD34 antibody onto the SPA-modified surface was ~ 112 ng/cm². Because these were the same surface area of quartz crystal, the mass of anti-CD34 antibody by random immobilization (on the DA-modified surface, black line) was 3.7 times higher than by oriented immobilization (on the SPA-modified surface, red line). The number of antibody molecules immobilized onto both these surfaces was in close association with the number of SPA or DA; meanwhile, the number of DA molecules on SS-DA was far higher than the number of SPA on SS-DA-SPA. Thus, there were more antibodies on the DA-modified surface than on the SPA-modified surface.

The stability of the coating was the most important factor of the technique applied and could affect the biocompatibility of the coating.³⁰ So after being prepared, the quartz crystals were rinsed with ultrapure water at high flow velocity of 500 μ L/min for more than 12 h (Figure 3). No significant mass change occurred, indicating that these coatings were very stable and antibody conjugation on both these surfaces was not based on physical adsorption.

The IBA of immobilized antibody depends on the exposure of F(ab') fragment on the surface, so the amount of F(ab') fragment exposure on the surfaces was determined by ELISA using rabbit anti-goat IgG F(ab')₂-peroxidase antibody which specifically reacts with goat IgG F(ab') but

Figure 4. F(ab') fragment of anti-CD34 antibody exposed on the different surfaces determined by ELISA.

Figure 5. Water contact angle of each surface.

not with the Fc fragment. As shown in Figure 4, the optical densities of SS, SS-DA-SPA-CD34, and SS-DA-CD34 were 0.009 ± 0.003 , 0.134 ± 0.004 , and 0.147 ± 0.007 , respectively. According to the optical densities, the amount of F(ab') fragment exposure on oriented antibody-immobilized surface (SS-DA-SPA-CD34) was 0.91 times the amount of the random immobilized surface (SS-DA-CD34).

Rating the amount of accessible F(ab') fragments on the surface (ELISA data) to the antibody mass immobilized on the surfaces (QCM data), the IBA of antibody on SS-DA-SPA-CD34 (oriented immobilization) was 3.48 times higher than on SS-DA-CD34 (random immobilization), indicating that control of antibody orientation retains the antibody activity.^{12,13}

The measurement of the WCA is well known as a useful technique to investigate surface characteristics. WCA of the different surfaces is shown in Figure 5. Compared with 316L SS, the WCA of the other surfaces decreased significantly. Relative to the DA-modified surface, the WCA of SS-DA-CD34 decreased significantly. Compared with the SPA-modified surface, the WCA of SS-DA-SPA-CD34 decreased significantly. These results indirectly indicate that anti-CD34 antibodies were successfully immobilized onto the DA surface and the SPA-modified surface, respectively. This indicated that the antibody immobilization may also be helpful for the enhancement the hydrophilicity. The dominating molecular mechanisms are still not fully understood, but we believe that both the amino acid residue and surface charge might contribute to the two dominating mechanisms.

Figure 6. Relative amount of fibrinogen and platelets on the material surface compared with 316L SS. The results showed that oriented immobilization resists fibrinogen adsorption and platelet adhesion, compared with others.

Figure 7. Representative P-selectin stain images showing activated platelets that are labeled with a red fluorescent dye. The results showed that oriented immobilization prevented platelet activation, compared with others (bar = 20 μ m).

Fibrinogen, one of the most important proteins in the blood coagulation, has several cell adhesion and protein interaction domains. The fibrinogen adsorption was monitored both by QCM-D (Figure 3) and ELISA (Figure 6). As shown in Figure 3, the mass densities of adsorbed fibrinogen on SS-DA-CD34 and SS-DA-SPA-CD34 were ~ 1560 and ~ 840 ng/cm², respectively. So the mass of adsorbed fibrinogen on the random SS-DA-CD34 was 1.86 times higher than on the oriented SS-DA-SPA-CD34. Because the fibrinogen adsorption from the single protein solution as determined by QCM-D may be different from the physiological condition, the samples were incubated with PPP, and then the relative amount of adsorbed fibrinogen was determined by ELISA using a goat anti-human fibrinogen IgG HRP-conjugated antibody. As shown in Figure 6, the amount of adsorbed fibrinogen on the random SS-DA-CD34 was 2.29 times higher than on the oriented SS-DA-SPA-CD34. Both these results of fibrinogen adsorption confirmed that the oriented immobilization of anti-CD34 antibody resists fibrinogen adsorption better than the random immobilization.

The platelet adhesion and the surface-induced platelet activation were quantified by determination of the LDH release (Figure 6) and fluorescent staining for P-selectin (Figure 7). The oriented antibody immobilization did suppress platelet adhesion, compared with random immobilization.

Similar to platelet adhesion, oriented immobilization also prevented platelet activation (red fluorescence), compared with random immobilization.

Graft thrombosis is the most common cause of failure, so it is very important that the CD34-modified surfaces have good blood-compatibility. When a biomaterial is implanted in the human body, the fibrinogen adsorption onto the surface of the implant plays an important role in the process of thrombosis, as it is converted by thrombin into fibrin.³¹ Then, platelet adhesion and platelet activation happen and lead to thrombus formation. Here, fibrinogen adsorption, platelet adhesion, and platelet activation were measured and the results indicated that oriented immobilization of anti-CD34 antibody improves the blood-compatibility. In recent studies, some anti-CD34 antibody-modified surfaces with random immobilization containing anticoagulant or antifouling agents such as polyethylene glycol (PEG),^{6,32} polyacrylic acid,⁹ or heparin⁷ exhibited good blood-compatibility. But before antibody immobilization, these surfaces with anticoagulant or antifouling agents possessed better blood-compatibility. These studies also indicated that random immobilization of antibody does not improve blood-compatibility.

It is puzzling that SPA has no anticoagulant properties and the blood-compatibility of the SPA exposed surface (SS-DA-SPA) was very poor,¹⁶ but the blood-compatibility of SS-DA-SPA-CD34 was improved remarkably. Although the theory has been proved that hydrophilic surfaces possess good thromboresistant properties,³³ the surface of SS-DA-CD34 with the best hydrophilicity did not show the best blood-compatibility. This indicated that the oriented antibody immobilization may also be helpful for the enhancement the blood-compatibility. The dominating molecular mechanisms underlying the antibody-induced blood-compatibility are still not completely understood, but we believe that both the specific antibody-antigen interactions and the steric hindrance might constitute the two dominating mechanisms.

It is known from other *in vivo* experiments that true long-term blood-compatibility cannot be achieved only by preventing platelet adhesion.^{34,35} Rapid re-endothelialization at the disease site and on the surface of devices not only provides an inherent anti-thrombogenic potential but also interrupts cytokine-driven activation of SMCs leading to restenosis.³ The concept of rapid re-endothelialization actually was the primary motivation to immobilize anti-CD34 antibody onto a surface to capture EPCs. But the IBA and EPC-CE of anti-CD34 antibody have not been studied until now. Additionally, the EPC-capturing experiment was usually performed in static conditions, which are different from the physiological condition. Thus, the difference between the static system and the flow system deserved analysis.¹⁷

Figure 8 shows that EPCs express CD34 membrane mark but not SMCs, suggesting that SMCs are suitable negative cellular candidates to assess antibody activity. To evaluate the capturing capability of the CD34-modified surfaces for EPC versus SMC, three types of surfaces were incubated with EPC and SMC in static or under flow conditions for 2 or 12 h.

Figure 9 shows that the numbers of adherent EPC on the CD34 surfaces were significantly higher than on 316L SS at the initial attachment stage (2 h after seeding) and at the early growth stage (12 h after seeding), which suggests that the CD34-modified surfaces favored EPC adhesion. Compared with the random antibody-immobilized surface, the number of adherent EPC onto the oriented antibody-immobilized surface increased significantly under flow condition, but decreased significantly in static. The numbers of adherent EPC on the oriented SS-DA-SPA-CD34 were 1.35 and 1.64 times higher than on the random SS-DA-CD34 after seeding for 2 and 12 h under flow condition, respectively. But the numbers of adherent EPC on SS-DA-SPA-CD34 were only 0.87 and 0.90 times the number on SS-DA-CD34 after seeding for 2 and 12 h under static conditions, respectively. Quantitative result of EPCs' attachment by MTT assay was in accord with FITC-immunofluorescent micrographs of actin expression.

Figure 8. Characterization of endothelial progenitor cells: immunostaining demonstrates only EPC expressing CD34 membrane mark, but not SMC. Cell nuclei were counterstained with DAPI (blue).

Figure 9. FITC-immunofluorescent micrographs of actin expression of EPCs' attachment on different surfaces in static and under flow conditions for 12 h culture (bar 100 mm) and the number of EPCs' attachment on different surfaces in static and under flow conditions after 2 and 12 h culture measured by MTT assay. n = 5.

These results indicated that the two types of CD34-modified surfaces had their own advantages on EPC-CC in static or under flow condition. Speculation the reason is that EPC-CC under flow condition and in static, respectively, depends on the immediate accessibility of F(ab') fragment (no measurements) and total amount of exposed F(ab') fragment on the surfaces (see Figure 4). High antibody density on the randomly immobilized surface caused shielding of some F(ab') fragments by other antibodies. They were not exposed for capturing EPC when the cells flow over the surface. In contrast, the low antibody density on the oriented immobilized surface had an ordered arrangement and the F(ab') fragments were completely exposed for capturing EPCs (see Figure 1). So the immediate accessibility of F(ab') fragments was higher on SS-DA-SPA-CD34 than on SS-DA-CD34, and the surface with oriented antibody immobilization could capture more EPCs

Figure 10. FITC-immunofluorescent micrographs of actin expression of SMCs' attachment on different surfaces in static and under flow conditions for 12h culture (bar 100mm) and the number of SMCs' attachment on different surfaces in static and under flow conditions after 2 and 12h culture measured by MTT assay. n=5.

than a randomly immobilized surface under flow condition. The situation is somewhat different in the static condition, because cells could settle on the surface and F(ab') fragments have enough time to expose for capturing EPC by rearrangement of the antibodies, and then the number of adherent EPC on the surface depends on the total amount of exposed F(ab') fragment on the surface. So the number of adherent EPC correlates better with the total amount of F(ab') fragment under static incubation condition. This assumption has been verified by the amount of exposed F(ab') fragments and the numbers of adherent EPC. Figure 4 shows that the amount of F(ab') fragment exposure on SS-DA-SPA-CD34 was only 0.91 times the amount on SS-DA-CD34, and Figure 9 shows that the numbers of adherent EPC on SS-DA-SPA-CD34 were 0.87 and 0.90 times the number on SS-DA-CD34 after seeding for 2 and 12 h in static, respectively.

In general, the static system has higher throughput and greatest ease of use but obtains less-detailed information, while the flow-based test is more difficult to set up but is closest to physiology if one is interested in the dynamics of adhesion in the vasculature.¹⁷ Although static evaluation of EPC-CC has been accepted and widely used, our results indicate that it is better to do this experiment under flow for simulating the physiological condition.

Additionally, the EPC-CE, which is defined as the ratio of the number of adherent EPC to the mass of immobilized antibody, was analyzed. The EPC-CE of each antibody on the oriented SS-DA-SPA-CD34 was 5.16 and 6.26 times higher than on the random SS-DA-CD34 after seeding for 2 and 12 h under flow condition, respectively. Meanwhile, the EPC-CE of each antibody on SS-DA-SPA-CD34 was 3.32 and 3.44 times higher than on SS-DA-CD34 after seeding for 2 and 12 h in static conditions, respectively. Although the two kinds of CD34-modified surface have their own advantages on the EPC-CC in static or under flow condition, the EPC-CE determination indicates that oriented immobilization of the anti-CD34 antibody had higher efficiency for EPC-capturing than random immobilization.

Figure 10 shows that there was no significant difference between all surfaces concerning the number of adherent SMC, except between the random antibody-immobilized surface and the control (316L SS) at the early growth stage (12 h after seeding) under static conditions. Although the random antibody-immobilized surface showed more SMC adhesion than the oriented immobilization, there was no significant difference.

Although the CD34-modified surfaces were unable to resist SMC adhesion compared with 316L SS, they could significantly promote EPC attachment, especially the surface with oriented antibody immobilization. Continuous supplement of EPC from blood ensures that these surfaces become covered rapidly by EPC, because the time needed for SMC proliferation should be longer than the time needed for EPC attachment. The clinical application of the anti-CD34-coated Genous™ Bio-engineered R stent (OrbusNeich, Hong Kong) indicated that capturing of EPCs on a surface promotes rapid re-endothelialization and inhibits SMCs' proliferation and thromboresistance.³⁶

Conclusion

In this contribution, with SPA surface and poly DA surface as platform, we investigated the anti-CD34 antibody orientation-induced changes in IBA, EPC-CC and EPC-CE of anti-CD34 antibody. The results showed that the IBA of the oriented immobilized antibody was 3.48 times higher compared to random immobilization. The EPC-CC of the oriented antibody-immobilized surface was higher than random immobilized surface under flow condition, which is close to the physiological condition. In addition, EPC-CE of the oriented immobilized antibody was higher than of the random immobilized. Meanwhile, oriented immobilization of anti-CD34 antibody could improve blood-compatibility, but could not resist SMC attachment. In total, oriented immobilization can obtain high biocompatibility at a low anti-CD34 antibody dosage. These results clearly revealed the significance of antibody orientation and may revolutionize the antibody-immobilization protocols used in cardiovascular and other blood-contacting biomedical devices.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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