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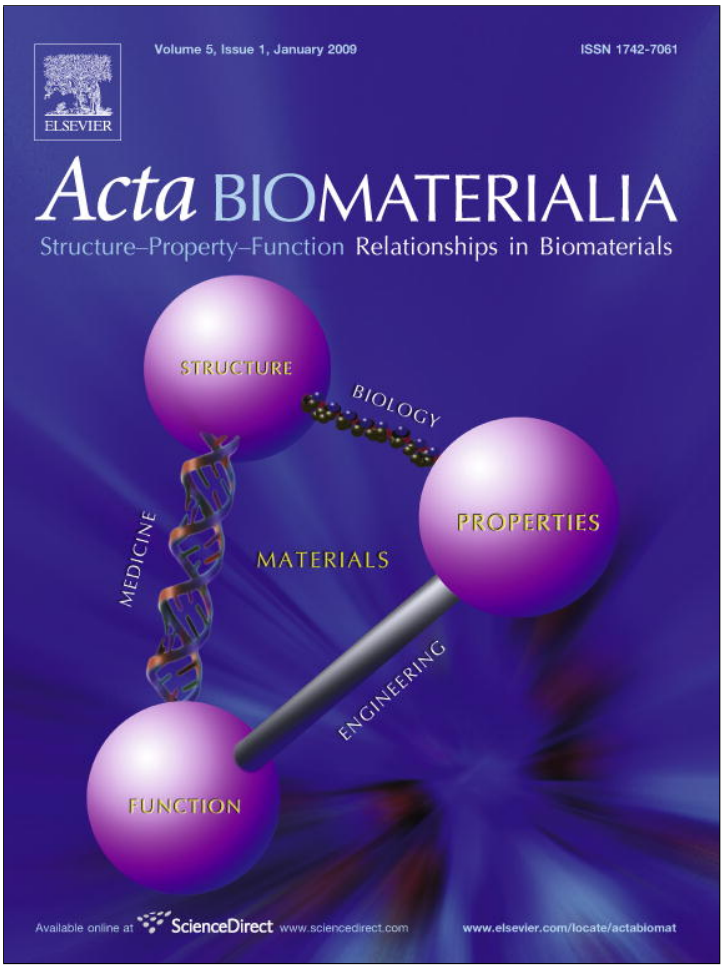

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Hemocompatibility of surface-modified, silicon-incorporated, diamond-like carbon films

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Abstract

The hemocompatibility of plasma-treated, silicon-incorporated, diamond-like carbon (Si-DLC) films was investigated. Si-DLC films with a Si concentration of 2 at.% were prepared on Si (100) or Nitinol substrates using a capacitively coupled radiofrequency plasma-assisted chemical vapor deposition method using a mixed gas of benzene (C_6H_6) and diluted silane (SiH_4:H_2 = 10:90). The Si-DLC films were then treated with O_2, CF_4 or N_2 glow discharge for surface modification. The plasma treatment revealed an intimate relationship between the polar component of the surface energy and its hemocompatibility. All in vitro characterizations, i.e. protein absorption behavior, activated partial thromboplastin time measurement and platelet adhesion behavior, showed improved hemocompatibility of the N_2- or O_2-plasma-treated surfaces where the polar component of the surface energy was significantly increased. Si–O or Si–N surface bonds played an important role in improving hemocompatibility, as observed in a model experiment. These results support the importance of a negatively charged polar component of the surface in inhibiting fibrinogen adsorption and platelet adhesion.
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Keywords: Diamond-like carbon; Surface treatment; Hemocompatibility; Polar component; Platelet adhesion

1. Introduction

Diamond-like carbon (DLC) film has emerged as a promising coating layer for blood-contacting applications owing to its superior mechanical properties, chemical inertness and hemocompatibility [1–7]. Comparative studies have reported that DLC is more hemocompatible than other biomaterials such as Ti, TiN, TiC, CN and polymethylmethacrylate (PMMA) [1,2,8,9]. Because biological reactions essentially occur on the surface, the effects of the atomic bond structure or the surface properties of DLC films on their hemocompatibility have been thus a major concern [1,2,10]. However, no consistent relationship has been found between hemocompatibility and the atomic bond structure of the films or the wettability of their surfaces. For example, Kwok et al. [11] and Huang et al. [12] reported that surfaces with lower wetting angles had improved blood compatibility. Ma et al. also reported the higher albumin to fibrinogen absorption ratios on surfaces with higher surface energy [13]. However, Leach et al. observed self-contradictory behavior in hydrophilically coated guide wires and catheters [14]. They reported that hydrophilically coated guide wires suppressed clot deposition while the same coating on catheters enhanced clot deposition. In contrast, Hasebe et al. observed that hemocompatibility was improved in fluorine-incorporated DLC

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films [15,16], where the surface energy decreased with fluorine incorporation. Jones et al. explored platelet attachment on Ti, TiN, TiC and DLC surfaces [17]. The greatest platelet spreading was seen on the more hydrophilic surfaces, even if these authors did not consider the chemical effects of different materials.

In this study, we investigated the hemocompatibility of surface-modified silicon-incorporated DLC (Si-DLC) films using a plasma surface treatment method. The plasma surface treatment enabled us to address the role of surface chemical bonds. It was recently reported that Si incorporation into DLC films improves both corrosion resistance in body fluid conditions and mechanical reliability, with higher interfacial toughness [18,19]. Si-DLC coating is thus a strong candidate as a protective layer for implant materials to avoid the release of metal ions. Plasma treatment of the surface using O_2, N_2 or CF_4 glow discharge resulted in a variety of surfaces ranging from hydrophilic to hydrophobic, corresponding to changes in the polar and dispersive components of the surface energy [20]. In vitro hemocompatibility tests revealed that hydrophilic surfaces with greater polar components of the surface energy suppressed fibrinogen adsorption and platelet adhesion and activation. A model experiment revealed that Si–O or Si–N bonds on the surface play an important role in improving hemocompatibility, presumably due to the negatively charged polar component.

2. Materials and methods

2.1. Si-DLC deposition

The Si-DLC films were deposited on Si (100) or electrochemically polished Nitinol (NiTi) substrates using a radio-frequency plasma-assisted chemical vapor deposition (RF–PACVD) method. Details of the deposition system have been reported previously [18,19]. The films on the Si wafers were used for surface property characterization via wetting angle measurement and X-ray photoelectron spectroscopy (XPS) analysis. All in vitro hemocompatibility tests were performed using the films deposited on the Nitinol plates. A mixture of benzene and diluted silane (SiH_4:H_2 = 10:90) was used as the precursor gas. The substrates were initially cleaned with an argon discharge for 15 min at a bias voltage of –400 V and a pressure of 0.49 Pa. An interlayer of amorphous silicon (a-Si:H) with a thickness of nearly 5 nm was deposited on the substrates to ensure better adhesion of the Si-DLC films. The Si-DLC film was then deposited at a bias voltage of –400 V for 11 min 40 s at a pressure of 1.33 Pa. The film thickness of the Si-DLC films was 0.55 μm as measured using an alpha step profilometer. Rutherford backscattering spectrometry showed that the Si concentration in the films was 2 at.%. The structure and mechanical properties of Si-DLC film have previously been investigated in detail [18].

The Si–DLC films were then treated with RF glow discharge of various gases, i.e. such as O_2, N_2 and CF_4, in the same PACVD chamber. The plasma treatments were performed for 10 min at a bias voltage of –400 V and a pressure of 1.33 Pa. The plasma treatment did not change the film thickness significantly except for the O_2 plasma treatment. The O_2 plasma treatment etched the Si-DLC films and reduced the film thickness to 0.31 μm. The surface properties and chemical bonds of the plasma-treated Si-DLC films were reported by Roy et al. [20].

2.2. Wetting angle and XPS measurements

The surface energies of the samples were characterized according to the method of Owens [20–22]. Young's equation for wetting of a solid surface by a liquid can be expressed in terms of the dispersive and polar components of the surface energy of liquid and solid as follows:

$$1+\cos\theta = 2\left(\frac{\sqrt{\gamma_{sv}^{d}}\sqrt{\gamma_{lv}^{d}}}{\gamma_{lv}}+\frac{\sqrt{\gamma_{sv}^{p}}\sqrt{\gamma_{lv}^{p}}}{\gamma_{lv}}\right), \quad (1)$$

where θ is the contact angle between the solid and liquid and γ_{tv}, γ_{sv} are the free energies of the liquid and solid against their saturated vapor, respectively. The superscripts d and p refer to the dispersive and polar components, respectively. The wettability of the film surfaces was characterized by measuring the θ of two different liquids with known values of dispersive and polar components of the surface energy by solving the simultaneous equations. In the present work, deionized water and formamide were used [21]. The contact angle measurements were performed using a contact angle goniometer (Rame-Hart Inc., USA). The reported contact angles in this paper correspond to an average of 10 measurements.

XPS measurements of the surface were performed using a Physical Electronics PHI 5800 ESCA system. The X-ray source used was Al K_α at 1486.6 eV and the anode was maintained at 250 W, 10 kV and 27 mA. The XPS measurements were done at a chamber pressure of 2×10^{-8} Pa. For high-resolution measurement, analyzer pass energy was 58.70 eV (energy resolution 0.125 eV). The spot size of the beam was 400 μm × 400 μm. The calibrations of peak position were done by taking the C1s peak at 284.6 eV. The curve fittings were carried out with a mixture of Gaussian and Lorentzian functions. The percentages of individual peaks were determined using the peak areas of individual peaks and the corresponding atomic sensitivity factor of the element [23].

2.3. Protein adsorption tests

Plasma protein adsorption tests were performed by treating the samples with bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA) and fibrinogen (Sigma–Aldrich) solutions and measuring the absorbance through enzyme-linked immunosorbent assay (ELISA) analysis. The proteins were dissolved separately in phosphate-buf-

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fered saline (PBS, pH 7.4) at concentrations of 3 and 0.2 mg ml^{-1}, respectively. The above concentrations of albumin and fibrinogen were chosen based on their concentrations in human blood (albumin: 35–55 mg ml^{-1} and fibrinogen: 2–4 mg ml^{-1}). The samples (1 cm × 1 cm) were initially rinsed with a PBS solution and placed in the protein solutions at 37 °C for incubation times of 5 and 60 min. After incubation, the samples were carefully washed in distilled water to completely remove unadsorbed proteins. The washed samples were treated with 5% sodium dodecyl sulphate and rinsed at 37 °C overnight followed by ultrasonication for 10 min to desorb the proteins from the specimen. The resultant solutions were loaded into 96-well plates along with Micro BCA working solution and incubated at 60 °C for 1 h. The absorbance of each well was measured at 562 nm using an ELISA reader and the adsorbed protein concentrations were evaluated using calibration curves.

2.4. Activated partial thromboplastin time (aPTT) measurements

Fresh human blood mixed with sodium citrate was centrifuged at 2000*g* for 10 min to obtain platelet-poor plasma (PPP). The specimens (1 × 1 cm) were incubated in PPP at 37 °C for 30 and 60 min. The reaction plasma was treated with Actin FS solution and 0.025 M $CaCl_2$ solution in an analyzer at 37 °C to determine the aPTT. The aPTT measurements were done using a Sysmex CA-50 Instrument.

2.5. Platelet adhesion test

Human whole blood (45 ml) was collected from healthy volunteers who had not taken any medication for at least 10 days. The blood was mixed with 5 ml of acid–citrate–dextrose, and platelet-rich plasma (PRP) was isolated by centrifugation at 180*g* for 15 min, which separated blood cells. Subsequently, a portion of the PRP was centrifuged at 2000*g* for 10 min to obtain PPP. The PRP platelet density was adjusted to 3.0×10^5 μl^{-1} by dilution with PPP. Sample disks were washed with PBS and then incubated in 24-well plates containing 1 ml of adjusted PRP at 37 °C for 30 min in an atmosphere containing 5% CO_2 gas. Thereafter, the supernatant was discarded and the samples were washed with PBS. Adherent platelets were then fixed for 60 min at room temperature in 0.8 ml of freshly prepared 1% glutaraldehyde. After fixation, the samples were washed and dehydrated in a graded ethanol series (20%, 40%, 60%, 80%, 90% and 100% for 15 min each) as described by Frank et al. [24]. All dried materials were examined by *scanning electron microscopy* (SEM; Sirion FEI) and fluorescence microscopy (Eclipse 50*i*, Nikon, Tokyo, Japan). The morphology of adherent platelets was observed by SEM. The platelet-covered area per unit area (67,500 μm^2) was investigated via photographs using computer-aided image analysis software (Image-Pro-Plus, Media Cybernetics, USA). Measurements were performed at 10 randomly selected areas on each surface. The results of the experiments are expressed as the means of coverage/unit area ± standard deviation. Values were compared by *t*-test (Student's *t*-test in Microsoft Excel), and differences were considered statistically significant when $P < 0.05$.

3. Results and discussion

Fig. 1 shows the water contact angles and surface energies of the Si substrate and Si-DLC and plasma-treated Si-DLC films. The O_2-plasma-treated Si-DLC films have the most hydrophilic surface, with a water contact angle of 13.4°, whereas a hydrophobic surface was obtained by CF_4 plasma treatment. The Si substrates had a high polar component in their surface energies, due to the native silicon oxide layer on their surface. The Si-DLC coatings decreased the polar component while the dispersive component was slightly increased. These plasma treatments of the Si-DLC coatings mostly affect the polar component of surface energies. The dispersive component was found to remain nearly constant for all the films except the O_2-plasma-treated films. The hydrophobicity of the CF_4-plasma-treated surfaces originates from a substantial decrease in the polar component of the surface energy. N_2 plasma treatment increases the polar component by about three times compared to that of the deposited Si-DLC films, which results in a lower contact angle. O_2 plasma treatment increased the polar component by about five times compared to that of deposited Si-DLC films, while the dispersive component was reduced to half of the untreated value. The polar components attract the electric dipoles of water, which minimizes the interfacial energy and lowers the water contact angle. Because of a possible aging effect of the hydrophilic surface, we performed the following in vitro hemocompatibility tests within 12 h of the surface treatment.

The interaction of biomaterials with blood starts with the adsorption of plasma proteins onto their surfaces.

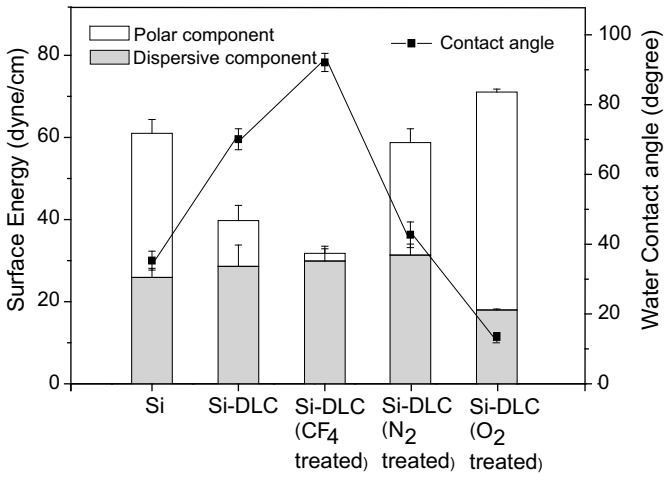

Fig. 1. Water contact angle and surface energies of plasma-treated Si-DLC films.

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The first proteins that are adsorbed on the surface of biomaterials are albumin, fibrinogen and fibronectin. Adsorption of albumin retards the adhesion and activation of platelets, while adsorption of fibrinogen promotes platelet adhesion and activation. The adsorbed fibrinogen is converted into insoluble fibrin polymer, which finally results in the formation of thrombus [13,25]. Fig. 2a and b show the amounts of albumin and fibrinogen adsorption, respectively, on Si-DLC and plasma-treated Si-DLC films after incubation for 60 min. The plasma protein adsorption tests showed higher albumin adsorption on all the O_2-, N_2- and CF_4-plasma-treated films without any considerable difference between them (see Fig. 2a). Fig. 2b shows the fibrinogen adsorption data for various Si-DLC surfaces. Because of large error in the fibrinogen adsorption on the untreated Si-DLC surface, no significant difference was observed between the untreated and the plasma treated Si-DLC coatings. However, fibrinogen adsorption on the O_2-plasma-treated Si-DLC films is distinctly smaller than those on the CF_4- and N_2-plasma-treated Si-DLC films (Fig. 2b).

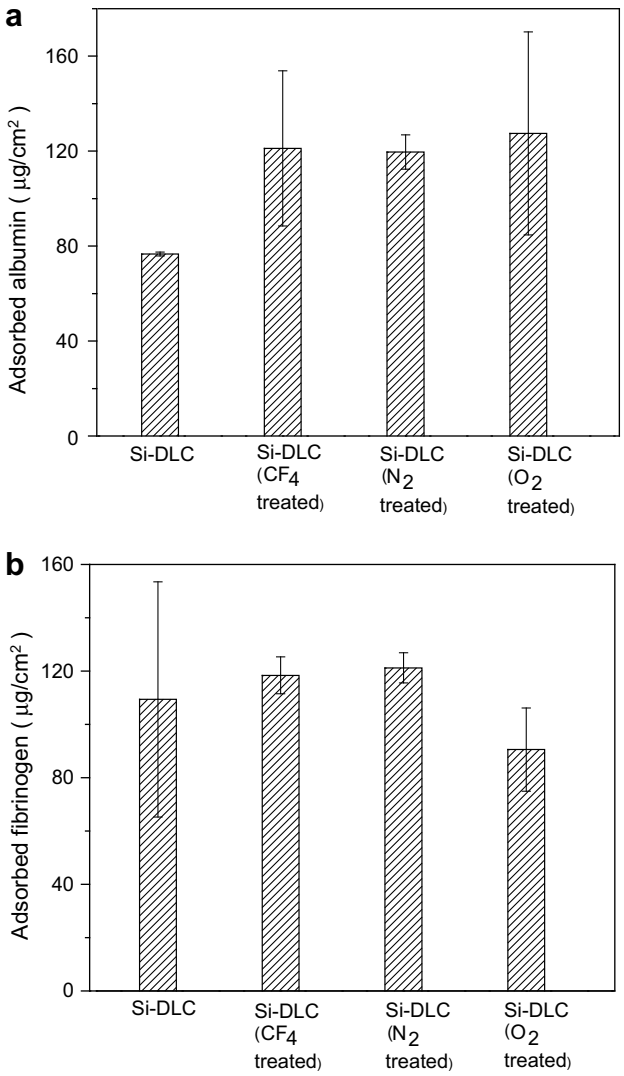

Fig. 2. (a) Albumin adsorption of Si-DLC and plasma-treated Si-DLC films after incubation for 60 min. (b) Fibrinogen adsorption of Si-DLC and plasma-treated Si-DLC films after incubation for 60 min. (c) Albumin to fibrinogen ratios of Si-DLC and plasma-treated Si-DLC films after incubating for 5 and 60 min.

Docoslis et al. observed similar behavior of albumin adsorption for hydrophobic talc and hydrophilic silica films [26]. They indicated that the amount of adsorbed protein on a material surface is related to the ratio of favorable macroscopic attractive forces to unfavorable microscopic repulsive forces and to the average ratio of favorable (attractive) and unfavorable (repulsive) orientations of the protein molecules. Protein adsorption on a biomaterial surface is also dependent on the specific adsorption and desorption rate constants. For hydrophobic talc, they obtained a higher ratio of attractive to repulsive forces, which gave rise to the higher albumin adsorption of talc. The high albumin adsorption of CF_4-plasma-treated Si-DLC films in this study can be explained by the strong hydrophobic interactions between the protein molecules and hydrophobic film surfaces. Increases in binding energy of 5.3 and 12.8% for human serum albumin (HAS)–talc and HAS–silica, respectively, have also been observed [27]. It can be thus said that the higher albumin adsorption of N_2- and O_2-plasma-treated Si-DLC films is caused by a higher polar component in their surface energy, as shown in Fig. 1. Albumin with its hydrophilic nature is attracted to these surfaces through electrostatic interactions.

While albumin adsorption increased for both hydrophobic and hydrophilic surfaces, fibrinogen adsorption is more strongly dependent on surface properties, as proposed by Slack and Horbett [28] and Ta et al. [29]. Slack and Horbett observed that fibrinogen adsorption was lower on hydrophilic glass compared with hydrophobic materials such as polystyrene, polyethylene and silicone rubber. Protein adsorption from blood plasma is governed by the Vroman effect, which involves a complex series of adsorption and displacement steps [30]. It was reported that adsorbed fibrinogen molecules undergo a transition from a weakly bound (displaceable) to a tightly bound (non-displaceable) state most rapidly or effectively on polystyrene followed by silicon rubber, polyethylene and finally glass [30]. Ta et al. [29] proposed a strong hydrophobic interaction between the D domains of fibrinogen and the highly ordered pyrolytic graphite (HOPG) surface, leading to a tightly bound fibrinogen film. Conversely, the electrostatic interaction between the negatively charged hydrophilic surface and the positively charged αC domains of fibrinogen is weak in nature due to the low degree of hydrophobicity. The present results are consistent with these arguments, i.e. the high fibrinogen adsorption of CF_4-plasma-treated Si-DLC films is due to the strong hydrophobic interaction between fibrinogen molecules and the hydrophobic film surface, whereas the low fibrinogen adsorption of O_2-plasma-treated Si-DLC films originates from the weaker electrostatic interactions between fibrinogen molecules and the hydrophilic film surface.

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The aPTT determines the ability of blood to coagulate through the intrinsic coagulation mechanism. It measures the clotting time from the activation of factor XII through to the formation of fibrin clot [31]. The aPTT also governs how a biomaterial affects the coagulation time. The enzymatic activities that lead to clot formation are measured through aPTT measurement. In Fig. 3, the aPTT of untreated Si-DLC film is compared with those of the plasma-treated films for an incubation time 60 min. It is evident that the O_2-plasma-treated Si-DLC films had a higher aPTT. This indicates that the O_2-plasma-treated Si-DLC films have a tendency to retard the intrinsic coagulation activities of blood compared with the other samples, which is consistent with the protein adsorption behavior. Similar behavior was observed with N_2-plasma-treated Si-DLC films. In contrast, CF_4 plasma treatment did not induce a notable change in aPTT when compared with untreated Si-DLC film.

Platelet adhesion and activation on the surface of a biomaterial is the most essential character in determining the hemocompatibility of a biomaterial. Low platelet adhesion and activation denotes good hemocompatibility, while a higher degree of platelet adhesion and activation should result in the formation of a thrombus. Fig. 4 shows the morphology of adherent platelets on the Nitinol and plasma-treated Si-DLC surfaces. Activation of platelets can be judged by changes in their morphology: as activation proceeds, the platelets lose their round shape, form pseudopodia and spread on the biomaterial surface. In the case of Nitinol substrates, most platelets on the surface remained round, with only a few of the platelets seen in high-magnification SEM losing their round shape (Fig. 4a). Pseudopodia formation is observed in the activated platelets, as indicated by an arrow. On the Si-DLC film, most platelets are clearly activated, as seen in Fig. 4b: platelets spread on the surface (arrow in Fig. 4b) with tangled pseudopodia. Similar behavior was observed on the CF_4-plasma-treated Si-DLC surface shown in Fig. 4c. However, the degree of platelet activation was much smaller on the N_2- and O_2-plasma-treated Si-DLC surfaces, as shown in Fig. 4d and e, respectively. Low-magnification SEM microstructures clearly show that most platelets kept their round shape. Activation of platelets is barely observed, although platelets of irregular shape can be seen in the high-magnification SEM image. The platelet adhesion area ratios are summarized in Fig. 5. The N_2- and O_2-plasma-treated Si-DLC films were found to have considerably reduced platelet adhesion compared with untreated and CF_4-plasma-treated Si-DLC films. This result is consistent with the results of the protein adsorption and aPTT measurements.

The present results show that N_2 or O_2 plasma treatment improved the hemocompatibility of Si-DLC film. The major difference in the N_2- or O_2-plasma-treated Si-DLC surfaces from the untreated surfaces is the surface chemical bonds: N_2-plasma-treated surfaces have C–N bonds and Si–N bonds in addition to C–C and C–Si bonds, while O_2-plasma-treated surfaces have C–O and Si–O bonds [20]. In order to explore the effects of each surface bond on hemocompatibility, pure hydrogenated amorphous carbon (a-C:H or DLC) and hydrogenated amorphous silicon (a-Si:H) were prepared and treated with N_2 and O_2 glow discharge. a-C:H films were deposited using benzene as the precursor gas. Diluted silane was used to

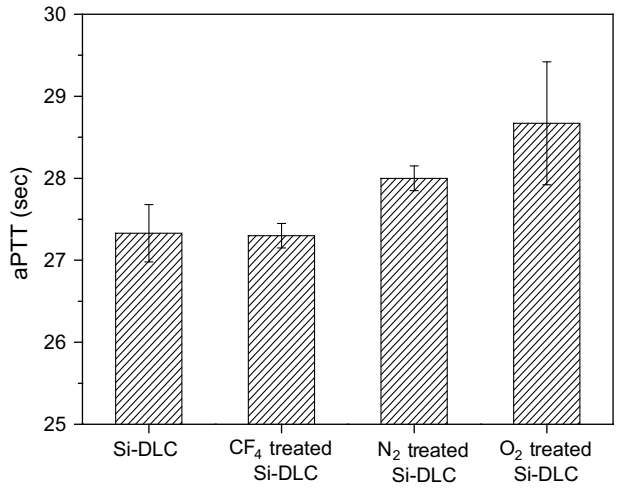

Fig. 3. The aPTTs of as deposited and O_2-plasma-treated Si-DLC films. The times in parenthesis are the incubation times in PPP.

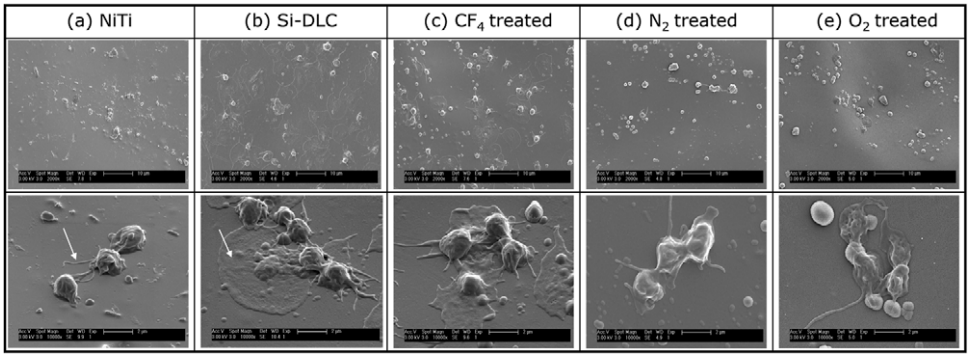

Fig. 4. The morphology of blood platelets on (a) Nitinol substrate, (b) as-deposited Si-DLC, (c) CF_4-plasma-treated Si-DLC, (d) N_2-plasma-treated Si-DLC films, and (e) O_2-plasma-treated Si-DLC films.

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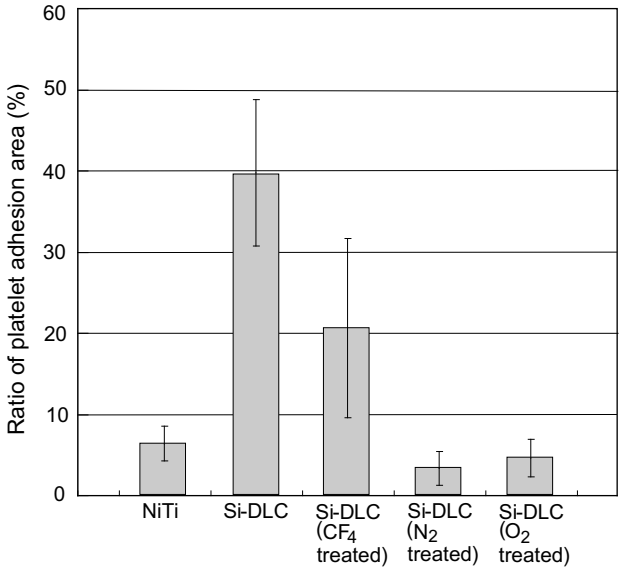

Fig. 5. The platelet adhesion area ratios of Nitinol substrate, as-deposited Si-DLC film, and Si-DLC film plasma-treated with CF_4, N_2 and O_2.

prepare the a-Si:H film. Other deposition and plasma-treatment conditions were the same as those for the Si-DLC films.

Fig. 6 shows the XPS analysis of the as-deposited and the plasma-treated a-C:H and a-Si:H films. A small amount of oxygen was observed in all films due to exposure

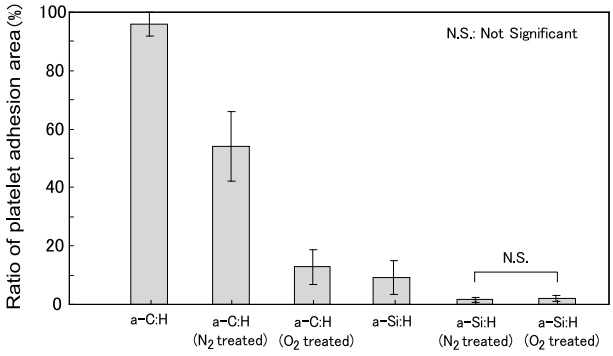

Fig. 7. The platelet adhesion area ratios of a-C:H and a-Si:H surfaces with and without O_2 or N_2 plasma treatment.

to ambient air before XPS measurement. However, it is evident that O_2 plasma treatment of a-C:H results in the formation of C–O surface bonds (Fig. 6b), while N_2 plasma treatment results in C–N and C≡N bonds on the surface (Fig. 6c). Similar behavior was observed with a-Si:H film as shown in Fig. 6d–f. Si–O bonds and Si–N bonds dominate on the surfaces that have undergone O_2 and N_2 plasma treatment, respectively. All the oxide and nitride peaks (C–O, C≡N, Si_x–O_y and Si_x–N_y) have large values of full-width at half maximum (FWHM). The large value of FWHM is presumably due to the distortion of the bond by ion bombardment during plasma treatment. Fig. 7 shows the platelet adhesion surface area ratios of these

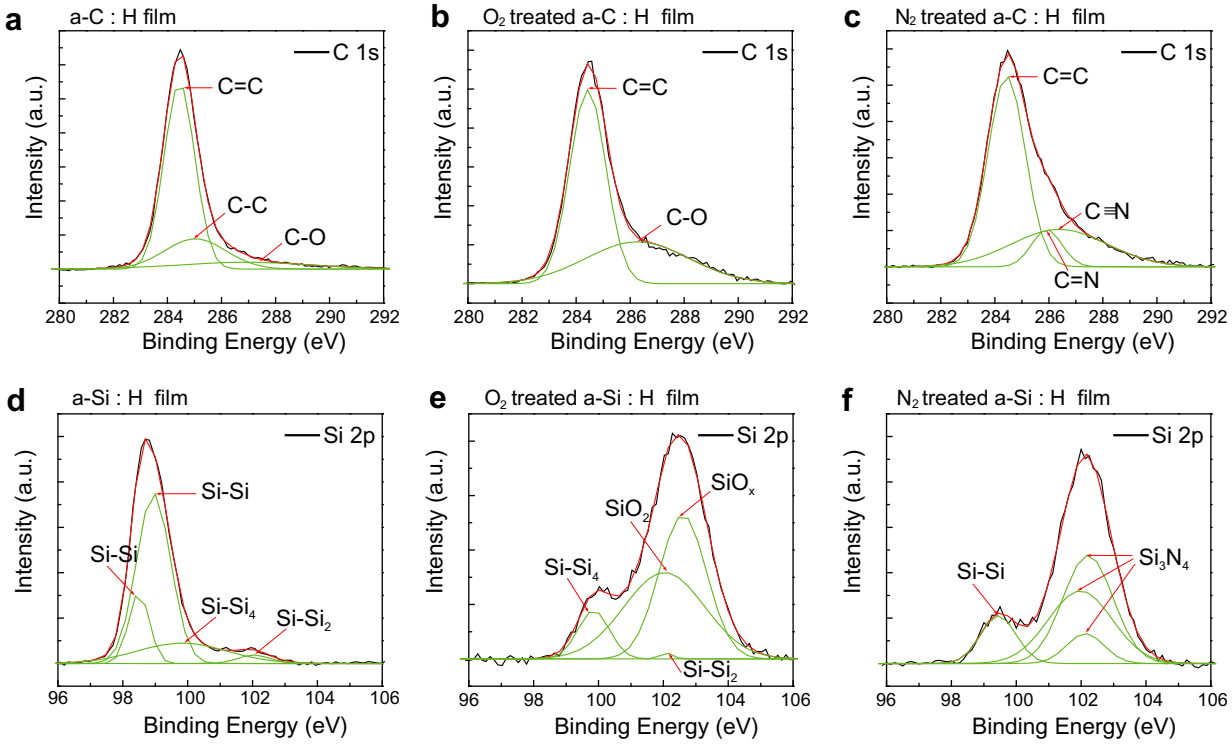

Fig. 6. High-resolution C1 s XPS spectra of as-deposited a-C:H (a), O_2-plasma-treated a-C:H (b) and N_2-plasma-treated a-C:H (c). High-resolution Si 2p XPS spectra of as-deposited a-Si:H (d), O_2-plasma-treated a-Si:H (e) and N_2-plasma-treated a-Si:H (f). Chemical bonds are identified according to Ref. [34].

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specimens. Both N_2 and O_2 plasma treatment improve the hemocompatibility of a-C:H and a-Si:H films. However, the plasma treatment of a-Si:H films had more significant effects in suppressing platelet adhesion, which reveals the importance of the Si–N or Si–O bonds. This observation can be understood if one considers the larger difference in the electronegativity between Si and N ($\Delta\chi = 1.14$ on the Pauling scale) and between Si and O ($\Delta\chi = 1.54$) than those between C and N ($\Delta\chi = 0.49$) and between C and O ($\Delta\chi = 0.89$). A higher degree of negatively charged polarity is expected in Si–O and Si–N bonds than in C–N and C–O bonds. The negatively charged polarity would suppress platelet adhesion and fibrinogen adsorption as the platelets and proteins tend to have a net negative zeta potential [16]. In this study, N_2- or O_2-plasma-treated Si-DLC surfaces include Si–O and Si–N surface bonds, which results in a higher degree of negative charge polarity.

Recently, Okpalugo et al. [32] and Ong et al. [33] studied the effect of Si incorporation into DLC films on hemocompatibility. They observed that platelet adhesion was suppressed as the Si concentration increased. This observation is closely related with the Si–O surface bonds, because the surface oxygen concentration was observed to increase with increasing Si concentration [33]. Ong et al. further reported that the polar component of the surface energy increased with Si concentration [33]. It is thus possible to have higher surface polarization in films of higher Si concentration. Their observations support the present suggestion that the negatively charged polar component caused by Si–O surface bonds improves the hemocompatibility.

4. Conclusions

Surface modification of Si-DLC films by plasma treatment enabled us to explore the effects of surface chemical bonds on hemocompatibility. A hydrophilic surface was obtained using N_2 and O_2 plasma treatment due to a significant increase in the polar component. In contrast, CF_4 plasma treatment gives rise to a hydrophobic surface with a low polar component. It was evident from the results of the protein adsorption tests, aPTT measurement and platelet adhesion test that the hydrophilic surface with a large polar component of the surface energy exhibits improved hemocompatibility. It was further observed that Si–N and Si–O surface bonds are more effective in improving the hemocomptibility than C–O or C–N bonds. These observations support the importance of the negatively charged polar component of the surface energy in inhibiting fibrinogen adsorption and platelet adhesion.

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