In-vitro cellular behavior on amorphous carbon containing silicon

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Abstract

Amorphous carbon has been studied for its biological properties. Doping can alter the film structure to achieve certain desirable properties, like lowering of stress. The incorporation of a secondary element however also alters the film surface properties, which in turn, the biological response. We have investigated the response of fibroblast and osteoblast-like cells on amorphous carbon and amorphous carbon containing silicon. The amorphous carbon with the highest silicon concentration exhibits higher surface energy, with higher polar component. Both cell types adhered, spread and proliferated well on all films, especially on the one with the highest silicon content.

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1. Introduction

Amorphous carbon (a-C) traditionally known for its high hardness and elastic modulus, low roughness, low coefficient of friction and stiction [1], and high corrosion resistance [2], is now being identified as a potential candidate for biological applications. This synthetic carbon film has shown to be compatible with a variety of living cells [3–9], and has low thrombogenicity as well [10–16]. However, a-C is not without its shortcoming. The film is highly stressed after the synthesis, and this cannot be avoided, as it is the by-product of the formation of diamond-like phase in the a-C [17]. If the stress issue is left untreated, film delamination and loss of material can occur, which will render the film useless for any practical application. It is particularly more important in biological applications, as film failure threatens to impair the function of devices and compromise the health of the patient. Impurity doping is one way to reduce the stress. Silicon incorporation into a-C is found to reduce the stress significantly [18,19]. And the compatibility of silicon-doped hydrogenated a-C with living cells is found to be promising [20,21] as well. Surface properties like surface energies and roughness of the materials are crucial aspects when considering cell-material interaction. In this study, we study the affinity of fibroblast and osteoblast-like cells on unhydrogenated amorphous carbon containing silicon based on the influence of the surface energies of the films.

2. Experimental

2.1. Film deposition and roughness

Unhydrogenated amorphous carbon films containing silicon were sputtered from graphite (99.995% purity) and silicon (99.999% purity) targets on (100) monocrystalline silicon wafers. Prior to deposition, the wafers were chemically cleaned in piranha bath and treated with Ar plasma for 20 min at a radio frequency (RF) induced substrate bias of −300 V to remove surface oxides. The graphite target power density was 7.4 W/cm\textsuperscript{2}, and the silicon target power densities were 1.0 and 2.5 W/cm\textsuperscript{2} to vary Si concentration. The base pressure (4.5×10\textsuperscript{−3} Pa), process pressure (800×10\textsuperscript{−3} Pa), Ar flow rate (50 sccm) and substrate bias voltage (−10 V) all remained constant. The surface roughness was characterized by Shimadzu 9500J2 atomic force microscope under constant force in contact mode.

2.2. Film composition and structure analysis

Atomic concentration of C and Si was characterized by X-ray photoelectron spectroscopy (XPS) using Kratos AXIS X-
ray photoelectron spectrometer equipped with a monochromatic Al–Kα (1486.71 eV) X-ray radiation operating at 15 kV and vacuum of 10⁻⁶ Pa. The bonding of a-C(Si) was characterized by Raman spectroscopy using Renishaw Raman Spectroscope RM1000 excited with a HeNe laser at a wavelength of 633 nm by Raman spectroscopy using Renishaw Raman Spectroscope RM1000 excited with a HeNe laser at a wavelength of 633 nm and laser power of 1 mW. The peak deconvolution was done using Gauss–Lorentz distribution function, and the fitted curves have reduced Chi-square of 1.2 or less to ensure convergence.

2.3. Surface energy determination

The surface free energy of the films was determined by contact angle measurements. The Lifshitz–van der Waals/acid–base (van Oss) approach [22] was employed to interpret the contact angle measurements. In this method, the surface tension is differentiated into three components: the apolar Lifshitz–van der Waals (LW) component, the polar acid (+) and base (−) components. The total surface tension is given by

\[ \gamma_{\text{tot}} = \gamma_\text{LW} + 2\sqrt{\gamma_+ \gamma_-} \]  

(1)

Where \( \gamma^+ \) is the acceptor and \( \gamma^- \) the donor part of the surface energy. The interfacial tension was postulated both of solid–liquid and liquid–liquid systems as

\[ \gamma_{12} = \gamma_1 + \gamma_2 - 2\sqrt{\gamma_1 \gamma_2} - 2\sqrt{\gamma_1^+ \gamma_2^-} - 2\sqrt{\gamma_1^- \gamma_2^+} \]  

(2)

For solid–liquid systems, combining Eq. (2) with Young’s equation: \( \gamma_\text{lv} \cos \theta = \gamma_\text{sv} - \gamma_\text{sl} \) [23] (where \( \theta \) is the measured contact angle, \( \gamma_\text{lv}, \gamma_\text{sv} \) and \( \gamma_\text{sl} \) are the liquid–vapor, solid–vapor and solid–liquid interfacial tension respectively) yields

\[ \gamma_1 (1 + \cos \theta) = 2\sqrt{\gamma_1 \gamma_2} - 2\sqrt{\gamma_1^+ \gamma_2^-} - 2\sqrt{\gamma_1^- \gamma_2^+} \]  

(3)

Eq. (3) can be used to determine the three surface tension components (\( \gamma_\text{LW}^+, \gamma_\text{LW}^- \) and \( \gamma_\text{s} \)) by measuring contact angles and solving three simultaneous equations. To do this, three liquids of known surface tension are required. We used deionized water, formamide and diiodomethane with known surface tension values [24]. Contact angle measurements were conducted using sessile drop technique with First Ten Angstroms 200 Goniometer.

2.4. Cell culture

Two types of cell-line were used in this study: African green monkey kidney fibroblast COS7; and human osteosarcoma MG63. Both cell-lines were obtained from American Type Culture Collection (ATCC, Rockwell, MD). Cell cultures were conducted at 37 °C in a humidified 5% CO₂ atmosphere. The standard medium used for culturing COS7 was Dulbecco’s modified eagle’s medium (DMEM, ATCC) supplemented by 10% fetal calf serum (FCS, ATCC) and 1% penicillin (ATCC), while for MG63 was Eagle’s minimum essential medium (EMEM, ATCC), also supplemented by 10% fetal calf serum (FCS, ATCC) and 1% penicillin (ATCC). For cell assay, the a-C samples were diced into 10×10 mm and subjected to UV exposure before being placed in 24-well culture dish for cell culturing. Seeding concentration of both cell-lines was 4×10⁴ cells/ml. Viable cells (unstained by the added trypan blue) were counted using hemacytometer. Statistical analysis was carried out on cellular test using one-way analysis of variance (ANOVA) at an average of three replicates to compare data between different periods of culture. Student’s \( t \)-test was carried out to compare the culture data between coatings on each count. Statistical significance was considered at \( p<0.05 \).

3. Results and discussion

The Si atomic concentration of the three a-C films as determined by XPS are determined to be 0 at.% Si, 16.6 at.% Si and 37.6 at.% Si after 5 min of Ar ion etching for the removal of surface oxides and contaminants.

Fig. 1 shows a typical Raman spectrum of a-C. The spectrum can be deconvoluted into two peaks; the graphitic G- and disorder D-peak, where the D-peak is the shoulder of the G-peak at lower wavenumbers. The presence of the D-peak is due to the presence of C sp² aromatic ring structures. Fig. 2 clearly
shows the decrease in the relative intensity of the D-peak. The breaking of the sp² aromatic rings bonding structure will cause a decrease in the intensity of the D-peak. This signifies an increase in the overall disordering of the C-network, and enhances the chance of sp³ formation. The decrease in $I_D/I_G$ intensity ratio with increasing Si corresponds to the decrease in the average crystallite size of sp²-bonded clusters [25], as well as the increase in sp³ fraction [26]. Raman spectroscopy results suggest Si atoms preferentially substitute the sp²-hybridized C atoms during sputter implantation; the incorporation of Si breaks the sp²-hybridized aromatic ring bonding structures, and removes two $\pi$ bonds for each C atom substitution and thus promotes the formation of sp³-hybridized C–C bonding configuration.

Surface energies are calculated from the contact angles of the three test liquids, and are presented in Fig. 3. The dispersive component of surface energy decreases initially at low Si concentration and reached a minimum at Si concentration of $\sim 16.6$ at.%, and then follow by a rise as the Si concentration increases further. The polar component, on the other hand, increases with Si concentration.

There are relatively fewer dangling bonds at the surface of hydrogenated a-C when most are terminated by H, which has only one electron available for bonding. The tendency for the surface atoms to bond with other atoms in the atmosphere is hence reduced. Unlike hydrogenated a-C, the surface atoms of hydrogen-free a-C films are not fused and are free to bond with atoms in the atmosphere, especially oxygen. After the surface adsorption of atmospheric oxygen, polarity increases because Si–O and C–O bonds are polar in nature. The greater difference in electronegativities between oxygen ($\sim 3.5$) and silicon ($\sim 1.8$) as compared to carbon ($\sim 2.5$) implies that there is a higher affinity of oxygen with silicon. Therefore, there is an increase in the surface oxygen level when Si concentration is increased, hence resulting in greater polar component.

<table>
<thead>
<tr>
<th>Film</th>
<th>O (at.%)</th>
<th>C (at.%)</th>
<th>Si (at.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-C</td>
<td>9.3</td>
<td>90.7</td>
<td>0</td>
</tr>
<tr>
<td>a-C(16.6at.%Si)</td>
<td>25.1</td>
<td>59.9</td>
<td>15.0</td>
</tr>
<tr>
<td>a-C(37.6at.%Si)</td>
<td>26.5</td>
<td>46.0</td>
<td>27.5</td>
</tr>
</tbody>
</table>

Fig. 4. Surface roughness of a-C and a-C(Si) films.

Fig. 5. Cell attachment number of (a) COS7 fibroblast; and (b) MG63 osteoblast-like cells. The values are mean of three replicates, while the error bars denote the standard error. ANOVA shows that both cell-types proliferated on a-C and a-C(Si) with $p<0.001$. *denotes significant difference between COS7 count on a-C(Si16.6at.%) and a-C (*$p<0.001$); and ** denotes significant difference between MG63 count on a-C(Si37.6at.%) and a-C (**$p<0.05$).
surface composition (scanned prior to Ar ion etching) as determined by XPS is presented in Table 1.

The dispersive component on the other hand is directly related to the dispersive or induced dipole-to-dipole forces of the atoms in the films, and this force is governed by the following relationship

$$F \propto \frac{\alpha}{r^3} \left[ \frac{d}{dr} \left( \frac{1}{r^2} \right) \right].$$

(4)

Where $\alpha$ is the polarizability, and $r$ the inter-atomic distance. It may be apparent that the dispersive component is affected by the surface roughness, but study has shown independency of wetting and surface morphology when the roughness is below 100 nm [27]. As shown in Fig. 4, the roughest surface is around 2 nm on the a-C (Si 16.6 at.%) film. Therefore, the dispersive component is mainly affected by the density of the film. The density is low at Si concentration of $\sim$ 16.6 at.% and increases as Si concentration is increased. The drop in density means an increase in inter-atomic distance. Since the dispersive force is inversely proportional to the inter-atomic distance, the increase in the distance thus resulted in a decrease in the dispersive forces and ultimately the dispersive component of the surface energy. The increment of the dispersive component above Si atomic concentration of 16.6 at.% is caused by an increase in density. In this case, more Si atoms bond with the sp$^2$-hybridized C atoms, hence turning them into sp$^3$-configured C–Si and/or C–C bondings. This will get rid of more of the longer bond length $\pi$-bonds (3.35 Å), thereby increasing the global density and result in a smaller inter-atomic distance as the Si concentration increases. The evolution of surface roughness with film density is similar to results obtained from undoped a-C [28].

The number of attached COS7 fibroblast and MG63 osteoblast-like cells is presented in Fig. 5. ANOVA has shown significance of $p<0.001$, implying the cells are growing and proliferating well on both a-C and a-C(Si) films. While $t$-test has shown significant difference of cell count between a-C(Si37.6at.%) and a-C for both cell-lines on the sixth day of incubation.

Both COS7 and MG63 seem to proliferate better on a-C film with the highest Si concentration ($\sim$ 37.6 at.%). a-C(Si37.6at.%) has the highest polar/dispersive component ratio, and also has the highest surface energy among the three films. The result shows that both cell-types prefer a more hydrophilic surface. In a physiological environment, protein adsorption always precedes cellular adhesion. Pre-adsorbed proteins, in combination with proteins produced by the cell, and depending on the coating properties, determine the strength and type of adhesion. There exist several proteins in physiological environment and in vitro culture medium, i.e. albumin, fibronectin, vitronectin, globulin, etc. for example, the presence of fibronectin on either the cellular or the solid surface will exponentially increase adhesion and spreading of cells. Cell spreading is a combined process of continuing adhesion and cytoplasmic contractile meshwork activity. At first, the lamellar protrusions are formed, and microfilaments can always be observed in these lamellae. In a later stage, the cytoplasmic flaps in between the protrusions also expand, completing cell spreading. Fig. 6 shows the living cells morphology on a-C(Si37.6at.%). The long and fine cytoplasmic extensions in multiple directions show that there
is excellent adhesion behavior of the cells to the film. The cells also exhibit long lamellipodes indicating active cell migration leading to a homogenous colonization (c.f. Fig. 6b and d).

Cell adhesion and spreading are influenced by the physico-chemical characteristics of the underlying solid surface. The substrate surface free energy can affect the cell spreading. Poor spreading on hydrophobic surface and good spreading on hydrophilic surface can be observed in both the absence and presence of pre-adsorbed serum proteins [29]. Therefore, the substrate characteristics extend through the adsorbed proteins and affect the cell adhesion and spreading behavior. Cells can reach the underlying substrate by pseudopodia protruding through the pre-adsorbed protein layer, cells can consume pre-adsorbed proteins to make direct contact, and/or the substrate characteristics are reflected in the composition and conformation of adsorbed proteins, thus presenting different molecular groups to adhering and spreading cells. All proteins have NH2 and COOH groups at the ends. The NH tends to be positively charged and the COOH groups negatively charged [30]. a-C(Si) contain C=O and Si–O bonds at the surface and are polarized due to the difference in electronegativity between each element. Thus, the proteins can electrostatically bond with the film surface. Since a-C(Si37.6at.%) has the highest polar component of the surface energy, it has better affinity with the proteins; and in turn attracts more cells to adhere, spread and proliferate.

4. Conclusions

Unhydrogenated a-C and a-C(Si) films are deposited by magnetron sputtering. Raman spectroscopy results show a decrease in C sp²-hybridized bondings with the incorporation of Si. But it does not translate to a more hydrophilic surface due to the polar Si–O and C–O bonds on the surface. The films become more hydrophilic, and with higher polar/dispersive surface energy component ratio when Si concentration is increased. Both COS7 and MG63 can adhere, spread and proliferate well on a-C and a-C(Si) films. The more hydrophilic film a-C(Si37.6at.%) seems to be more conducive for cellular growth when the count is highest at the end of the incubation period.

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References

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