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Simple surface modification of a titanium alloy with silanated zwitterionic phosphorylcholine or sulfobetaine modifiers to reduce thrombogenicity

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Abstract

Thrombosis and thromboembolism remain problematic for a large number of blood contacting medical devices and limit broader application of some technologies due to this surface bioincompatibility. In this study we focused on the covalent attachment of zwitterionic phosphorylcholine (PC) or sulfobetaine (SB) moieties onto a TiAl₆V₄ surface with a single step modification method to obtain a stable blood compatible interface. Silanated PC or SB modifiers (PCSi or SBSi) which contain an alkoxy silane group and either PC or SB groups were prepared respectively from trimethoxysilane and 2-methacryloyloxyethyl phosphorylcholine (MPC) or *N*-(3-sulfopropyl)-*N*-(methacryloyloxyethyl)-*N,N*-dimethylammonium betaine (SMDAB) monomers by a hydrosilylation reaction. A cleaned and oxidized TiAl₆V₄ surface was then modified with the PCSi or SBSi modifiers by a simple surface silanization reaction. The surface was assessed with x-ray photoelectron spectroscopy (XPS), attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and contact angle goniometry. Platelet deposition and bulk phase activation were evaluated following contact with anticoagulated ovine blood. XPS results verified successful modification of the PCSi or SBSi modifiers onto TiAl₆V₄ based on increases in surface phosphorous or sulfur respectively. Surface contact angles in water decreased with the addition of hydrophilic PC or SB moieties. Both the PCSi and SBSi modified TiAl₆V₄ surfaces showed decreased platelet deposition and bulk phase platelet activation compared to unmodified TiAl₆V₄ and control surfaces. This single step modification with PCSi or SBSi modifiers offers promise for

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improving the surface hemocompatibility of TiAl_6V_4 and is attractive for its ease of application to geometrically complex metallic blood contacting devices.

Keywords

surface modification; phosphorylcholine; sulfobetaine; blood compatibility; cardiovascular devices

1. Introduction

Platelet deposition still occurs on the metallic surfaces utilized in cardiovascular applications such as vascular stents, heart valves, and ventricular assist devices (VADs). As a result, patients implanted with these devices often require chronic anticoagulation or anti-platelet therapy to reduce the risks of thrombosis and thromboembolism. Unfortunately, this pharmacologic therapy comes with an increased risk of bleeding which can result in significant morbidity and mortality [1–5]. Enhancing the thromboresistance of metallic blood contacting surfaces could thus lead to more widespread application of cardiovascular devices with lower complication risks and potentially permit the development of new areas for device application.

To enhance the thromboresistance of VADs in particular, several types of coatings such as titanium nitride (TiN), diamond-like carbon (DLC), 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer, and heparin coatings have been applied to metallic blood contacting surfaces [5]. MPC-based coatings, are notable in that the biomimetic and zwitterionic phosphorylcholine (PC) group-bearing polymers have demonstrated attractive levels of blood compatibility by inhibition of protein adsorption, platelet adhesion and platelet activation on modified surfaces [6–11] and they have been applied onto a variety of metallic surfaces such as vascular stents and VADs [12–15].

In a previous study assessing the preclinical biocompatibility of VAD coatings [15], a physically-adsorbed MPC copolymer coating showed superior performance to a DLC coating, a more common coating for VADs. While DLC coatings have also demonstrated good hemocompatibility and durability independently of comparative studies with MPC, they also carry the risk of microcrack formation [16]. Unlike heparin coated surfaces, MPC copolymer coatings have not been shown to present a potential risk for heparin-induced thrombocytopenia and should be less susceptible to degradative enzymatic process that can act on heparin [17–19]. However physically adsorbed PC group-bearing polymer coatings are not as stable as DLC coatings and the concern of surface stability in long-term applications may offset its perceived advantages. MPC coatings that are covalently linked onto metallic surfaces would thus be more attractive to ensure sustained non-thrombotic properties in long-term cardiovascular applications [13, 16].

Along these lines, we have recently demonstrated that a PC group-bearing polymer could be covalently bound to a titanium alloy (TiAl_6V_4) surface by a condensation reaction or with plasma initiated graft polymerization after the TiAl_6V_4 surface was treated with a functional silane coupling agent [20, 21]. However, the required pre-modification steps for these reactions may have resulted in diminished control of the uniformity and coverage of the PC groups on the modified surface. A simplified surface modification technique would be attractive as it could potentially result in better control of the coating process and increase the ease and reproducibility of the coating process for bulk manufacturing as well as reduce the amount of MPC necessary for coating and thereby reduce the overall cost of the coating process.

The aim of our study was to develop a surface modification strategy to obtain a stable blood compatible interface on a TiAl_6V_4 surface. This surface has relevance for a number of cardiovascular devices, particularly in the rotary blood pump field where there is interest in extending this type of device therapy to the pediatric population [22]. In the present study, we focused on developing a simple modification method to covalently attach hemocompatible moieties onto a TiAl_6V_4 surface in a process that would be amenable to complex surfaces such as one would encounter in a rotary blood pump. For this, we prepared a silanated PC modifier (PCSi) which contains an alkoxy silane and PC groups to modify a clinically-relevant TiAl_6V_4 surface in a single-step. Additionally, in an effort with similar objectives, a silanated sulfobetaine (SB) modifier (SBSi) was also prepared. Surfaces modified with SB group bearing polymers with zwitterionic side groups $[-\text{N}^+(\text{CH}_2)_n \text{SO}_3^-]$ have also exhibited anti-bioadherent properties and non-thrombogenicity due to the ability of the surface to resist protein adsorption and platelet adhesion similar to PC group modified surfaces [23–27]. The modification effect of PCSi and SBSi modifiers on a TiAl_6V_4 surface was characterized and the blood compatibility of the modified surfaces was assessed in terms of platelet adhesion and activation following acute blood contact in vitro.

2. Materials and Methods

2.1 Materials

Titanium alloy (TiAl_6V_4) was purchased (California Metal & Supply Inc., Gardena, CA) and polished with 3.0, 1.0, 0.25, and 0.1 micron diamond pastes (Electron Microscopy Sciences, Washington, PA). The polishing methodology utilized with increasingly fine pastes was matched to that employed for rotary blood pumps under development by Launchpoint Technologies (Goleta, CA). The MPC was obtained from NOF Corporation (Tokyo, Japan), and synthesized by the same method described in a previous report [6]. *N*-(3-sulfopropyl)-*N*-(methacryloxyethyl)-*N,N*-dimethylammonium betaine (SMDAB), trimethoxy silane (TMSi) and platinum 10 wt% on activated carbon (Pt/C) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Synthesis of silanated zwitterionic modifier

Silanated zwitterionic surface modifiers (PCSi or SBSi) were prepared from TMSi and either MPC or SMDAB monomers by a hydrosilylation reaction. A round bottom flask equipped with magnetic stirrer was charged with anhydrous MeOH (10 mL), and MPC or SMDAB monomer (1 mmol) was dissolved under Ar gas for 30 min. TMSi (10 mmol) was then added in excess and Pt/C (0.1 g) was added as a catalyst followed by flushing with Ar gas for 10 min and sealing of the flask. The mixture was reacted at 40°C for 24 h in an oil bath. Unreacted TMSi monomer and solvent were removed by a rotary evaporator at 40°C under reduced pressure. After evaporation, anhydrous MeOH was added and the product filtered with a 25 mm syringe filter (poly(tetrafluoroethylene) (PTFE), 0.45 μm , Corning Inc., Corning, NY) to remove the Pt/C. MeOH was removed again by rotary evaporation. The brown, oil-like reaction product was stored under refrigeration after sealing the container to exclude moisture (Figure 1). The chemical structures of the silanated PC and SB (PCSi and SBSi) were confirmed with ^1H NMR (300MHz, Bruker Biospin Co., Billerica, MA).

2.3 Surface modification with the silanated MPCSi and SBSi

TiAl_6V_4 was polished and cleaned ultrasonically 3 times for 5 min each with ethanol and acetone after samples were cut to a predetermined size (1×2.5 cm) from a TiAl_6V_4 sheet. Titanium surfaces were passivated with a 35% nitric acid solution for 1 h and rinsed with distilled water for 24 h. Then, silanized titanium surfaces with the PCSi or SBSi were prepared by a hydrous liquid phase deposition method. The synthesized PCSi or SBSi was

diluted at 3 % concentration in MeOH and stirred for 30 min after adding the amount of necessary distilled water and HCl (0.05 M) to hydrolyze the methoxy groups of the PCSi or SBSi modifiers under acidic conditions (pH 4–5). Then the TiAl_6V_4 sample was immersed in the activated PCSi or SBSi solution and stirred for 30 min to adsorb the activated PCSi or SBSi on the titanium surface via weak hydrogen bonding. After that, the sample surfaces were dried in an oven for 1 h at 110 °C to silanize the surfaces with the PCSi or SBSi through covalent bonding. Samples treated in this manner were referred to as Ti-PCSi or Ti-SBSi. TMSi modified TiAl_6V_4 samples (Ti-TMSi) were also prepared by the same silanization reaction as a control. The modified samples were rinsed by stirring in deionized water for 24 h before using.

2.3 Surface characterization

The surface composition of the modified and unmodified TiAl_6V_4 samples was analyzed by x-ray photoelectron spectroscopy (XPS) using a Surface Science Instruments S-probe spectrometer at the University of Washington (Seattle, WA). The surface composition on a given sample was averaged from three composition spots. The mean value for three different samples was determined. The modified TiAl_6V_4 surfaces were also analyzed with an attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR, Shimadzu, Columbia, MD). The spectra were collected with 1024 scans at a resolution of 4 cm^{-1} . The static contact angle of water on the surfaces of unmodified and modified titanium samples was measured at room temperature using a contact angle goniometer (VCA optima, AST Product Inc., Billerica, MA) by placing 1 μL of distilled water on the surfaces. The contact angle was also measured after four weeks in the surface modified samples that underwent continuous stirring under deionized water to test the long term stability of the surface modification. The TiAl_6V_4 surfaces were stained with rhodamine 6G (Sigma-Aldrich, St. Louis, MO) by immersing in rhodamine 6G aqueous solution (0.2 mg/mL) for 30 sec, followed by washing in distilled water for 30 sec and drying [28, 29]. The surfaces were observed with fluorescence microscopy (ZEISS, Carl Zeiss, Inc. Thornwood, NY) and obtained images were analyzed with an Image-J program (National Institutes of Health, Washington, DC).

2.4 Surface protein adsorption

Surface protein adsorption on modified and unmodified TiAl_6V_4 samples was assessed by a micro-bicinchoninic acid (BCA) assay [30]. Ovine fibrinogen (Sigma-Aldrich) was prepared in phosphate buffer solution (PBS; BD Biosciences, San Jose, CA) at a concentration of 0.03 g/dL. The samples were immersed in the fibrinogen solution at 37 °C for 3 h followed by washing with 50 mL PBS. A protein analysis kit (Quantipro-Micro BCA kit, Sigma-Aldrich) based on the BCA method was used to quantify adsorbed fibrinogen. The mean value of fibrinogen adsorption from 3 independent samples, each measured in triplicate, was determined.

2.5 Blood collection and blood contact test

NIH guidelines for the care and use of laboratory animals were observed. Whole blood was collected from healthy ovines by jugular venipuncture using an 18 gauge $1\frac{1}{2}$ " needle after discarding the first 3 mL, and 2.7 mL was then immediately added to monovette tubes containing 0.3 mL of 0.106 M trisodium citrate (Sarstedt, Newton, NC). Whole ovine blood was also collected by jugular venipuncture directly into a syringe containing heparin (3.0 or 6.0 U/mL) after discarding the first 3 mL for blood contacting experiments. Then, modified titanium and unmodified TiAl_6V_4 samples were placed into Vacutainer® blood collection tubes without additives (BD Biosciences, Franklin Lakes, NJ), filled with citrate or heparinized ovine blood and incubated at 37 °C on a hematology mixer (Fisher Scientific, Pittsburgh, PA). Although some anticoagulation is necessary to perform the blood contact

testing, citrate and heparin were both used to provide a comparison between stronger (citrate) and weaker (heparin) inhibitors of platelet deposition.

2.6 Scanning electron microscopy of platelet adhesion and morphology

After contact with citrated or heparinized ovine blood, surfaces were rinsed with PBS and immersed in a 2.5% glutaraldehyde solution for 2 h at 4°C to fix the surface adherent platelets, and treated for 1 h in 1% (w/v) OsO₄. The samples were serially dehydrated with increasing ethanol solutions and sputter coated with gold/palladium. Each sample surface was observed by scanning electron microscopy (SEM; JSM-6330F, JEOL USA, Inc., Peabody, MA).

2.7 Quantification of platelet adhesion and activation

Modified and unmodified titanium samples were incubated with heparinized ovine blood for 2 h at 37 °C with continuous rocking as above. The surfaces were rinsed thoroughly after blood contact with 50 mL of PBS and immersed in 1 mL of 2% Triton X-100 solution (Sigma) for 20 min to lyse surface adherent platelets. The number of deposited platelets on each sample was then quantified by a lactate dehydrogenase (LDH) assay [31] with an LDH Cytotoxicity Detection Kit (Takara Bio, Tokyo, Japan). Calibration of spectrophotometer absorbance results to platelet numbers was accomplished using a calibration curve generated from known dilutions of ovine platelet rich plasma in the lysing solution. The percentage of activated ovine platelets in the bulk phase of the blood contacting the surface samples was quantified by a flow cytometric assay using fluorescein conjugated Annexin V protein [32]. Activation levels from 5 independent samples were averaged for each surface type after subtracting the level of activation found for tubes filled with ovine blood that were rocked in the absence of a metallic surface specimen.

2.10 Statistical analyses

Data are presented as means with standard deviation. Statistical significance between sample groups was determined using ANOVA followed by post-hoc Newman-Keuls testing of specific differences. Statistical significance was considered to exist at $p < 0.05$.

3. Results

3.1 Surface modification and characterization of the modified TiAl₆V₄ with silanated zwitterionic modifier PCSi or SBSi

To achieve one step surface modification of TiAl₆V₄ with non-specific protein adsorption, silanated zwitterionic modifier PCSi or SBSi were synthesized by hydrosilylation between trimethoxysilane and MPC or SMDAB. The hydrosilylation in this study occurred on Si-H to alkene group in the methacryloyl group of MPC and SMDAB in the presence of a platinum catalyst. The chemical structure of the synthesized PCSi and SBSi was confirmed by ¹H NMR. For PCSi (in deuterated ethanol) the peaks were: δ (ppm) 1.07–1.10 (SiCH₂CHCH₃, 2H), 1.15–1.20 (SiCH₂CHCH₃, 1H), 1.90–2.11 (SiCH₂CHCH₃, 3H), 3.21–3.26 (N(CH₃)₃, 9H), 3.42–3.53 (Si(OCH₃)₃, 9H), 3.79–3.83 (CH₂N(CH₃)₃, 2H), 3.96–4.01 (OCH₂, 2H), 4.05–4.15 (CH₂PO₄CH₂, 4H) ppm, and for SBSi (in deuterated ethanol) the peaks were: δ (ppm) 0.92–0.94 (SiCH₂CHCH₃, 2H), 1.17–1.29 (SiCH₂CHCH₃, 3H), 1.37 (SiCH₂CHCH₃, 1H), 2.18–2.28 (CH₂CH₂S, 2H), 2.82–2.86 (CH₂CH₂S, 2H), 3.22–3.28 (N(CH₃)₂, 6H), 3.28–3.38 (Si(OCH₃)₃, 9H), 3.69–3.83 (CH₂N(CH₃)₂CH₂, 4H) and 4.54–4.60 (OCH₂, 2H).

The surface composition analyzed by XPS is also shown in Table 1. The surfaces modified with TMSi which were prepared as a control showed a decrease in Ti composition and increased Si composition in comparison with unmodified TiAl₆V₄ (Ti) ($p < 0.05$). The data

also support the successful modification of TiAl_6V_4 surfaces with the PCSi or SBSi based on an increased phosphorus composition ($\text{P} = 1.5 \pm 0.2\%$) on the surface of Ti-PCSi and an increased sulfur composition ($\text{S} = 2.5 \pm 1.1\%$) on the surface of Ti-SBSi in comparison to unmodified TiAl_6V_4 (Ti) and Ti-TMSi ($p < 0.05$). An attenuated total reflection FTIR spectrum for each surface is shown in Figure 2. On the surface of Ti-PCSi and SBSi, there are new absorbance peaks at 1723 cm^{-1} (C=O stretching vibration), $1480\text{--}1300\text{ cm}^{-1}$ (CH_2 , CH_3 bending), $1250\text{--}1020\text{ cm}^{-1}$ (Si-O-Si asymmetric stretching vibration), $1150\text{--}1050\text{ cm}^{-1}$ (PO_4CH_2^- stretching vibration) 1172 , 1040 cm^{-1} (SO_3^- vibration) 970 cm^{-1} ($\text{N}(\text{CH}_3)_3$ vibration), $925\text{--}950\text{ cm}^{-1}$ (Si-O-Ti siloxane bond to titanium) [33, 34].

Fluorescent micrograph images of unmodified TiAl_6V_4 (Ti), Ti-TMSi, Ti-MPCSi and Ti-SBSi observed after staining with rhodamine 6G and generating 3D plots of the fluorescence intensity are seen in Figure 3. Both the PCSi modified surface (Ti-PCSi) and the Ti-SBSi stained strongly with the rhodamine and coverage was relatively uniform. Unmodified TiAl_6V_4 (Ti) and Ti-TMSi control surfaces did not stain positively and yielded dark images.

Table 2 shows the surface contact angle before and after surface modification. The surface contact angles significantly decreased on both the Ti-PCSi and Ti-SBSi in comparison with TiAl_6V_4 (Ti) and Ti-TMSi attributable to the presence of the hydrophilic PC and SB groups on the surfaces. The contact angles did not significantly change after continuous rinsing with deionized water over a four week period.

The amount of adsorbed ovine fibrinogen on the unmodified and modified titanium surfaces is shown in Figure 4. Both the Ti-PCSi and Ti-SBSi showed a significant decrease in adsorbed fibrinogen relative to Ti and Ti-TMSi surfaces.

3.2 In vitro surface blood compatibility of the modified TiAl_6V_4

Electron micrographs of platelet deposition from citrated ovine blood for 3 hr at 37°C for unmodified TiAl_6V_4 (Ti) and modified titanium samples (Ti-TMSi, Ti-PCSi and Ti-SBSi) are shown in Figure 5. There were many adhered platelets on the unmodified Ti and Ti-TMSi surfaces and some platelet aggregation was seen. The deposited platelets exhibited an activated morphology, as demonstrated by extended pseudopodia and surface spreading. In contrast, platelet deposition was sparse on the Ti-PCSi and Ti-SBSi surfaces and those platelets found were in a discoid morphology without signs of surface activation. Platelet adhesion and morphology was also observed after contact with heparinized ovine blood (3 U/mL) for 2 hr at 37°C (Figure 6). Platelet deposition and surface aggregation appeared to increase on the Ti and Ti-TMSi surfaces with the heparinized blood when compared to surfaces in contact with citrated blood. The marked decrease in platelet deposition on the Ti-PCSi and Ti-SBSi surfaces compared to the Ti and Ti-TMSi samples was again observed with the heparinized blood and few platelets were deposited.

The number of deposited platelets as quantified by the lactate dehydrogenase (LDH) assay after heparinized ovine blood contact is shown in Figure 7. The Ti-PCSi and Ti-SBSi modified surfaces showed large decreases in the number of deposited platelets relative to unmodified TiAl_6V_4 and Ti-TMSi ($p < 0.01$). However, the Ti-PCSi was not significantly different than the Ti-SBSi surfaces. Platelet activation in the bulk phase, as evidenced by Annexin V binding, for ovine blood after contact with the unmodified and modified titanium samples is shown in Figure 8, where platelet activation levels following contact with Ti-PCSi and Ti-SBSi samples were significantly lower than that for unmodified TiAl_6V_4 (Ti) and Ti-TMSi control samples.

4. Discussion

There is increasing interest in zwitterionic moieties including carboxybetaines (CB), sulfobetaines (SB) and phosphobetaines (PC group-bearing polymer) to design biocompatible polymeric materials since surfaces modified with these moieties are less supportive of untoward cell adhesion or enzymatic activation based on their minimization of protein adsorption [35]. Kitano et al. [36–38] showed common effects of zwitterionic group-bearing polymer surfaces on the structure of surrounding water in that PC, SB or CB groups did not disturb the hydrogen-bonding network structure of water. It was further suggested that the resistance of these surfaces to protein adsorption related to the local water structure [39].

To initiate chemical modification of a polymeric biomaterial surface, there are many approaches that have been pursued, including direct grafting with a zwitterionic group containing monomer [24, 25, 40–42] or the use of alkoxysilane compounds which have PC, SB or CB groups such as PCSi or SBSi as surface modifiers [43, 44]. However, previous studies have required a complicated synthesis route to generate the appropriate PC or SB groups and attach these onto the surface. In this report we simply prepared PCSi and SBSi as titanium alloy surface modifiers by a hydrosilylation reaction using MPC or SMDAB. This reaction between a hydride-siloxane (Si-H) and vinyl (C=C) compounds with a catalyst such as Pt/C is relatively simple and is widely used in the silicone industry to prepare monomers, silicone-carbon compounds and crosslinkable polymers [45]. In this study, there was concern about the lower reactivity of methacrylate groups in comparison with vinyl groups [46], however the hydrosilylation of methacrylate groups was confirmed at almost 100 % completion under mildly increased temperature (40 °C), provided the Si-H and catalyst were in excess and the reaction time was sufficient. The NMR analysis of synthesized PCSi and SBSi did not show any double bond peaks at 5.2–5.5 ppm and 6.0–6.2 ppm, which would be associated with unreacted monomers. The purified PCSi and SBSi product could be collected and stored without aggregation of the siloxane compounds by keeping anhydrous conditions during the synthesis process.

West, *et al.* [26] evaluated two copolymers containing SB or PC moieties for use as potential biocompatible coatings. They showed that both SB and PC group-bearing polymer coatings reduced cell adhesion (bacterial adhesion, human macrophages, and granulocytes) with respect to the uncoated materials, and that PC group-bearing copolymer coatings were superior to the SB-based copolymer coatings in reducing cellular adhesion. In this study, we prepared a titanium alloy surface that was modified with PC or SB groups using silanated PC or SB modifiers (PCSi or SBSi) and the modified surfaces (Ti-PCSi or Ti-SBSi) were compared in terms of fibrinogen adsorption, platelet deposition and platelet activation. Both the Ti-PCSi and Ti-SBSi surfaces experienced significantly reduced fibrinogen adsorption, platelet deposition and bulk phase platelet activation relative to unmodified Ti and Ti-TMSi control samples. Although there was a trend towards lower average values for platelet deposition, fibrinogen adsorption, and platelet activation for the Ti-PCSi relative to the Ti-SBSi, none of these differences were found to be statistically significant.

The PCSi and SBSi modified surfaces were prepared by hydrous liquid phase deposition so that the three methoxy groups of the PCSi and SBSi were changed to hydroxyl groups and the hydrolyzed PCSi and SBSi were deposited in the bulk state. The hydrolyzed modifiers had three reactive sites (hydroxyl groups) and could react with each other as well as the desired hydroxyl groups of the titanium surface. This alternative reaction by the PCSi and SBSi with tri-hydroxyl groups could result in non-uniform surfaces (bulk deposition of the PCSi and SBSi) as was seen to some extent with rhodamine staining. A relatively large variation was also observed in the SBSi composition of Ti-SBSi samples as evidenced by

the XPS results (Table 1). The surface uniformity might be better controlled by changing the PCSi or SBSi modifiers concentration, silanization conditions or the modifier reactivity. A more uniform surface could be obtained by an anhydrous liquid phase deposition method where the PCSi and SBSi could deposit on the titanium surface in anhydrous toluene with a catalyst at room temperature by stirring for 24 h without hydrolysis of the methoxy groups. Toward this end we also prepared a PCSi or SBSi monolayer deposited surfaces with these surface modifiers and mono-functional silanated PCSi and SBSi modifiers from 1-chlorodimethylsilane with the same hydrosilylation reaction, and then reacted with the titanium surface to prepare a more uniform surface. The mono-functional silanated PCSi and SBSi modification on a TiAl_6V_4 surface also showed a significant decrease in platelet adhesion and activation compared to the control surface. However, it was difficult to prepare a highly covered monolayer with these mono-functional modifiers and the modified surfaces did not show improved performance in decreasing platelet deposition and activation relative to the bulk deposited PCSi or SBSi modified surfaces (data not shown). This may have been due to difficulty in achieving monolayer coverage with PCSi and SBSi modifiers because of imperfect monolayer availability of hydroxyl groups on the alloy substrate. In this study, we did not apply a water plasma treatment before the PCSi and SBSi modification, though we demonstrated that water plasma treatment could increase the surface reactivity of the titanium alloy in previous studies [20, 21]. Better monolayer deposition may have resulted from further surface treatment, such as water plasma exposure or other chemical pretreatments, to increase hydroxyl group numbers on the surface before silanization with the PCSi and SBSi modifiers. However, the bulk deposition of PCSi or SBSi prepared under the hydrous liquid phase deposition conditions may have increased the coverage and modification density of the PC or SB groups on the surface without needing to increase the reactive hydroxyl groups on the bare titanium surface.

Previously we reported that PC group-bearing polymers could be attached onto a titanium alloy surface by immobilizing an MPC copolymer (poly(MPC-co-methacryl acid) (PMA)) or by plasma induced MPC grafting polymerization after the titanium surface was pre-modified with a functional silane coupling agent [20, 21]. Those surfaces showed significant improvement over non-modified surfaces in terms of decreased platelet deposition and bulk phase platelet activation. However, those techniques required multiple steps to pre-functionalize the surface in order to immobilize or graft the PC group-bearing polymer onto the titanium surface. There was no significant difference in terms of the inhibition of platelet deposition and activation when comparing the current one-step procedure with these previous titanium surfaces modified with PC group-bearing polymers [20, 21]. The simpler and equally effective surface modification method using a small silanated PC molecule (PCSi) thus is more attractive in that this coating could be applied in a direct manner under mild conditions and could be readily applied to the assembled, complex geometries of a cardiovascular device such as a rotary blood pump [47].

While in our study the SB-based surface modification did not exhibit a significantly greater biological effect than that found with PCSi, and in fact trended to less of an effect, further investigation of this surface modifier is warranted due to its cost being orders of magnitude lower than for the PC-containing modifier. Earlier studies by Bernards et al [48] prepared nonfouling polymer brushes composed of varying mixtures of positively $[-\text{N}^+]$ and negatively $[-\text{SO}_3^-]$ charged monomers by surface initiated atom transfer polymerization (ATRP) on a gold-coated surface. Their results demonstrated that the polymer brush surface coating composed of oppositely charged monomers exhibited low protein adsorption. Their best nonfouling surface coating was copolymer brushes formed from a 1:1 homogeneous reaction mixture of two oppositely charged monomers. Furthermore, Zhang et al [49] prepared a “superlow” fouling surface on glass slides by grafting SB- or CB-bearing polymers by surface initiated ATRP. Better controlled surface modification techniques such

as offered by surface initiated ATRP might maximize the surface modification effect of sulfobetaine and result in further improvements in surface blood biocompatibility.

Some limitations of the current report should be noted. First, in this study, we polished a raw titanium alloy sheet by hand with diamond pastes of 3, 1, 0.25 and 0.1 μm size. While this level of polish was utilized to mimic the materials being employed industrially for a blood pump, the roughness of the polished, unmodified titanium surface was of a scale that additional texture added from the surface modification would not likely be detectable by AFM and ellipsometry, as noted in our previous study [21]. A more complete surface modification and more extensive surface analysis would be possible if a more highly polished surface were to be employed, although this would not likely match the types of surfaces utilized with many devices being employed clinically. Second, the amount of adsorbed protein estimated by the micro-BCA method (Figure 4) was higher than for previous reports where the zwitterionic PC or SB groups have been attached in monolayer to more idealized surfaces [27, 48]. In these reports more accurate quantification methods, such as a quartz crystal microbalance (QCM) and surface plasmon resonance (SPR), were employed. In this study, the micro-BCA method was used to provide for a relatively simple comparison between unmodified and variably modified surfaces and the levels of fibrinogen adsorption measured were generally consistent with previous reports employing the micro-BCA method [10, 20]. Finally, the efficacy of the generated surfaces was evaluated in an in vitro setting, with ovine blood, for a relatively limited period of blood contact. While blood mixing was present, the hemodynamic environment would not match either the high flow regimes experienced in, for instance, a rotary blood pump, nor would there be the non-ideal flow conditions that one might experience in a crevice formed by connecting metallic parts or in regions of flow recirculation behind impeller blades [47]. Next steps for surface evaluation would involve the coating of cardiovascular devices for extended blood contact under relevant hemodynamic conditions with in vitro perfusion loops, and with testing in appropriate large animal models. A challenge with such experiments is the expense of generating multiple devices for comparative testing and the maintenance of implanted animals, although such experiments are possible and have shown the potential benefits of MPC-based coatings on blood pump biocompatibility in vivo [15].

5. Conclusions

Silanated zwitterionic surface modifiers (PCSi or SBSi) were successfully prepared from trimethoxysilane and MPC or SMDAB by a hydrosilylation reaction. Non-thrombogenic interfaces were simply achieved on a TiAl_6V_4 surfaces by the covalent attachment of PCSi or SBSi onto the surface in a single step modification reaction. Platelet deposition and bulk phase platelet activation were significantly decreased on PCSi or SBSi modified TiAl_6V_4 surfaces in comparison with unmodified and trimethoxysilane modified control surfaces. This single step modification with PCSi or SBSi modifiers offers promise for improving the surface hemocompatibility of blood contacting devices utilizing TiAl_6V_4 and is attractive for its ease of application to potentially complex device surfaces.

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Figure 1.
Synthetic scheme for zwitterionic surface modifiers (PCSi or SBSi).

Figure 2.
Attenuated total reflectance (ATR) FT-IR spectra on the unmodified titanium (TiAl₆V₄ (Ti)), Ti-PCSi and Ti-SBSi.

Figure 3.

Fluorescent micrograph images of unmodified TiAl₆V₄ (Ti), Ti-TMSi, Ti-PCSi and Ti-SBSi observed after staining with rhodamine 6G and digital image processing to create a 3D plot where the z-dimension is proportional to pixel intensity.

Figure 4.

Ovine fibrinogen adsorption from buffer at 37°C for 3 h onto surfaces of control tissue culture polystyrene, unmodified and modified TiAl₆V₄ samples as determined by micro-BCA assay (n=5).

Figure 5.

Low (top row) and high (bottom row) magnification scanning electron micrographs of TiAl_6V_4 (Ti), Ti-TMSi, Ti-PCSi and Ti-SBSi samples after contact with fresh ovine blood (citrate) under mixing for 3 h at 37 °C.

Figure 6.

Low (top row) and high (bottom row) magnification scanning electron micrographs of TiAl₆V₄ (Ti), Ti-TMSi, Ti-PCSi and Ti-SBSi samples after contact with fresh ovine blood (with heparin at 3 U/mL) under mixing for 2 h at 37 °C.

Figure 7. Platelet deposition onto surfaces after contact with ovine blood (with heparin at 6 U/mL) for 2 h under mixing as determined by lactate dehydrogenase (LDH) assay (n=5).

Figure 8.

Quantification of activated platelets in the bulk phase of ovine blood after surface contact under continuous rocking. Platelet activation was quantified by flow cytometric measurement of Annexin V binding onto platelets (n=5). The background platelet activation level was determined from a rocked tube into which no test surface was placed. This background value was subtracted from all tests where a surface was included.

Table 1

Atomic percentage at listed binding energy (eV) as determined by X-ray photoelectron spectroscopy

	C 1s at 285 eV	O 1s at 532 eV	Ti 2p at 455 eV	Al 2p at 74 eV	Si 2p at 106 eV	N 1s at 403 eV	P 2p at 133 eV	S 2p at 168 eV
TiAl ₆ V ₄ (Ti)	42.0 (±8.0)	41.1 (±5.2)	9.5 (±1.1)	4.3 (±3.1)	1.0 (±1.0)	1.0 (±0.5)	0.1 (±0.2)	0.0 (±0.0)
Ti-TMSi	44.8 (±12.9)	33.3 (±7.7)	2.3 (±1.2)	2.7 (±1.5)	13.1 (±2.7) *	0.8 (±0.7)	0.0 (±0.0)	0.0 (±0.0)
Ti-PCSi	32.3 (±4.9)	47.4 (±3.9)	0.3 (±0.5)	0.5 (±0.8)	16.2 (±3.7) *	1.7 (±0.4) *	1.5 (±0.3) *	0.0 (±0.0)
Ti-SBSi	37.0 (±8.2)	40.3 (±6.8)	0.0 (±0.0)	1.2 (±2.1)	17.6 (±4.9) *	2.0 (±0.7)	0.0 (±0.0)	2.0 (±0.9) *

* p<0.05 vs. Ti surfaces

N=7, ± standard deviation for Ti

N=3, ± standard deviation for other samples

Table 2

Contact angle with distilled water on the unmodified and modified titanium samples

	TiAlV ₄ (Ti)	Ti-TMSi	Ti-PCSi	Ti-SBSi	Ti-PCSi-after 4 weeks of rinsing	Ti-SBSi-after 4 weeks of rinsing
Contact Angle(°)	53.7 (±4.1)	95.2 (±4.1)	16.4 (±3.9) *	21.8 (±5.5) *	22.9* (±3.2)	24.9* (±4.4)

* p<0.05 vs. Ti and Ti-TMSi surfaces

N=3, ± standard deviation