Synthesis of a Zwitterionic Silane and Its Application in the Surface Modification of Silicon-Based Material Surfaces for Improved Hemocompatibility

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ABSTRACT A phosphorylcholine-like silane coupling agent bearing zwitterionic molecular structure was synthesized and studied. The chemical structure of this silane coupling agent was characterized by FTIR, ^{1}H NMR and ^{31}P NMR. The zwitterionic structure was successfully constructed onto the surface of silicon as a self-assembled layer (SAL). Static water contact angle, and atomic force microscopy (AFM) were used to investigate the wettability and surface topography of the modified silicon surfaces. Static water contact angle results indicated that the hydrophilicity of the surfaces could be effectively improved by the modification with this zwitterionic silane coupling agent. The changes of the topography and water contact angle of the modified surfaces with different incubation periods in PBS solution were also measured to evaluate the stability of the SALs. Blood compatibility of the modified surfaces were evaluated by testing the full-blood activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT), as well as by observing the adhered blood platelets onto the surface. The modified surfaces showed prolonged clotting time and fewer adherent platelets, revealing that the blood compatibility was evidently improved by the modification using this zwitterionic silane.

KEYWORDS: phosphorylcholine • self-assembled layer • atomic force microscopy • hemocompatibility

1. INTRODUCTION

Silicon and silicon-based inorganic materials are widely used in fabrication of biomedical devices, because of their nontoxicity, good mechanical properties, easy processing, and relatively low cost. However, silicon is not regarded as an optimally biocompatible material for the fabrication of implantable microdevices in contact with the biological matrix (1). Despite the chemical and mechanical stability of these materials in the biological environment, when the microdevices such as cardiovascular stents are implanted into the body, unfavorable responses such as protein adsorption and platelet adhesion may be elicited and fatal thrombus might be initiated. Therefore, the biological effects of silicon-based microdevices are sometimes limited.

There is an increasing interest in modification of the surfaces of different materials to obtain better biocompatibility and hemocompatibility. Attempts of such modification included heparin immobilization (2), adsorption or grafting of hydrophilic polymers such as poly (ethylene glycol) (3) and poly (vinylpyrrolidone) (4, 5). Another effective approach to obtain such biocompatible surfaces was to introduce zwitterionic groups such as carboxybetaine, sulfobetaine and phosphobetaine to the substrate surfaces (6). Among these, phosphobetaine-based materials were considered as biomimetic fouling-resistant materials, because they contain phosphorylcholine (PC) headgroups, which were present in high concentration in the outer leaflet of the lipid bilayer of cell membranes (6b, 6d, 7). Amphiphilic polymers bearing PC groups were reported to be able to form a surface similar in structure to the outer surface of the cell membrane. Such biomimetic surfaces could resist protein adsorption (8, 9) and platelet adhesion (10, 11), and consequently exhibit significantly reduced thrombus formation compared with the unmodified substrate surfaces (12, 13).

In the early 1980s, the first PC-containing vinyl monomer was synthesized (14–16). Later, Ishihara, Ueda, and Nakabayashi reported an improved method for the synthesis of PC-bearing monomer, 2-methacryloyloxyethyl phosphorylcholine (MPC) (17). Since then, efforts have been focused on the application of MPC in the field of biomaterials, because of its good capability to suppress protein adsorption, platelet adhesion, thrombus formation, and cell adhesion. MPC can easily undergo free-radical polymerization as other methacrylate monomers do. It can also copolymerize with other methacrylate monomers such as *n*-butyl methacrylate (BMA), *n*-hexyl methacrylate, and *n*-dodecyl methacrylate

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(DMA), etc. (6d, 18). MPC-based copolymers have been used extensively as matrix biomaterials or as coatings on substrates and, as such, have shown potential in a wide range of medical device applications including biosensors, drug carriers, soft contact lenses, vascular stents, urological devices, and so forth (6d, 19–21).

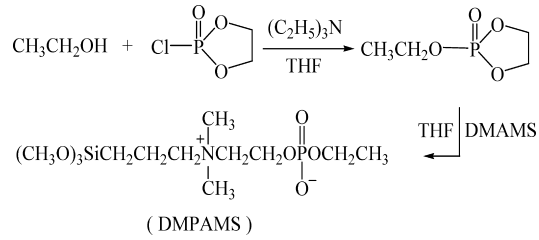
Regarding the mechanism of the good biocompatibility and hemocompatibility of amphiphilic polymers bearing PC groups, Hayward and Chapman suggested that it was the PC headgroup, rather than the whole molecules of phosphatidylcholine, that was responsible for the blood compatibility. Importantly, it was the zwitterionic nature of the PC group that could attract a large number of water molecules, producing a hydrated surface that leads to a large excluded volume and thus could interact reversibly with proteins without causing conformational changes or binding to the surface (22). Until now, most of the researches on the PC modification were focused on polymeric biomaterials. For the phosphorylcholine modification of inorganic surfaces, there are few reports. Lu et al. developed a two-step strategy of the covalent immobilization of PC groups onto silicon surfaces (23, 24). First, a monolayer of hydroxyl or amino-functionalized silane molecules was applied on the silicon surface, and PC groups were then coupled with the surface through these terminal functional groups. The PC-modified silicon surfaces showed reduced protein adsorption. Qiu et al. reported the synthesis of a silane coupling agent bearing sulfobetaine group, and the nonthrombogenicity of glass slide was improved by the simple one-step coating process of this zwitterionic silane coupling agent (25). The relatively sophisticated surface-initiated atom transfer radical polymerization (ATRP) of MPC on silicon wafers was also reported by different researchers (26).

In this study, we tried to explore another way of phosphobetaine-modification of inorganic surfaces. The synthesis of a new zwitterionic silane coupling agent bearing phosphorylcholine-like group was reported. A self-assembly process was utilized to improve the hydrophilicity as well as biocompatibility of silicon and glass surfaces using this novel functional silane. The changes in hydrophilicity and topography of the modified surfaces were confirmed by the covalent bonding of the PC-like groups onto the silicon surfaces. Clotting time tests and platelet adhesion observation were carried out to evaluate the hemocompatibility of the modified glass surfaces.

2. MATERIALS AND METHODS

2.1. Materials. 2-Chloro-2-oxo-1, 3, 2-dioxaphospholane (COP) was synthesized according to the methods of Lucas et al. (27). The structure of the resulting compound was characterized using the GC-MS technique (Voyager), yielding a 96.95% purity of COP. *N*,*N*-Dimethylaminopropyl trimethoxysilane (DMAMS) (purity >97%) was purchased from Aldrich Chemical Co.. Hydrogen peroxide (30%) and sulphuric acid (98%) were obtained from Shanghai Chemical Co. Silicon wafers (p-type, boron doped with ⟨100⟩ orientation) were purchased from Shanghai Kaiqing Photoelectric Materials Co. All the other raw materials and solvents were dried and purified according to standard methods.

Scheme 1. Synthesis Process of DMPAMS



2.2. Synthesis of *N*,*N*-Dimethyl, *N*-(2-Ethyl phosphate ethyl)-aminopropyl-trimethyoxysilane (DMPAMS). The synthesis route is illustrated in Scheme 1. A mixture of 14.25 g COP and 11.1 g triethylamine (TEA) was dissolved in 70 mL of dried tetrahydrofuran (THF) in a 250 mL round-bottomed flask. The solution of 4.6 g of purified ethanol and 20 mL of dried THF was slowly added into the flask dropwise by a predried dropping funnel with magnetic stirring for 1 h. All the steps above were performed at −15 °C. The reaction was continued for 2 h with continuous stirring, and the system was then slowly heated to room temperature and stirred for another 2 h. The white precipitate was filtrated before rotary evaporation to remove the solvent from the intermediate. A solution of 20.7 g of DMAMS in 80 mL of dry acetonitrile was then added to the round-bottom flask above. The solution was slowly heated to 70 °C and stirred magnetically for 24 h. After that, most of the THF was removed through rotary distillation, and the solution was then precipitated with anhydrous ether. DMPAMS, the viscous mahogany precipitate, was obtained after the solution was dried in a vacuum oven for 1 day.

2.3. Immobilization of DMPAMS on Silicon Surface. To remove the organic impurities from the substrate surface, silicon wafers (100) cut into strips of 1 × 1 cm^2, or glass plates (used for clotting time test) were treated by immersing in the freshly prepared piranha solution of 70% conc. H_2SO_4 (aq) and 30% H_2O_2 (aq) (V/V) at 90 °C for 0.5 h (29). The substrates were washed by immersing in deionized water under sonication for 0.5 h, and then rinsed with sufficient deionized water, dried in N_2 stream, and immediately used for the following chemical immobilization within 1 h.

The DMPAMS was dissolved in ethanol to make solutions of different concentrations (10, 30, 50, and 200 mM). The cleaned silicon wafers were immersed in these solutions for different coupling time from 0.5 to 5 h to immobilize DMPAMS onto the surfaces. The wafers were then washed thoroughly with ethanol and sonicated in ethanol for 15 min to remove nongrafted DMPAMS molecules. A self-assembled layer of DMPAMS was obtained after curing in an oven at 80 °C for 1day.

2.4. Structure Characterization. FTIR spectrum (KBr disk) was obtained with a Magna-550 FTIR spectrometer (Nicolet) at room temperature. Spectrum was recorded with a resolution of 4 cm^{-1} in the range of 4000−400 cm^{-1}. ^1H NMR and ^{31}P NMR spectra were recorded with a Bruker model AVANCE DMX-500 spectrometer, using tetramethylsilane (TMS) as the standard for ^1H NMR and NaH_2PO_4 as the standard for ^{31}P NMR, and $CDCl_3$ as the solvent.

2.5. Static Contact Angle Measurements. Static water contact angles were measured in air by the sessile drop method using a contact angle goniometer (OCA15, Data Physics Inc., Germany) on DMPAMS modified silicon surfaces with neat silicon surface as control. Readings were made after a water droplet was dropped onto the surface for 1 min. For each sample, the data were collected 10 times to get an average value.

2.6. Atomic Force Microscopy (AFM). To investigate the morphology of the self-assembled surfaces, we obtained AFM images with a NanoScope IV (Digital Instruments Inc.) in tapping mode using a silicon cantilever, which has a typical spring

constant of around 40 N/m with a resonance frequency around 160−165 kHz. The AFM images were acquired by scanning the samples in air under ambient laboratory conditions at a scan rate of 1.5 Hz and 1 μm × 1 μm in size. The roughness of the surface was determined by measuring the root-mean-square (rms) roughness parameter R_{rms}, defined as the root-mean-square average of the height (z) taken from mean data plane, expressed as

$$R_{rms} = \sqrt{\frac{1}{N}\sum_{i=1}^{N} z_i^2}$$

where z_i is the current z value and N is the number of points within the box cursor.

2.7. Hemocompatibility Evaluation. The experimental procedure to evaluate the hemocompatibility of modified surfaces was described in our previous report (28). Briefly, human full-blood activated partial thromboplastin time (APTT), prothrombin time (PT), and thromboplastin time (TT) of DMPAMS modified glass-plate surfaces and neat glass surfaces were measured on a sysmex CA-1500 using DADE BEHRING Actin, DADE BEHRING Thromburel's, and DADE BEHRING Test-Thrombin reagents accordingly. Full human blood obtained from healthy volunteers was mixed with 0.2 mL of trisodium citrate (109 mM) and transferred into glass-plates grafted with DMPAMS of different concentrations, for a contact time of 1 h. Plasma was prepared by centrifuging the treated blood at 3000 rpm for 15 min and then used for testing. Each sample was measured 3 times to give an average value.

Because platelet adhesion is one of the important steps during blood coagulation on the surfaces of biomaterials, platelet adhesion study was also performed to evaluate the interactions between blood and modified surfaces. Full-blood was drawn from a healthy volunteer and mixed with a 1/9 volume of 3.8 wt % trisodium citrate solution immediately. Platelet-rich plasma (PRP) was obtained by centrifugation of the anticoagulated blood at 1200 rpm for 5 min. Then, it was placed on the DMPAMS-modified silicon surfaces and neat silicon surfaces, and incubated for an assigned period of time at 37 °C. After the incubation, the samples ($n = 3$) were rinsed three times with PBS to remove the nonadherent platelets. The adhered platelets were then immersed in 1% glutaraldehyde in PBS for 60 min at room temperature for fixing. After the glutaraldehyde fixation, the samples were dehydrated sequentially with aqueous ethanol solution of 25, 50, 75, 90, and 100% (V/V) for 30 min each. The samples were then air-dried, sputter-coated with gold, and observed with scanning electron microscopy (TESCAN 5136MM).

3. RESULTS AND DISCUSSION

3.1. Static Water Contact Angle Measurement. Static water contact angle measurement is commonly used to evaluate the surface hydrophilicity of materials. As shown in Figure 1, the surface of neat silicon had a contact angle of 47.8°. A steep decrease in contact angle by 21.5° could be observed after the silicon substrates were grafted with 10 mM DMPAMS solution for only 0.5 h. The surface became much more hydrophilic as a result of the introduction of the zwitterionic structure with strong hydrophilicity which was similar in the structure of phosphorylcholine groups (PC). It also indicated that the DMPAMS molecules could be easily self-assembled from the solution onto the silicon surfaces within 0.5 h after being immersed into the 10 mM DMPAMS solution. When the soaking time was prolonged from 0.5 to 1 h, the surface contact angle slightly decreased by 2.8°. After that, when the soaking time further increased from 1 to 5 h, no apparent decrease in contact angle could be observed. These results indicated that grafting of DMPAMS molecule from the solution onto the substrate surface might reach the plateau within 1 h, resulting in a complete SAL.

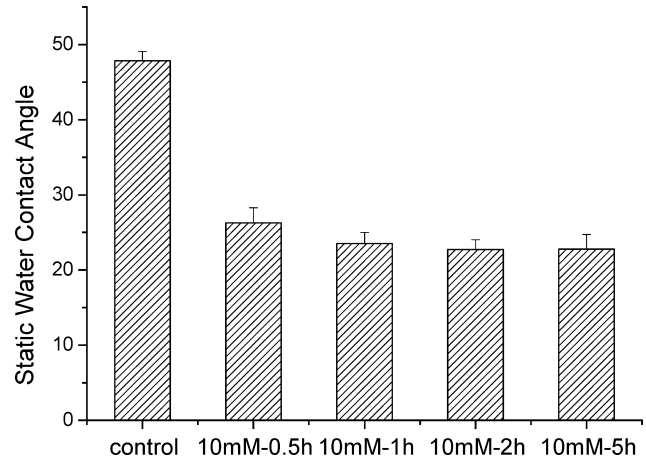


FIGURE 1. Static contact angle of DMPAMS-modified silicon samples formed at different soaking time (solution concentration: 10 mM).

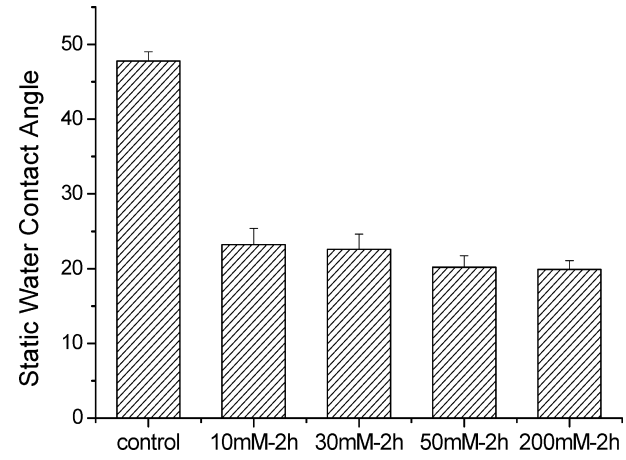


FIGURE 2. Static contact angle of DMPAMS modified silicon samples formed at different solution concentration (soaking time = 2 h).

From Figure 2, when the soaking time was fixed at 2 h, however, the contact angle decreased only 3.3° while the solution concentration was increased from 10 mM to 200 mM. It suggested that a saturated layer of DMPAMS on silicon surfaces could be obtained in a solution of relatively lower concentration of DMPAMS for an adequate coupling time.

According to Brunius' theory (5), the surface energy would be lowered while improving the hydrophilicity of the material, and would greatly influence the interactions and activations between organism and material surface when in contact. To some extent, the hydrophilicity of the surface has a direct relationship to the biocompatibility or hemocompatibility of biomaterial.

3.2. Surface Morphology. Because the implantable microdevices are always used directly in contact with the tissue, the surface topography of substrate greatly affects the tissue and biologic fluid protein adsorption, and further

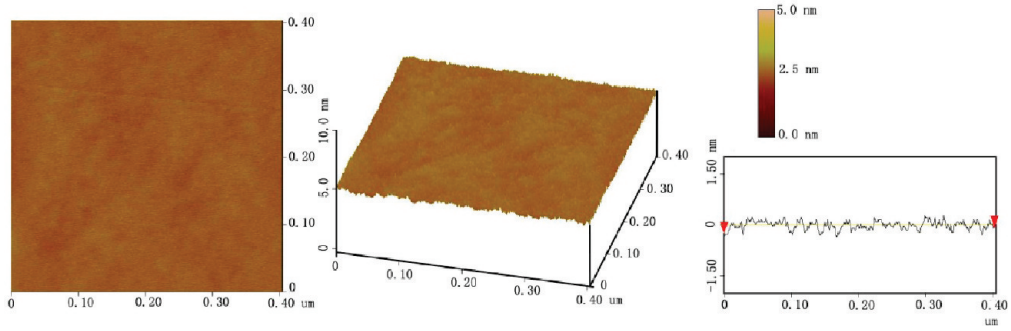


FIGURE 3. Surface morphology and section plot of untreated silicon wafer (Size: 0.4 μm $\times$ 0.4 μm).

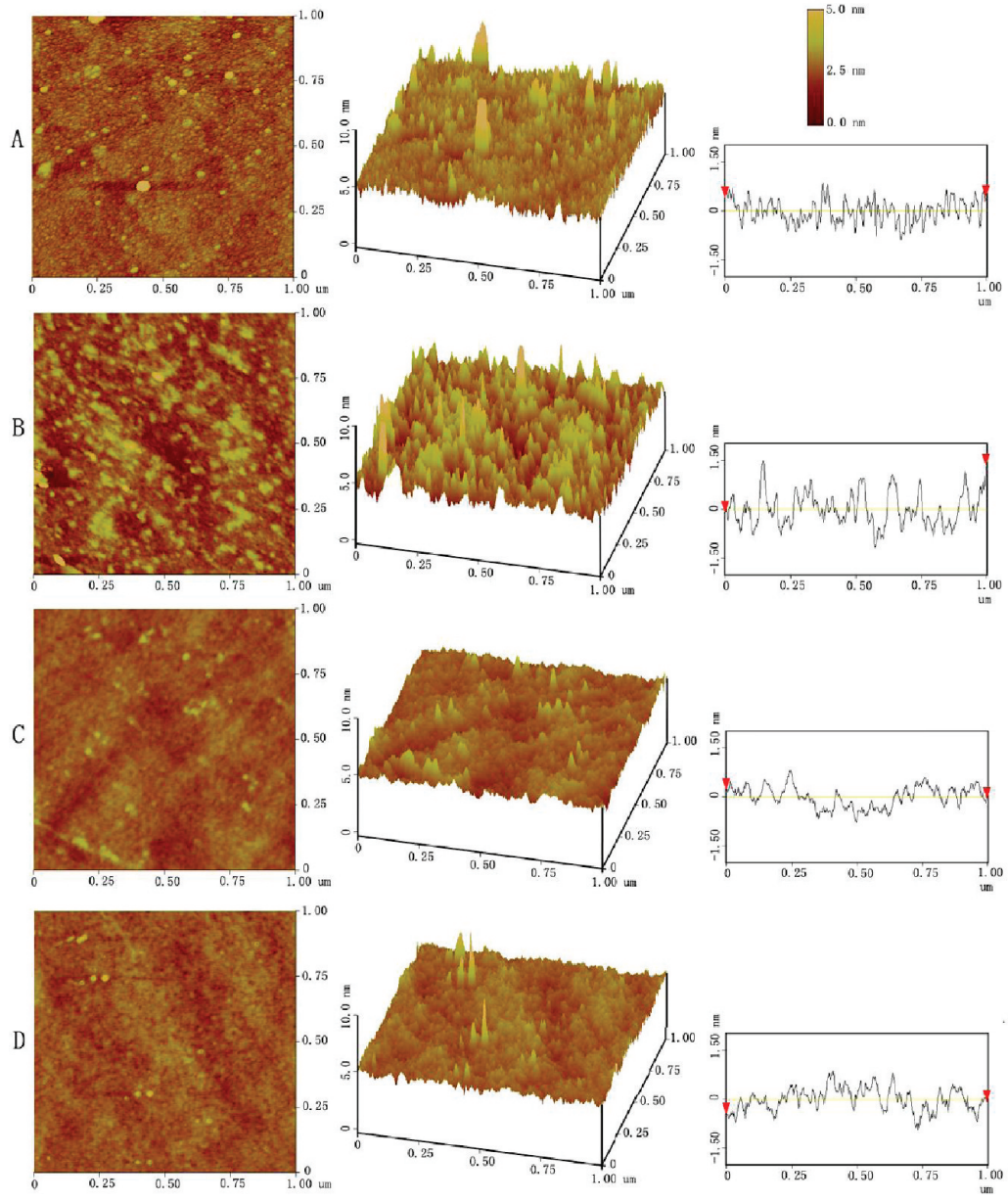


FIGURE 4. AFM images and section plots for DMPAMS modified silicon surfaces formed at different soaking times in 10 mM DMPAMS: (A) 0.5, (B) 1, (C) 2, and (D) 5 h.

affects the cell adhesion and proliferation (31, 32). In this study, the morphology of various DMPAMS modified silicon surfaces prepared in different conditions was investigated using AFM. AFM is an effective tool for studying the topography as it can display the real-space morphology and nanostructure of the surfaces. The information on the homogeneity of the surfaces could also be obtained in terms of the analysis of the roughness values (1).

The surface morphology of the silicon wafer as a control was shown in Figure 3. The surface looked flat and smooth

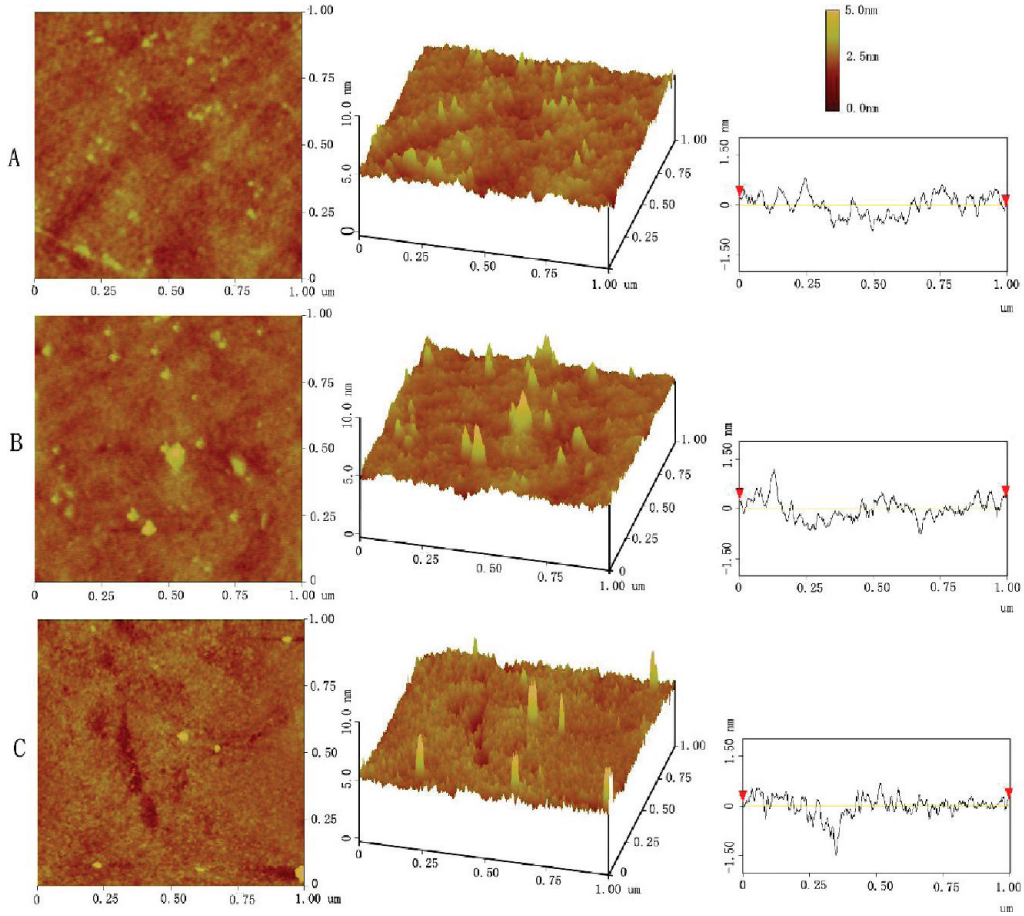


FIGURE 5. AFM images and section plots for modified silicon surfaces formed using DMPAMS of different solution concentrations (coupling time = 2 h): (A) 10, (B) 50, and (C) 200 mM.

without peaks and valleys, with a low rms value of only 0.127 nm and a surface undulation below 0.5 nm. Figure 4 showed AFM images of the surfaces modified with 10 mM DMPAMS for different coupling times. After the silicon surface immobilized in the 10 mM DMPAMS solution for 0.5 h, it became much rougher, with the rms value increasing to 0.686 nm (Figure 7). Some peaks appeared on this surface suggested that DMPAMS molecules were covalently coupled onto the surface and that a SAL was formed but with a low coverage. As the coupling time increased to 1 h, rms value increased to 0.768 nm, with more peaks being suggestive of enlarged surface coverage. However, although the coupling time was further prolonged, the surface became smoother with rms lowering, it suggested that the amount of DMPAMS molecules coupled onto the surface increased and the coverage increased. This trend could be observed from the section plots. There were more peaks on the surfaces in 10 mM DMPAMS with the coupling times of 0.5 and 1 h than with coupling times of 2 and 5 h. After the coupling time arrived at 2 h, the surface undulation flattened.

Figure 5 showed the AFM images of surfaces grafted with DMPAMS of different concentrations for 2 h. Surfaces were mainly covered with uniform and smooth coatings of DMPAMS without significant difference in their morphology. However, from the surface images, it could be found that the surface coverage rose with the increase in DMPAMS

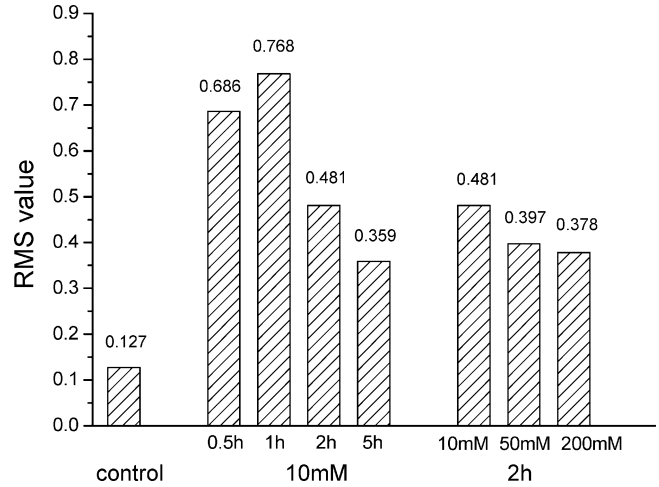


FIGURE 6. Rms values of different samples immobilized in DMPAMS solution with different conditions.

concentration (Figure 6). Accordingly, the surface became much flatter, with the rms value decreased from 0.481 to 0.378 nm.

3.3. In vitro Anticoagulation Properties. In this study, the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) were tested to evaluate the anticoagulation effect of the DMPAMS modified surfaces. The PT indicates the extrinsic pathway of the blood-clotting system; and APTT detects the intrinsic coagu-

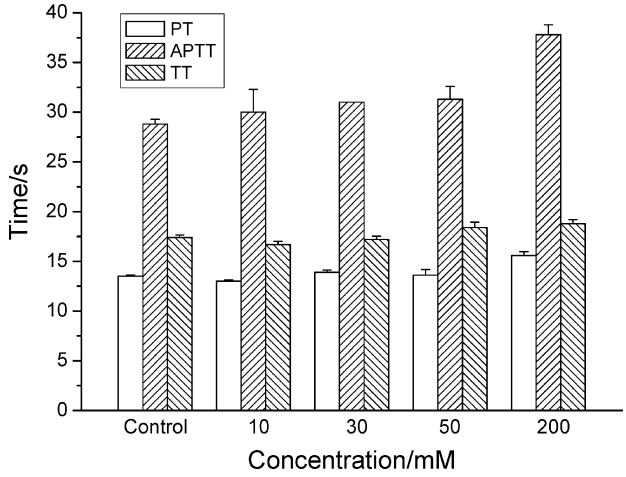


FIGURE 7. Anticoagulation properties of DMPAMS-modified samples formed by soaking in DMPAMS solution with different concentrations (soaking time = 2 h).

lation, i.e., an influence on Factors XIIa, XIa, IXa, VIIIa, and the high-molecular-weight kininogen. TT is the assay for the last step of coagulation, i.e., the thrombin-mediated fibrin formation (30). Therefore, blood plasma APTT, PT, and TT test are commonly used to evaluate the in vitro anticoagulation properties of different biomaterials.

The normal ranges of APTT, PT, and TT for healthy plasma were regarded to be 34 ± 7 s, 14 ± 2 s, and 17.5 ± 2.5 s, respectively. Data of PT, APTT, and TT for the samples grafted with DMPAMS were shown in Figure 7. As for the samples grafted with DMPAMS at relatively low concentration of 10, 30, and 50 mM, no significant changes of the APTTs could be observed along with the increase in the DMPAMS concentrations, compared to the control. When the concentration arrived at 200 mM, the average PT and APTT were significantly prolonged from 13.6 ± 0.12 s and 28.8 ± 0.5 s for control to 15.6 ± 0.36 s and 37.8 ± 1 s, respectively. It demonstrated that the anticoagulation property was evidently improved after the surface was modified with DMPAMS.

3.4. Platelet Adhesion Studies. The adhesive proteins in blood, including fibrinogen (Fg) and von Willebrand's factor (vWf), adsorb to a biomaterial's surface within seconds upon exposure to blood (33). Blood clotting on biomedical devices is the result of a multistep process that involves plasma protein adsorption, platelet adhesion and activation, formation of thrombus, and embolization (34). The adhesion and activation of platelets on the material surface usually lead to coagulation. The activated platelets could further activate a line of coagulation factors, resulting in thrombus on the material surface. Therefore, the in vitro platelet adhesion test could be utilized to investigate the blood compatibility of the material surface. The shapes of the adhered platelets could be carefully observed by the scanning electron microscopy. Once the platelets are activated, "pseudopods" will stretch out accompanied by the aggregation of the platelets. Therefore, the platelet adhesion test is regarded as a valid method to estimate the fundamental hemocompatibility (30).

Figure 8 showed typical SEM photographs of platelet adhesion results for surfaces modified with DMPAMS of

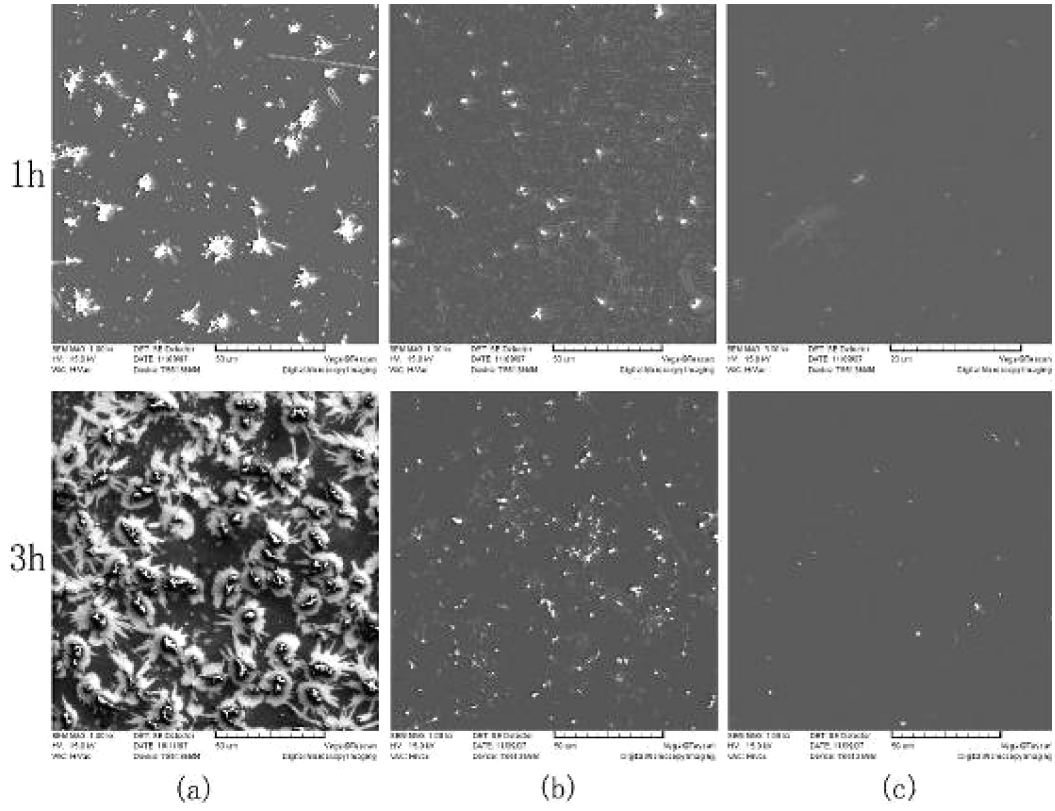


FIGURE 8. SEM micro photographs of surfaces modified by DMPAMS with different solution concentrations in contact with human platelet-rich plasma for 1 and 3 h (magnification = 1000): (a) untreated silicon surface; (b) 30 mM; (c) 200 mM.

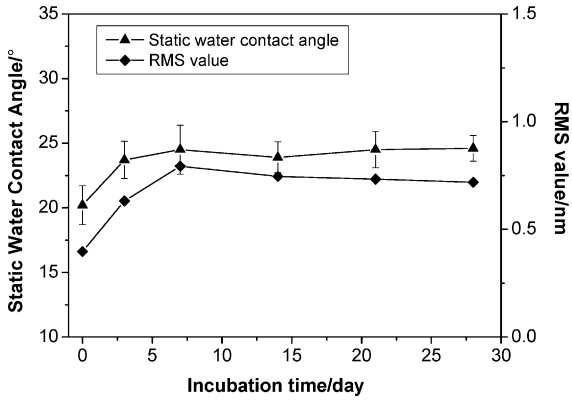


FIGURE 9. Static contact angle and rms value of surfaces modified by DMPAMS (solution concentration = 50 mM, soaking time = 2 h) vs incubation time in PBS ($n = 6$; paired t-test was used and the significant level was set to $P < 0.03$).

different concentrations. It could be seen that the control surface incubated in PRP for 1 h showed a large number of adhered platelets with lots of "pseudopods" stretching out. The amount of platelets adhered on the control surface apparently increased when the contact period prolonged to 3 h, with platelets' shape changed remarkably on control surface at 3 h with a feature of initiated aggregation. Furthermore, thrombus could be found on some areas of the surface. In contrast, the surface grafted with 30 mM DMPAMS effectively reduced the amount of adherent platelets at both 1 and 3 h. Much less shape change of the adherent platelets was observed on DMPAMS modified surface. Almost no platelet attachment could be found on the surfaces grafted with 200 mM DMPAMS even when the contact time arrived at 3 h. From these results, it was suggested that the surface was not completely covered when the concentration of DMPAMS solution was 30 mM which resulted in more adhesion of platelets on the surface. The results proved that platelet adhesion could be effectively prohibited by zwitterionic DMPAMS SAL, resulteding in an improved hemocompatibility.

3.5. Stability of the DMPAMS Self-Assembled Layers. Both the good performance and stability of the SAL are required from its effective applications. The stability of the immobilized DMPAMS self-assembled layer on the silicon surface was examined by the immersion of the modified silicon substrates in a phosphate-buffered solution (PBS) (pH 7.4) for a predetermined period of times. The stability of SAL on the modified substrate was investigated with surface water contact angle and rms value change of the grafted DMPAMS surfaces during the incubation. As shown in Figure 9, the contact angle and rms value increased during the 7 day incubation. The contact angle increased from 20.2 to 24.5°, whereas rms increased from 0.397 to 0.793 nm. This change might relate to the desorption of DMPAMS molecules from the surfaces that were not substantially coupled onto the substrate. However, during the following three weeks of incubation, both the contact angle and rms value maintained a stable low level, indicating that the DMPAMS molecules were covalently coupled to the substrate, resulted in a stable film.

CONCLUSIONS

In this study, a novel zwitterionic silane coupling agent, *N*,*N*-dimethyl, *N*-(2-ethyl phosphate ethyl)-aminopropyltrimethyoxysilane (DMPAMS), was synthesized. Its structure was confirmed using FTIR spectra, ^{1}H NMR, and ^{31}P NMR. The SAL of DMPAMS was effectively constructed onto the silicon surfaces by a simple dip-coating process. The surface hydrophilicity of the modified substrate was improved significantly by this zwitterionic silane coupling agent. The AFM investigation results of the modified silicon surfaces indicated that it took 30 min to obtain a fully covered layer in 10 mM DMPAMS solution. When the soaking time prolonged, the grafting density increased accordingly. In addition, the morphology of DMPAMS-modified silicon surface became much flatter with the increase of the concentration of DMPAMS. The incubation experiment indicated that the self-assembled DMPAMS film was stable in the PBS solution after incubating for one month. The PT and APTT of human blood on modified surface prepared from 200 mM DMPAMS were significantly prolonged from 13.5 and 28.8 s for control to 15.6 and 37.8 s, respectively. From the SEM images, the platelets adhesion onto the modified surface was prohibited, indicating an improved hemocompatibility of silicon surface by this novel zwitterionic silane coupling agent.

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Supporting Information Available: Structural characterization of DMPAMS, including FTIR spectrum, ^{1}H, and ^{31}P NMR spectrum (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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