Original Research

**Nano-structure TiO$_2$ film coating on 316L stainless steel via sol-gel technique for blood compatibility improvement**

Mohammadreza Foruzanmehr$^1$, Seid Mohammad Hosainalipour$^1$, Shamsoddin Mirdamadi Tehrani$^1$, Mahnaz Aghaeipour$^2$

$^1$Department of Materials Science and Engineering, Iran University of Science and Technology, Narmak, Tehran, Iran
$^2$Iranian Blood Transfusion Research Centre, Tehran, Iran

**Abstract**

**Objective(s):** Titanium oxides are known to be appropriate hemocompatible materials which are suggested as coatings for blood-contacting devices. Little is known about the influence of nanometric crystal structure, layer thickness, and semiconducting characteristics of TiO$_2$ on blood hemostasis.

**Materials and Methods:** Having used sol-gel dip coating method in this study, TiO$_2$ thin films were deposited on nano-scale electro-polished stainless steel 316L with 1 to 5 nano-sized layers. Surface morphology and structure of the film were studied with X-ray diffraction and atomic force microscopy. Blood compatibility was also determined by measuring the platelet activation (CD62P expression), platelet adhesion (Scanning Electron Microscopy), and the blood clotting time on these samples.

**Results:** The films were compact and smooth and existed mainly in the form of anatase. By increasing the number of TiO$_2$ thin layer, clotting time greatly extended, and the population of activated platelet and P-selectine expression changed according to the surface characteristics of each layer.

**Conclusion:** The findings revealed that stainless steel 316L coated with nano-structured TiO$_2$ layer improved blood compatibility, in terms of both blood platelet activity and coagulation cascade, which can decrease the thrombogenicity of blood contacting devices which were made from stainless steel.

**Keywords:** Blood compatibility, Flowcytometry, Nano-structured, Sol-gel, Titanium oxide

---

$^*$Corresponding author: Mohammadreza Foruzanmehr, Department of Materials Science and Engineering, Iran University of Science and Technology, Narmak, Tehran 16765-163, Iran. Tel.: +98-21-88778288, Email: sunforouz@metaleng.iust.ac.ir
Introduction

For medical implants, such as heart valves or vascular stents, which are in contact with blood, it is important to minimize the propensity of the surface in order to adsorb blood proteins, provoke blood clotting, and hence, reduce the risk of thrombosis (1). Titanium alloys are usually used for fabrication of synthetic heart valves. They possess blood compatibility and high resistance to degradation, wear, and fatigue; however, there are also some serious problems associated with these materials (2). Stainless steels are distinctively qualified not only because of their long service life, availability, and fabric ability, but also because they are non-corroding and non-contaminant, strong and rigid, appropriate to be polished to very smooth finishes. Stainless steels can withstand heat and chemical sterilization and are easily welded. The base materials of the stents are biocompatible and haemocompatible alloys. The most widely used materials are AISI 316L and 316LVM types of stainless steel, which are proved to be the most reliable in clinical applications (3). Early observational trials highlighted problems associated with the use of stents, in particular, a high incidence of subacute occlusion, despite aggressive anticoagulation regimens that prolonged hospital stay and were difficult to control, and occasionally led to severe events (4). In-stent restenosis results from a series of complex interactions involving the presence of a thrombogenic surface and the activation of platelets and coagulation proteins. Clinical studies have shown a significant reduction in acute occlusion and restenosis after antiplatelet therapy and have provided evidence that platelet activation may play an important role in in-stent restenosis (5). Therefore, the thrombogenicity of stents may be an important factor in prevention of in-stent restenosis. Surface modification has appeared to be a main method to improve anticoagulation of biomaterials contact with blood (6). Various biologically inert surface coatings, such as carbon, platinum, phosphorylcholine, and gold, have been applied to stainless steel stents to reduce thrombosis and restenosis; nonetheless, the effectiveness of these strategies has not been proven in clinical trials. In fact, gold coatings have resulted in increased rates of restenosis (7). For example, Baurschmidt and Coworkers studied the blood compatibility of SiC to use this material as artificial heart valves (8). A tilting flat disk-type ceramic valve has been developed by Mitamura et al (9). The valve comprises a single-crystal alumina ceramic disk and TiN valve cage. The blood compatibility of diamond-like carbon (DLC) was also studied by Dion and colleagues (10). In 1980s, Schaldach and coworkers researched the blood compatibility of Ta5+ doped rutile-type TiO2 Ceramics (11). Of all these materials, only TiO2 ceramics showed good hemocompatibility in comparison to LTI-carbon. In spite of the enhanced hemocompatibility of this doped semiconducting rutile ceramic compared to LTI-carbon, technological problems associated with the surface roughness of this material make the manufacturing costs prohibitive (10). Huang and associates observed that whenever the thickness of the titanium oxide layers increased, the blood compatibility of these layers noticeably improved (11). Materials in contact with blood have frequently shown different behaviour concerning the activation of blood platelets or the clotting cascade. Tsyganov and colleagues found that low-dose phosphorous ion implantation into rutile TiO2 could reduce the activation of both hemostatic systems. Surface roughness below 50 nm or crystal structure had only minor effects on blood compatibility (8). TiO2 film can be prepared by methods such as ion beam-assisted deposition, CVD, plasma immersion, etc. However, these methods are complex and expensive for depositing a uniform layer of TiO2 film on the...
substrate with complex shapes or geometry. Sol-gel technology is a low-temperature method which is independent of substrate shape and can achieve a good control of surface properties such as composition, thickness, and topography (12). In this paper, the blood compatibility of stainless steel stents coated by bare and one-to-five nano-structured TiO$_2$ layers was investigated. Furthermore, some correlation between platelet and coagulation cascade activation was developed.

**Materials and Methods**

**Film preparation**

Titanium tetra-iso-peropoxide (TTIP) was used as a starting material (Aldrich 99.99%), and nitric acid (Merck), as a peptizer. The water used in preparing TiO$_2$ sols was doubly distilled and deionized. The TiO$_2$ sol was synthesized as follows: a mixture of 30 mL (0.12 mol) of TTIP and 5 mL of ethanol (Merck) was added to 180 mL of water in a 500-mL jacketed Erlenmeyer flask to provide a TTIP-ethanol-water molar ratio of 1:0.75:83. Then, 2 mL of nitric acid was added to the TTIP solution and maintained at 20°C by circulating a coolant with a circulating chiller. The resulting sol was refluxed at 80°C for 12 hours under vigorous stirring (using a magnetic stirrer), which resulted in a milky solution. Thereafter, it aged for 48 hours at room temperature in the air. AISI 316L stainless steel plates with dimensions of 20 × 20 × 3 mm were used as substrates. Before coating, the samples were ground with successive SiC papers down to grit size 1500 and polished with a 5-µm diamond paste. In order to achieve better blood compatibility and resembling to stent manufacturing process, all of the samples were electropolished by electrolytes composed of deionized water, chromic acid, phosphoric acid, and sulfuric acid (conditions shown in Table 1). The samples were afterwards ultrasonically cleaned with acetone. The coatings were conducted by dip-coat method. The substrates were immersed into the sol and then withdrawn at a speed of 8 cm/min following each coating in order to avoid cracking. The samples were dried in an oven at a temperature of 100°C. The samples were coated by 1 to 5 layers and the obtained films were annealed at 500°C in nitrogen-purged furnace for 1 hour. The temperature was raised slowly, at a rate of approximately 1°C/min, from room temperature to 500°C.

<table>
<thead>
<tr>
<th>Table 1. Electro polish conditions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current density (A.cm$^{-2}$)</td>
</tr>
<tr>
<td>Temperature (°C)</td>
</tr>
<tr>
<td>Time (S)</td>
</tr>
</tbody>
</table>

**Surface characterization**

Phase composition was determined by XRD measurements using (SIESERT 3003TT) difractometer with settings of 40-kV and 30-mA Cu K$_\alpha$ radiation ($\lambda = 0.1540510$ nm) and range of 20 between 10° to 120°, on the powder obtained from dried sol which also was annealed as mentioned for the normal samples. To measure the single-layer thickness, first a stainless steel substrate was primed to reach a flat and smooth surface as explained before. Then, the sample was dipped up to the middle line and subsequently pulled out of the sol. It was processed afterwards as it was in normal samples. A contact mode AFM (DME Scanner95200E Dual Scope 30-26) was used to investigate boundary of bare and coated metal. Ten fields (10 ×10 µm) were chosen and average R$_y$ introduced as the film thickness. Non-contact mode AFM (DME Scanner 95200E Dual Scope 30-26) was employed to reveal topographic characterization and roughness specification of 1 to 5 layers TiO$_2$ thin films coated on stainless steel.

**Blood compatibility assessment**

Stainless steel 316L is clinically proved biocompatible to use in blood contacting devices such as coronary stents and heart valves and it was employed as a proper
control for the coming biological experiments (3). Glass is also used a thrombogenic reference. For blood compatibility tests, all samples were ultrasonically cleaned subsequently in ethanol and water, each for 10 minutes. The thrombogenicity of the samples was evaluated using a whole blood kinetic clotting time method, as previously described (13, 14). Additional samples with adherent platelets were prepared for scanning electron microscopy as described by Grunkemeir and colleagues (15) and observed with a Cambridge stereoscope 320. Scanning electron microscopy. The expression of the platelet activation marker CD62P was measured by flow cytometry. Blood (4.5 mL) was obtained by venipuncture from a healthy male blood donor who had denied the use of any drugs, especially aspirin. The blood was anticoagulated with 0.5 mL of sodium citrate (110 mM) in a sterile system (Vacutainer, Becton Dickinson). Fifty milliliter of anticoagulated blood was immediately mixed with 450 mL of fixing buffer (phosphate-buffered saline (PBS) with 2% paraformaldehyde and 0.1% sodium azide) at 4°C. Platelet-rich plasma PRP (6.68-6.86 × 10^4 PLT/µL) was obtained from the rest of the blood by spinning down at 200 g, 20°C, for 20 minutes. Fifty milliliter of PRP was mixed with 450 mL of fixing buffer at 4°C to obtain platelets in the resting state. PRP (450 mL) was incubated with 50 mL of adenosine diphosphate (0.2 mM) for 5 or 10 minutes at room temperature; then, 50 mL of these maximally activated platelets were fixed in 450 mL of fixing buffer. Before staining, platelets were kept in the fixing buffer for 2 hours at 4°C. They were washed once with PBS, 0.1% sodium azide, centrifuged at 1200 × g, resuspended with staining buffer (PBS, 0.1% sodium azide, 2% fetal Calf serum) and stained with fluoresceinisothiocyanate-labeled anti-mouse CD41a (Serotec, Clone Pm6.248) or with phycoerythrine-labeled anti-human CD62P (Serotec, Clone Psel.KO.2.12) for 30 minutes. The platelets were washed once again and then measured by flow cytometry (PASIII, PARTEC). Only single-labeled platelets were used to avoid problems with compensation. Platelets were gated in the forward scatter-side scatter plot. Five thousand events in this gate were recorded. The correct position of this gate was confirmed by the CD41a-labeled platelets and non-specific bonding border line determined by Iso type negative antibody IgG1 for each blood donation. Median values of the CD62P histogram were used for evaluation. The median CD62P signal of unstained platelets was set as 0% activation and that of the adenosine diphosphate-activated platelets were set as 100% activation. One hundred milliliters of PRP were dispersed on each sample on a circle with approximately 10-mm diameter. The platelets were allowed to adhere on the surface for 45 minutes at 37°C in humidified air with 5% CO₂. Then, the supernatant was aspirated and mixed with 900 mL of fixing buffer. The patch was carefully rinsed twice with 100 mL of PBS in order to remove less adherent platelets, and the rinsing solution was fixed together with the supernatant. The blood platelets were stained and measured by flow cytometry as described earlier. The experiment was performed with 1 blood sample.

Results and Discussion

Phase composition

The composition of TiO₂ films synthesized on stainless steel due to interaction of Ni and Cr peaks into background intensity was determined through preparing TiO₂ powder from sol and then measuring the powder by X-Ray diffraction. Figure 1 shows the XRD pattern of TiO₂ powder. It reveals that the original film is completely Anatase, and using Sherer’s equation, the crystallite size was determined to be 12 nm. Figure 2 shows the existence of film on stainless steel substrate.
Figure 1. XRD pattern of titanium oxide films synthesized by Sol-Gel.

Figure 2. AFM image shows different phase based on voltage change crossing from stainless steel 316L to TiO2 coatings.

Film thickness
The single layer of TiO2 film that was formed on 316L SS, measured to be 46 nm. Figure 3 shows nano-thickened layer which can be assumed as nanometric film (less than 100 nm); subsequently, 2, 3, 4, and 5 layers are approximately 92 nm, 138 nm, 184 nm, 230 nm in thickness. Previous investigations revealed that better hemocompatibility is achieved in case of thickness increase (16). However, others state that the uniformity of oxide layer on the surface of metallic implants was more important than its thickness for improving the biocompatibility of devices (17). All of the mentioned theories can be attributed to the multifaceted nature of blood compatibility, which is discussed further in this paper.

Figure 3. AFM image shows the height elevation which reveals the formation of TiO2 film in the range of nano sized coating.

Surface topography and morphology survey
Figure 4 Shows the AFM images of 1 to 5-layer TiO2 films coated on 316L SS, it can be seen that the films are composed of TiO2 particles which are tightly agglomerated with each other. Table 2 shows the mean surface roughness (Ra) of each sample while the roughness of electro-polished stainless steel was below 20 nm, so that the measurement of roughness may be little affected by the substrate. It is mainly related to the TiO2 film coated and seems that the roughness of the films is strongly affected by coating sequences. This means that for the first coating, the preferred sites for deposition of TiO2 particles were determined by surface tension and nano-scale inclination of the stainless steel. However, in the second coating, the substrate surface was covered with TiO2 particles, and in the next coating, gelation of TiO2 sol occurred in the valley of the spiky film, so that the height of peaks was reduced. This is why the 2-layer coated sample has a smoother surface in comparison to the 1-layer. This procedure continues for the next coatings and it can be assumed in the odd numbers of coatings the roughness of the samples slightly increased and in the even numbers of coatings decreased.
Figure 4. AFM images show topographic and morphologic characteristics of (A) one layer (B) two layers, (C) three layers, (D) four layers, and (E) five layers TiO2 nano sized coating.

Table 2. Mean surface roughness of samples.

<table>
<thead>
<tr>
<th>Number of coating</th>
<th>Ra (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20.1</td>
</tr>
<tr>
<td>II</td>
<td>17.7</td>
</tr>
<tr>
<td>III</td>
<td>23.5</td>
</tr>
<tr>
<td>IV</td>
<td>14.8</td>
</tr>
<tr>
<td>V</td>
<td>15.6</td>
</tr>
</tbody>
</table>

Whole blood clotting time

The blood compatibility of stainless steel coated with different number of TiO2 films was measured to obtain the clotting time and platelet adhesion. Figure 5 shows the blood profiles of tested materials. The absorbance (at 540 nm) of the hemolysed hemoglobin solution changes with time (the curves were fit to the values by exponential extrapolation). The higher absorbance results, the better thromboresistance. As a convention for comparison clotting times, the time at which the absorbance equals 0.1 is generally defined as the clotting time (13). Table 3 exhibits clotting times of the samples. These results indicate that increasing TiO2 film thickness extended the clotting time; however, C.T increased gradually from the sample with 2-layer TiO2 film. In 5-layer TiO2 film, it reached up to 63% more than that for bare metal.

It is suggested that the denaturing of fibrinogen is related to the charges of fibrinogen transferred to the material. During this process, fibrinogen decomposes and transforms into fibrinmonomer and fibrinpeptides, followed by crosslinking to form the irreversible thrombus (11).

Whole blood clotting time

The trends of platelet adhesion are in contrast to the results of the clotting time. The different behavior of clotting time and platelet adhesion demonstrate that these two hemostatic processes are different; based on their initiation.

Platelet activation and adhesion

The clotting cascade is activated mainly due to intrinsic pathway which is triggered via pre-kallikrein and factor XII activation, while, thrombocytes are mainly activated
on a surface by adsorbed protein like fibrinogen and von willebrand factor. Sunny and Sharma (16) found that with a thicker titanium oxide layer on titanium, the adsorption of albumin and fibrinogen increased by 6-fold. It can be inferred that by increasing film thickness more protein is adsorbed on the surface and consequently platelet adhesion is promoted as a result of this pre-adsorbed protein layer. Moreover, if surface roughness exceeds, it can induce protein adsorption (18).

This can be the reason that higher roughness in 3-layer coated sample led to more platelet adhesion. Another important mechanism which can affect platelet adhesion is surface charges of TiO2 layer. The isoelectric point of titania is pH 6.2 (21).

When samples are placed in vicinity with blood (pH 7.4), TiO2 will be negatively charged; therefore, thrombocytes with negative charges will not adhere to the surface. To accept this theory, it should be considered same trend of platelet adhesion and activation for all of the coated samples. Meanwhile, there are some differences in work functions between stainless steel and titanium oxide layer; thus, many electrons would transfer from stainless steel to the oxide layer (20). It seems that by increasing the TiO2 film thickness, the oxide layer would gradually obtain the semiconductive property and it can cause an enhancement of work function difference between stainless steel and TiO2 film. There would be an optimum thickness of the oxide layer, for surface negative charge density (11).

It can be speculated that there are various cross-talks between these two mechanisms which can act with each other competitively.

absorption effect. Figure 6 Compares bare and 4-layer TiO2-coated stainless steel on platelet adhesion; it showed the probability of postulated theory that bare stainless steel has less protein contamination, but, there is more platelet adhesion. On the contrary, coated sample had some protein adsorption with less platelet attachment.

Figure 7 reveals the expression of the P-selectine on non-adherent platelets showed mainly the same trend as the platelet adhesion.

The differences between values are not significant (analysis was done by SPSS software one-way analysis of variance).

Platelet activation Physiology can be initiated by several different ways such as trauma, contacting with adhered platelets, coagulation mediators. According to the test setup of platelet activation, it is a suspension of platelet rich plasma which is put on the samples so it can be said, that platelets first adhere to the surfaces and then they release some granules consists of adenosine diphosphate or Thromboxine A2 which activates the suspending platelets. Although the differences are not significant statistically the median line of each bar shows the amount of platelet activation and it can be seen in 4-layer TiO2 the median line has smallest amount among the others.

Table 4. Platelet adhesion.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Platelet density (number/µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>0.63</td>
</tr>
<tr>
<td>Bare</td>
<td>0.54</td>
</tr>
<tr>
<td>I Layer</td>
<td>0.32</td>
</tr>
<tr>
<td>II Layers</td>
<td>0.37</td>
</tr>
<tr>
<td>III Layers</td>
<td>0.45</td>
</tr>
<tr>
<td>IV Layers</td>
<td>0.23</td>
</tr>
<tr>
<td>V Layers</td>
<td>0.2</td>
</tr>
</tbody>
</table>
TiO2 film coating on 316L stainless steel for blood compatibility

**Conclusion**

Titanium oxide thin films have been successfully synthesized by sol-gel technique from 1 to 5 layers. Single-layer film thickness was determined 46 nm, and it consisted of embedded nm-scale TiO2 particles. Hemocompatibility was evaluated to reach 63\% longer clotting time for 5-layer TiO2 film, although there was no significant difference between platelet activation and adhesion. Four-layer TiO2 film had the lowest amount of platelet activity, which can be considered as the optimum condition in that protein adsorption and platelet repulsion interaction. It was found that the coated samples had better blood compatibility than bare 316L SS, and the sample with 4-layer TiO2 film showed the best behavior of blood compatibility. Towards this end, it will be necessary to conduct in vivo trial to establish the validity of these preliminary findings.

**References**

13. Maitz MF, Pham MT, Wieser E, Tsyganov I. Blood compatibility of titanium oxides with various crystal
structure and element doping. J Biomater Appl. 2003; 17: 303-.