In vitro Cyto and Blood Compatibility of Titanium Containing Diamond-Like Carbon Prepared by Hybrid Sputtering Method

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Abstract In recent years, diamond-like carbon films (DLC) have been given more attention in research in the biomedical industry due to their potential application as surface coating on biomedical materials such as metals and polymer substrates. There are many ways to prepare metal containing DLC films deposited on polymeric film substrates, such as coatings from carbonaceous precursors and some means that incorporate other elements. In this study, we investigated both the surface and biocompatible properties of titanium containing DLC (Ti-DLC) films. The Ti-DLC films were prepared on the surface of poly (ethylene terephthalate) (PET) film as a function of the deposition power level using reactive sputtering technique. The films’ hydrophilicity was studied by contact angle and surface energy tests. Their surface morphology was studied by scanning electron microscopy (SEM) and atomic force microscopy (AFM). Their elemental chemical composition was analyzed using energy dispersive X-spectra (EDX) and X-ray photoelectron spectroscopy (XPS). Their blood and cell compatibility was studied by in vitro tests, including tests on platelet adhesion, thrombus formation, whole blood clotting time and osteoblast cell compatibility. Significant changes in the morphological and chemical composition of the Ti-DLC films were observed and found to be a function of the deposition level. These morphological and chemical changes reduced the interfacial tension between Ti-DLC and blood proteins as well as resisted the adhesion and activation of platelets on the surface of the Ti-DLC films. The cell compatibility results exhibited significant growth of osteoblast cells on the surface of Ti incorporated DLC film compared with that of DLC film surface.

Keywords: Ti-DLC, hybrid reactive sputtering technique, surface analysis, cyto and blood compatibility

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1 Introduction

Polymeric material has been producing considerable interest in the synthesis of biomedical materials for tissue replacement, organ replacement, etc [1-2]. Recently, Poly (ethylene terephthalate) (PET) is one of the most significant polymeric materials used to produce biomedical materials because of its excellent mechanical properties and biocompatibility. In particular, it is widely adopted in cardiovascular implants, such as artificial heart valves and blood vessels [3-8]. However, the blood compatibility of the material is insufficient for the long-term antithrombogenic demand for in vivo applications and further attempts must be made to improve its blood compatibility [9], in addition to the large variety of physical and chemical methods used to obtain surfaces with required biocompatible properties.

In recent decades, diamond-like carbon (DLC) and amorphous carbon films have been proposed as potential biomedical coatings on polymeric surfaces due to their chemical inertness, low coefficient of friction, high wear resistance, flexibility and moderate biocompatibility [10,11]. This material is regarded as an excellent candidate for biomedical applications. Generally, DLC consists of an amorphous form of carbon containing both graphitic type bonding (sp^2) and diamond type tetragonal bonding (sp^3). One more advantage associated with DLC is that its properties can be tailored by the ratio of the tetrahedral bonding population to the trigonal bonding population (sp^3/sp^2 ratio) [11-13].

Furthermore, the incorporation of selective elements such as Ti, Ag, Cu and La on the surface of DLC film is a growing interest in achieving biocompatibility. Among the elements, titanium (Ti) is a highly biocompatible material. In biological surroundings, the adsorption of various proteins is related to the titanium
contents of DLC films [14–19]. The capability of adsorbing proteins will enhance cell attachment and subsequently induce cell proliferation and cell differentiation. Many methods such as direct ion beam deposition, pulsed laser ablation, filtered cathodic arc deposition, magnetron sputtering, RF plasma-activated chemical vapor deposition (PACVD) and plasma source ion implantation have been developed for the deposition of titanium containing DLC films [20]. Among these methods, reactive sputtering is one of the most suitable method for the preparation of Ti containing DLC film on polymers owing to its process at low temperature, avoiding any degradation of polymer substrates.

In this study, Ti containing DLC films were prepared on the surface of PET film substrate using hybrid reactive sputtering technique. Ti-DLC was deposited as a function of the power level. The surface properties of the Ti-DLC coatings were studied using different characterization techniques. The change in hydrophilicity of the Ti-DLC films was studied by contact angle and surface energy analysis. The morphological and chemical composition of the Ti-DLC was analyzed by scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and energy dispersive X-ray spectra (EDX). The hemocompatibility of the DLC and Ti-DLC films were also studied by in vitro analysis.

2 Experimental setup and methodology

The Ti-DLC films were deposited on PET substrates using reactive sputtering techniques. The hybrid depositions were performed in a Balzers PLS 500 sputter chamber. Before deposition, the substrates were cleaned in a supersonic bath using trichloroethylene, acetone and methanol. After cleaning they were kept in distilled water until being introduced into the chamber in order to avoid dust deposition before processing. Immediately before introduction the samples were dried in a nitrogen gas flow. Then the samples were attached overhead at the sample plate and moved by rotating the plate in the right position above the sputter heads and the magnetrons. Between the sample and the magnetron a movable baffle was used to uncover the sample when the correct process parameters were reached. The base pressure for all depositions was 2.5×10⁻⁵ mbar which was obtained using a turbo molecular pump. To deposit DLC, acetylene gas was passed into the chamber which was ionized by the (sputtering) Ar plasma. During the deposition of pure DLC, a carbon target was used, but solely acetylene was used as the working gas. The reason was to ensure that the carbon only originated from the acetylene plasma. For Ti doping a Ti target was at the second magnetron. Ti-DLC was coated using alternative deposition using both the carbon and Ti target and obtained titanium containing DLC (Ti-DLC) films. The Ti-DLC films were prepared on the PET film as a function of the power level. Typical operating parameters are listed in Table 1.

<table>
<thead>
<tr>
<th>Operating parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deposition time</td>
<td>C: 5 min, Ti: 30 s</td>
</tr>
<tr>
<td>Working pressure</td>
<td>1.0×10⁻² mbar</td>
</tr>
<tr>
<td>Target</td>
<td>Carbon and Titanium</td>
</tr>
<tr>
<td>Ar gas flow rate</td>
<td>30∼50 (sccm)</td>
</tr>
<tr>
<td>C₂H₂ gas flow rate</td>
<td>30∼50 (sccm)</td>
</tr>
<tr>
<td>Deposition power</td>
<td>50, 80 and 120 W</td>
</tr>
<tr>
<td>Temperature</td>
<td>60°C</td>
</tr>
</tbody>
</table>

2.1 Characterization of the coatings

2.1.1 Contact angle and surface energy

The hydrophilicity of the DLC film surface was studied by contact angle measurements. The contact angle was measured by the sessile drop method. A 5 µL drop of distilled water was put on the surface with a micro syringe and observed through a microscope. The height (h) and radius (r) of the spherical segment were measured and the angle was calculated by the following equation [21].

\[
\text{Contact angle } (\theta) = \sin^{-1}\left[\frac{2rh}{r^2 + h^2}\right].
\] (1)

Ten readings were taken at different places of the sample surface and an average was determined. The error in the measurement of the contact angle was estimated to be ±2°. Similarly, the contact angle measurements were carried out with respect to glycerol. The values of polar and dispersive components of the testing liquids are given in Table 2 [22].

Table 2. Surface energy of the testing liquids

<table>
<thead>
<tr>
<th>Test liquid</th>
<th>γLV (mJ/m²)</th>
<th>γSV (mJ/m²)</th>
<th>γLV (mJ/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>72.8</td>
<td>21.8</td>
<td>51.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>64.0</td>
<td>34.0</td>
<td>30.0</td>
</tr>
</tbody>
</table>

The total surface energy (γSV) of the Ti-DLC films was calculated using the geometric mean approach for adhesion work (W_A):

\[
W_A = (1 + \cos \theta) = 2\sqrt{\gamma_{LV}^p \gamma_{SV}^d} + 2\sqrt{\gamma_{LV}^d \gamma_{SV}^p},
\] (2)

where γSV^d and γSV^p are the dispersive and polar components of surface energy of solids (Ti-DLC film) and γLV^d and γLV^p are the polar and dispersion components of the surface energy of the testing liquids (distilled water and glycerol) respectively.

The interfacial tension (γSL) between the solid surface and biological fluids (fibrinogen, immunoglobulin G and albumin) was calculated using the following expression [22]:

\[
\gamma_{SL} = \left[\sqrt{\gamma_{LV}^p} - \sqrt{\gamma_{SV}^p}\right]^2 + \left[\sqrt{\gamma_{LV}^d} - \sqrt{\gamma_{SV}^d}\right]^2.
\] (3)

The energetic characteristics (polar and dispersive components of the surface energy) of the biological liquids are shown in Table 3 [22].
2.2.1 Thrombus formations

In vitro

films was analyzed using scanning electron microscopy. Surface morphology of the DLC concentration of the Ti-DLC was measured by energy dispersive spectra (EDX). Surface morphology of the DLC trination was adjusted to 4.0 pH. The cells were cultured in medium (DMEM-10% FBS containing 100 µg/mL penicillin and 100 µg/mL streptomycin). Cells were maintained in an incubator at 37 °C under a 5% CO2 atmosphere and passaged with the use of 0.25% trypsin. Cells were trypsinized from the culture flasks, washed once in the medium, and then collected by centrifugation. The pellets containing the cells were suspended in the medium. The cell density was adjusted to 4.0 × 10^4 cells/mL, and 1 mL of cell suspension was added to each sample surface. The cells were then allowed to attach to the surface of Ti-DLC films and remained undisturbed in an incubator at 37 °C under a 5% CO2 atmosphere for 2 days. The cultured cells were fixed in 2.5% glutaraldehyde solution. The morphology of the cells was studied using a Philips XL 30S scanning electron microscopy.

2.2.2 Platelet adhesion tests

Platelet-rich plasma (PRP) was prepared by collecting human blood in plastic syringes containing 3.8% sodium citrate phosphate buffered saline (PBS) solution to prevent coagulation. The mixture was centrifuged at 1300 rpm for 10 min at 4 °C and the supernatant collected. PET, DLC and Ti-DLC samples washed with PBS for 24 h were placed at the bottom of the wells of a multiwell tissue culture plate, and the PBS solution was removed from the multiwell tissue culture plate by pipetting. PRP (1 mL) was then seeded and incubated at 37 °C for 30 min. After incubation, the samples were recovered and rinsed three times with PBS to remove any weakly adsorbed platelets. After being fixed in 2.5% (v/v) glutaraldehyde PBS solution, the morphology of the adsorbed platelets was observed using a Philips XL 30S scanning electron microscopy.

2.2.3 Protein absorption analysis

Human albumin and fibrinogen were used to study the adsorption behavior of proteins on the film surfaces. All blood proteins were purchased from Sigma. Small disks (15 mm in diameter) of the Ti-DLC films were prepared with a punch and immersed in 1 mg/mL protein solutions in phosphate-buffered saline (PBS, pH 7.3–7.4) at 37 °C for 1 h. The disks were then recovered, and changes in the protein concentrations of the solution were determined using a UV-spectrophotometer.

2.2.4 Cell culture studies

To study the osteoblast biocompatibility of the sample films in bone formation, MC3T3 cells were chosen to test the cytocompatibility of the materials. MC3T3 cells were cultured in medium (DMEM-10% FBS containing 100 µg/mL penicillin and 100 µg/mL streptomycin). Cells were maintained in an incubator at 37 °C under a 5% CO2 atmosphere and passaged with the use of 0.25% trypsin. Cells were trypsinized from the culture flasks, washed once in the medium, and then collected by centrifugation. The pellets containing the cells were suspended in the medium. The cell density was adjusted to 4.0 × 10^4 cells/mL, and 1 mL of cell suspension was added to each sample surface. The cells were then allowed to attach to the surface of Ti-DLC films and remained undisturbed in an incubator at 37 °C under a 5% CO2 atmosphere for 2 days. The cultured cells were fixed in 2.5% glutaraldehyde solution. The morphology of the cells was studied using SEM.

3 Results and discussion

3.1 Chemical composition: XPS analysis

The chemical compositions of Ti-DLC film surfaces are determined from the results of XPS analysis. Fig. 1
shows the XPS survey scan spectra of Ti-DLC films formed as a function of the deposition power level and they mainly contain C1s and O1s components. The additional peak of Ti2p (458 eV) is presented on the sample deposited at 80 W and 120 W (Fig. 1(b) and (c)) while there is no evidence of Ti content observed on Ti-DLC film deposited at the power level of 50 W (Fig. 1(a)). Fig. 2(a) and 2(b) show the Ti2p core level spectra of Ti-DLC films which reveal a peak around 458 eV. Table 4 summarizes the elemental composition of Ti-DLC films which exhibits that the C1s component decreases with respect to the deposition power level whereas the content of O1s and Ti2p increases. The above results confirm that the oxygen and Ti2p component are incorporated into the DLC film surfaces.

Fig. 1 The XPS survey scan spectra of Ti-DLC films deposited as a function of power level (a) 50 W, (b) 80 W and (c) 120 W

Information on how oxygen and titanium are incorporated into the DLC film can be obtained from deconvolution of XPS signals using a Gaussian-Lorentzian fit. For calculating the XPS, PEAK version 4.1 fitting software was used. The C1s high-resolution XPS spectra of Ti-DLC films formed at 120 W are shown in Fig. 3(a). The C1s spectra of Ti-DLC film indicate the presence of four peaks with a binding energy of 282.0 eV, 284.5 eV, 285.3 eV and 287.7 eV due to TiC, sp², sp³ and CO [24]. The estimated fractions of the above components are 8.93, 45.18, 38.35 and 7.52 at % respectively. Fig. 3(b) shows the O1s spectrum of the Ti-DLC which is decomposed into three components at 530.2 eV, 532 eV and 533.4 eV due to TiO₂ and/or TiCxOy, C=O and C-O respectively. The above results confirm that Ti is bonded to the C and O atoms presented at the DLC film surfaces.

3.2 Elemental and morphological analysis: EDX, SEM and AFM results

The presence of C, O and Ti contents is also clearly confirmed by EDX spectra (Fig. 4(a)). Carbon and oxygen content are the major components of the spectra and there is also a small amount of Ti on the surface of DLC. These results confirm that there are five Ti atoms for 1000 C atoms. The results are in good agreement with XPS analysis.
In vitro Cyto and Blood Compatibility of Ti-DLC

Fig. 3 The XPS core level spectra: (a) C1s and (b) O1s spectra of Ti-DLC films coated at 120 W

Fig. 4 (a) EDX spectrum and (b) SEM image of Ti-DLC film coated at 120 W (color online)

Fig. 4(b) shows the SEM images of the Ti-DLC film coated at 120 W. It exhibits the uniform surface morphology of Ti-DLC films due to titanium and carbon atoms deposited homogeneously on the PET film substrate. However, we found small spherical particles on the surface of Ti-DLC films which may be due to the coatings for SEM preparation. Furthermore, the SEM image cannot give more information at the nanometer level. Therefore atomic force microscopy is more appropriate to investigate the surface morphology of the Ti-DLC film surfaces.

The detailed surface change induced by deposition power was investigated by atomic force microscopy (AFM). Fig. 5(a), (b) and (c) show the AFM images of Ti-DLC film coated as a function of the deposition power level. As shown in Fig. 5(a), the surface of Ti-DLC film is relatively smooth and has moderate roughness for the sample deposited at the power level of 50 W. The surface roughness of the Ti-DLC films was further increased with the increase in the deposition power level as can be seen in the AFM pictures (Fig. 5(b) and (c)). The root mean square roughness (RMS) of Ti-DLC films coated at different power levels is provided in Fig. 6. It is seen that the values of RMS gradually increase with the increasing deposition power level. The increase in surface roughness is mainly influenced by the residual stress due to the impact of both C, O and Ti atoms on the surface of PET film substrate during the deposition of Ti-DLC films [25–27].

(a) 50 W, (b) 80 W and (c) 120 W

Fig. 5 AFM topographic representation of Ti-DLC film surfaces for different deposition power levels (color online)
3.3 Contact angle, surface and interfacial energy of the DLC films

Contact angle measurements of the PET, DLC and Ti-DLC films are listed in Table 5. It is observed that the contact angle of the PET film is 86.2° for water and 76.6° for glycerol. After DLC coating the contact angle decreases to 64.1° for water and 55.6° for glycerol. The contact angle decreased further when Ti was incorporated to DLC. The total surface energy of the PET film was 26.84 mJ/m² which was increased further on the surface of DLC and Ti-DLC coatings (Table 5). Similarly, the surface polar component of the surface energy increases while the dispersive component decreases for DLC and Ti-DLC coating films. The wettability is related to the surface bonding energy of the interface between substrate and body fluid. Hence, the result reveals that the Ti containing DLC film could be more hydrophilic compared with the DLC and PET substrate. The interfacial tension between DLC films and water, blood and plasma proteins is listed in Table 6. It shows that the interfacial tension between the water and PET film is 22.25 mJ/m². A DLC coating reduces the interfacial tension to 6.82 mJ/m². The Ti containing DLC film further reduces the same to 3.15 mJ/m². The same patterns of results are obtained for the interfacial tension for blood, fibrinogen, IgG and albumin. The results indicate that Ti-DLC coating is strongly effective in reducing the plasma protein absorption which in turn may decrease the degradation of the protein structure and blood coagulation. Generally, the absorption of albumin in high amounts would inactivate the blood-material interface, while a fibrinogen adsorbed in high amounts would favor the platelet adherence and the activation of the blood coagulation system. For surface-coated PET films, the interfacial energy between Ti-DLC and fibrinogen is small which ensures low driving forces for protein deposition [27,28].

### Table 5. Contact angle and surface energy of the PET, DLC and Ti-DLC films

<table>
<thead>
<tr>
<th>Samples</th>
<th>Contact angle (degree)</th>
<th>Surface energy components (mJ/m²)</th>
<th>Water</th>
<th>Glycerol</th>
<th>γₚ</th>
<th>γₛ</th>
<th>γₛₚ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>86.2</td>
<td>26.84</td>
<td>5.89</td>
<td>20.95</td>
<td>26.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLC</td>
<td>64.1</td>
<td>20.04</td>
<td>55.6</td>
<td>19.53</td>
<td>39.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ti-DLC</td>
<td>54.3</td>
<td>13.54</td>
<td>51.7</td>
<td>32.11</td>
<td>45.65</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 6. PET and DLC-Blood and plasma proteins interfacial energy

<table>
<thead>
<tr>
<th>Samples</th>
<th>Interfacial energy components (mJ/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>PET</td>
<td>22.25</td>
</tr>
<tr>
<td>DLC</td>
<td>7.44</td>
</tr>
<tr>
<td>Ti-DLC</td>
<td>3.15</td>
</tr>
</tbody>
</table>

3.4 In vitro blood compatibility

Fig. 7 shows the thrombus formation on various surfaces under the same conditions, taking the thrombus formed on untreated PET during 30 min contact with blood as 100%. After DLC and Ti-DLC coatings, the thrombus formation is reduced significantly. The whole blood clotting time (WBCT) of the PET film is 258 s which is increased significantly on the DLC coated surface (Table 7). Furthermore the WBCT of the Ti-DLC is increased two times over the PET and DLC films. The results indicate that the Ti-DLC coating suppresses the mechanism of coagulation activity.
3.5 Adsorption of proteins on the DLC film surfaces

Prevention of protein adsorption and denaturation is one of the most important techniques for developing/enhancing a material’s anticoagulation properties. Many hypotheses have been proposed to describe the surface characteristics of the anticoagulating material. Table 8 shows the adsorption of albumin and fibrinogen onto DLC and Ti-DLC film surfaces from the protein solution (1 mg/mL). The adsorption of albumin and fibrinogen of PET film is 160 µg/cm² and 182 µg/cm². Furthermore, the adsorption amount of albumin and fibrinogen of DLC is decreased; however, Ti containing DLC decreases significantly the adsorption amount of both albumin (65 µg/cm²) and fibrinogen (137 µg/cm²). The results indicate that Ti-DLC film surface exhibits excellent inhibition of protein adsorption, which is in good agreement with platelet adhesion and thrombus formation test results.

### Table 8. Adsorption of proteins on the PET and DLC film surfaces

<table>
<thead>
<tr>
<th>Samples</th>
<th>Protein adsorbed (µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Albumin</td>
</tr>
<tr>
<td>PET</td>
<td>160</td>
</tr>
<tr>
<td>DLC</td>
<td>121</td>
</tr>
<tr>
<td>Ti-DLC</td>
<td>65</td>
</tr>
</tbody>
</table>

3.6 Cell culture studies

Fig. 9 shows the SEM images of osteoblast cells (MC3T3 cell) adhering to DLC and Ti-DLC film surfaces that were cultured for two days. It is clearly seen that the generation of pseudopods from cells adhering on the Ti-DLC film surface has spread and flattened better than that on DLC film surface (Fig. 9(a) and (b)), implying that the cell activity in Ti-DLC is higher than that in DLC. As investigated above, titanium plays a significant role in enhancing the cell and biocompatibility of the materials.

![Fig.8](image1.png)  
**Fig.8** SEM images of platelet adhesion on films (a) PET, (b) DLC and (c) Ti-DLC

![Fig.9](image2.png)  
**Fig.9** SEM micrographs of osteoblast cells (MC3T3) on (a) DLC and (b) Ti-DLC films

4 Conclusion

Titanium containing diamond like carbon (Ti-DLC) was prepared on the surface of polyethylene terephthalate (PET) film using reactive sputtering technique. The Ti-DLC was prepared as a function of the deposition power level. The surface morphology, topological, chemical composition and hydrophilicity of the Ti-DLC films were analyzed by using different methods of characterization such as SEM, AFM, XPS, EDX and contact angle techniques respectively. The blood and cell
compatibility of the Ti-DLC was studied by in vitro blood and cell compatibility tests. XPS results gave evidence for the incorporation of Ti on the surface of DLC; thus the concentration of the Ti content increases with the deposition power level increasing. Similarly, the EDX spectra confirmed the presence of Ti in the DLC film surface. Due to the residual stress induced by the impact of C, O and Ti atoms on the substrate, the surface roughness of the Ti-DLC films got increased as exhibited by AFM analysis. The results of contact angle and surface measurements revealed that the Ti containing DLC film surfaces became more hydrophilic in nature compared with the PET film substrate. The interfacial energy results revealed that the modified PET films resisted blood protein absorption. The in vitro tests exhibited that the blood and cell compatibility of the DLC films got increased, which has great potential for wide applications in several blood contact devices.

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References


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