In Vitro Investigation of Hemocompatibility of Hydrothermally Treated Titanium and Titanium Alloy Surfaces

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ABSTRACT: For decades, titanium and its alloys have been established as a biocompatible material for cardiovascular medical devices such as heart valves, stents, vascular grafts, catheters, etc. However, thrombosis is one of the reasons for implant failure, where blood clot forms on the implant surface, thus obstructing the flow of the blood and that leads to some serious complications. Various surface modification techniques such as heparin modification, albumin coating, surface anodization, plasma etching, and hydrothermal treatments have been explored to improve the hemocompatibility of titanium-based materials. However, there are several limitations related to the robustness of the surfaces and long-term efficacy in vivo. In this study, titanium and its alloy Ti−6Al−4V were hydrothermally treated to form nanostructured surfaces with the aim to enhance their hemocompatibility. These modified surfaces were characterized for their wettability, surface morphology, surface chemistry, and crystallinity. The hemocompatibility of these surfaces was characterized by evaluating blood plasma protein adsorption, platelet adhesion and activation, platelet−leukocyte complex formation, and whole blood clotting. The results indicate lower fibrinogen adsorption, cell adhesion, platelet activation, and whole blood clotting on hydrothermally treated surfaces. Thus, these surfaces may be a promising approach to prevent thrombosis for several titanium blood-contacting medical devices.

1. INTRODUCTION

Different types of blood-contacting medical devices such as mechanical heart valves, stents, vascular grafts, catheters, etc. are implanted in patients worldwide to treat cardiovascular diseases. It is estimated that the number of coronary procedures carried out in the United States to implant these medical devices in the year 2019 is approximately 1 055 000, out of which 335 000 (approximately 31%) are recurring procedures.1 Most of the recurring procedures are due to inappropriate interaction of the implant material with blood and its components, which results in infection, inflammation, and thrombosis.2,3 Thrombosis is one of the reasons for implant failure, where a blood clot forms on the implant surface, thus obstructing the flow of the blood. Fibrinogen is adhered on the surface immediately after blood comes in contact with the surface. Simultaneously, factor XII also adsorbs to the surface and gets autoactivated resulting in conversion of prekallikrein to kallikrein, thus initiating coagulation and thrombin formation on the surface. Fibrinogen reacts with thrombin and gets converted to fibrin and traps activated platelets and red blood cells to form blood clots. Hence, it is important to understand the blood−implant surface interactions and modify the implant surface characteristics to improve its compatibility with blood and its components, i.e., hemocompatibility.4

Several clinical approaches have been employed to prevent blood clotting on implant surfaces. The most common approach is to prescribe patients with blood thinners such as aspirin, clopidogrel, and vorapaxar to avoid clotting of the blood.5 However, there are significant risks associated with internal bleeding and weakened immune response that may compromise the overall health of these patients.6 Implants
coated with anticoagulants such as warfarin and heparin have also been widely used. However, there are risks associated with a decrease in plate count, body pain, and internal bleeding. Studies have also shown that the presence of nitric oxide reduces thrombosis as NO is a potent inhibitor of platelet function. Several other research approaches have also been investigated to the coating of implant surfaces with antifouling agents like poly(ethylene oxide) and albumin protein to inhibit adsorption of protein to the surface. However, the results show that there is no significant difference in blood clotting compared to unmodified surfaces. Other studies of different surface nanofeatures with varied roughness and wettability have also been investigated, and the results have shown significant differences in blood plasma protein adsorption. However, the effect of the size and shape of nanofeatures on protein adsorption and whole blood clotting is not well established. Different approaches on modifying the surface chemistry and topography combined have also been investigated. For example, superhydrophobic surfaces have shown a significant reduction in adsorption of blood plasma proteins and whole blood clotting. Nevertheless, these surfaces may not be stable for prolonged duration when exposed to blood. Also, superhydrophobic surfaces will not adhere endothelial cells, which plays a major role in the integration of many blood-contacting medical devices with the native tissue. Thus, there is a need to develop robust surfaces that prevents fibrinogen adsorption, controls blood clotting, and also have the potential to interact appropriately with the native tissue without compromising the implant integration in clinical environments.

Titanium (Ti) and its alloys have been established as a material of choice for different implantable medical devices such as heart valves, stents, dental, and orthopedic implants mainly due to its high specific strength to weight ratio, low modulus of elasticity and titanium oxide layers are biological inert. Furthermore, titanium is also considered to be biocompatible because of its low electrical conductivity, which leads to stable oxide layers. These oxide layers protect the implant from further corrosion. Surface modification on titanium surfaces has shown improved cytompatibility with different cells. Various nanostructures such as nanowires, nanotubes, and nanospheres have been developed on titanium surfaces using different techniques such as anodization, micromachining, and etching process where the results show improved cell adhesion. Even though titanium implants interact appropriately with the native tissue, they are prone to thrombosis when in contact with blood. In this study, titanium and its alloy Ti–6Al–4V were hydrothermally treated to form nanostructured surfaces with the aim to enhance their hemocompatibility. The hydrothermal treatment modified the surface of titanium and Ti–6Al–4V with different nanotopographies and wettability properties. Previous studies have shown that nanostructured titanium surfaces have led to better hemocompatibility when compared to nontextured surfaces. However, it is not well established whether it is the alteration of surface wettability or the nanostructure that majorly contributes to the difference in hemocompatible response. In this study, we have developed three different types of nanostructured surfaces by hydrothermal treatment on titanium and Ti–6Al–4V. The surfaces were characterized for their wettability, surface morphology, surface chemistry, and crystallinity. The hemocompatibility of these surfaces was characterized by evaluating blood plasma protein adsorption, platelet adhesion and activation, platelet–leukocyte complex formation, and whole blood clotting. The results indicate that surface topography and the size of the nanostructure play a major role in determining the hemocompatible response on hydrothermally treated surfaces and may have long-term implications in blood-contacting medical devices.

Figure 1. Representative SEM images of Ti and A surfaces treated by the hydrothermal treatment. Images were taken at two different magnifications (500× and 15 000×).
2. RESULTS AND DISCUSSION

Despite extensive research carried out to understand the interaction of blood and its components with the surface of implantable medical devices, implants fail majorly due to blood clotting. This is a major concern with blood-contacting biomaterials where protein adsorption, platelet adhesion and activation, which cause blood clotting, could be fatal for patients. In this study, titanium and its alloy, Ti6Al−4V, were exposed to an alkaline solution (NaOH), which leads to a corrosive reaction on the surface along with oxidation of the element(s) present (eq 1). Various permutations and combinations of variables such as NaOH concentration, temperature, and time for treatment were used to determine the optimal conditions for fabrication that resulted in uniform and repeatable nanostructures on different surfaces.

\[ 3\text{Ti} + 4\text{NaOH} + 4\text{H}_2\text{O} \rightarrow 2\text{Na}_2\text{TiO}_3 + \text{TiO}_2 + 6\text{H}_2 \]

(1)

The rate of the reaction is influenced by various factors such as the concentration of NaOH, reaction temperature, and time.38 These corrosive reactions led to unique nanostructures on the material surface.39 The modified surfaces were characterized for surface morphology, surface energy, surface chemistry, and crystal structure. The possible oxides states of titanium formed during the reaction in a NaOH solution according to the Pourbaix diagram40 for titanium are TiO2, TiO2HCl, and Ti3O5 (Ti1, Ti2, A1, A2). These titanium oxides when further treated with HCl (Ti3, A3) develop titanate structures (eq 2), and this modification led to unique surface topography that is different than the treatment with just NaOH.

\[ \text{Na}_2\text{TiO}_3 + 2\text{HCl} = \text{H}_2\text{TiO}_3 + 2\text{NaCl} \]

(2)

In this study, hemocompatibility of the treated surfaces was characterized by protein adsorption, cell cytotoxicity, cell adhesion, platelet adhesion and platelet activation, and whole blood clotting. Improved hemocompatibility may lead to fewer implant failures, thus increasing the implant life and decreasing recurring procedures in patients.

The morphology of different surfaces was visualized using scanning electron microscope (SEM). The results indicate that the unmodified surfaces as expected do not have any unique surface features. Whereas after the treatment with NaOH, the surfaces have developed unique nanostructures. Ti1 and A1 when exposed to 1 M NaOH for 4 h have developed nanoporous surfaces (Figure 1). There are also nanosized protrusions present on A1 with the length of single protrusions being approximately 250−430 nm. These protrusions are not present on Ti1. Ti2 and A2 when exposed to 5 M NaOH for 24 h have developed interconnected web-like nanoporous surfaces. The grain boundaries are also visible on the surface since they get etched faster than the rest of the surface. The web-like nanoporous architecture is more clearly visible on the A2 (approximate pore size 150−280 nm) as compared to that on Ti2 (approximate pore size 70−170 nm). Ti3 and A3 were exposed to 1 M NaOH for 2.5 h and later were further exposed to 0.6 M HCl for 1 h. This led to a dramatically different nanostructured surface, almost similar to nanosized granules. The average size of granules is approximately 160−220 nm for Ti3 and 90−120 nm for A3. All of the surfaces fabricated had respective uniform morphology (Figure 1). The mechanism for the formation of different nanostructures on surfaces is not well established. However, it is likely that titanium when exposed to NaOH solution at specific temperatures will start reacting to form TiO2 and Na+-O-Ti. The charge repulsion within Na+-O-Ti leads to protrusions in Ti1 and A1 (Figure 1). When the reaction time is significantly increased (Ti2 and A2), the TiO2 and Na+-O-Ti are denser on the surfaces and hence result in the absence of protrusions but a nanoporous surface. When the NaOH-treated surfaces are exposed to HCl, the surface becomes neutral due to the absence of Na+-O-Ti, resulting in formation of denser granule-like structures on the surface (Figure 1).

The contact angle on different surfaces was measured using a goniometer. There are two different configurations that define the contact angle on a nanostructured surface, namely, Wenzel and Cassie–Baxter.41 In the Wenzel state, there is a complete wetting of the surface. Whereas, Cassie–Baxter is a metastable state where air is trapped in between the surface and the liquid. Therefore, the contact angle is influenced by the area fraction of the liquid in contact with the solid and air. The apparent contact angles (\(\theta^*\)) for different surfaces were measured with deionized (DI) water, blood, and platelet-rich plasma (PRP) (Table 1). DI water is commonly used to characterize the surface wettability.42 However, the goal in this study is to understand how blood and its components interact with different surfaces, therefore contact angles of blood and PRP on different surfaces were also measured. Blood is a very viscous fluid that includes PRP (water, proteins, platelets, white blood cells, other biological factors) and red blood cells. However, when PRP is separated from blood, it is less viscous than blood, almost similar to DI water. All modified surfaces were hydrophilic (\(\theta^* < 90^\circ\)) with DI water (Table 1). The following trend for \(\theta^*\) was observed: Ti > A > Ti3 > A3 > A1 > Ti1 > A2 > Ti2. Ti2 and A2 are more hydrophilic compared to all of the other surfaces, almost close to being superhydrophilic (\(\theta^* < 10^\circ\)). This is mainly due to the etched grain boundaries that increase the surface area and thus more wetting on the surface. A1 and A3 had almost a similar contact angle despite different surface features and this could be due to the difference in surface chemistry that lead to similar polar interactions. Ti3 was least hydrophilic compared to all modified surfaces. In summary, titanium surfaces Ti, Ti1, and Ti2 are more hydrophilic than alloy surfaces A, A1, and A2, whereas A3 is more hydrophilic than Ti3 (Table 1). The surface wettability with PRP and blood is more influential for blood clotting. All of the modified surfaces are hydrophilic/hemophilic (\(\theta^* < 90^\circ\)) with PRP (Table 1). The following trend for \(\theta^*\) was observed: Ti > A > Ti2 > A3 > A2 > Ti3 >

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Table 1. Apparent Contact Angles of DI Water, Blood, and PRP on Different Surfaces

| Surface | DI Water | Blood | PRP |
|---------|----------|-------|-----|
| Ti      | 86       | 82    | 96  |
| A       | 79       | 87    | 94  |
| Ti1     | 32       | 25    | 12  |
| A1      | 46       | 22    | 8   |
| Ti2     | 13       | 41    | 29  |
| A2      | 17       | 38    | 18  |
| Ti3     | 64       | 47    | 16  |
| A3      | 46       | 39    | 28  |

*The values were rounded off to zero decimal places. There is a significant difference in the apparent contact angle (\(p < 0.05\)) between all surfaces except A1 and A3 and Ti and Ti3 (statistical differences are not shown in the table).
Ti1 > A1. Further, surface wettability characteristics were completely different from blood when compared to DI water and PRP (Table 1). The following trend for $\theta^\circ$ was observed: A > Ti > Ti3 > Ti2 > A3 > A2 > Ti1 > A1. This could be due to the difference in the liquid properties specifically the viscosity and surface tension.\(^{46–49}\) The results indicate that the interactions between different surfaces with different liquids were always in the Wenzel state, which is more stable than the Cassie–Baxter state.\(^{50}\)

The advancing contact angle ($\theta_{adv}$) for different surfaces with DI water (polar) and hexadecane (nonpolar) was measured (Table 2). The trend for $\theta_{adv}$ with DI water was similar to $\theta^\circ$. The $\theta_{adv}$ values were used to further calculate the surface energy using the Owens–Wendt equation. Higher surface energy relates to higher hydrophilicity, whereas lower surface energy relates to higher hydrophobicity of the surface. The following trend for surface energy was observed: Ti2 > A2 > Ti1 > A1 > Ti3 > A3 > Ti > A. Ti and A have non-zero surface energy. The following trend for surface energy was observed: Ti2 > A2 > Ti1 > A1 > Ti3 > A3 > Ti > A. Ti and A have non-zero surface energy compared to other surfaces. This is because of their low surface energy compared to other surfaces.

The surface chemistry was analyzed using X-ray photoelectron spectroscopy (XPS). Survey scans were obtained and processed using MultiPak and Origin software (Figure 2). All of the surfaces showed peaks for O 1s (529–530 eV for metal oxides), Ti 2p (458.5 eV for TiO$_2$), and C 1s (284.8 eV).

![Figure 2](https://dx.doi.org/10.1021/acsomega.0c00281)

Figure 2. XPS survey scans for different surfaces. Survey spectra were collected from 0 to 1100 eV with a pass energy of 187.85 eV.

indicating that the NaOH treatment removes some of the carbon impurities from the surfaces. Ti1 and A1 have higher Ti 2p peaks compared to Ti and A, respectively. This is due to the etching process that exposes more surface titanium. However, Ti2 and A2 have lower Ti 2p peaks compared to A1 and Ti1, respectively, due to the longer etching process that oxidizes the titanium on the surface. In contrast, Ti3 and A3 have higher Ti 2p peaks compared to all of the other surfaces due to the acidic environment that etches the oxide exposing more surface titanium (Table 3).

Table 3. XPS Elemental Composition Calculated from Survey Scans for Different Surfaces

|         | O 1s (%) | Ti 2p (%) | C 1s (%) |
|---------|----------|-----------|----------|
| Ti1     | 52.1     | 16.1      | 31.8     |
| Ti2     | 51.5     | 16.8      | 31.7     |
| Ti3     | 45.2     | 12.2      | 42.4     |
| A1      | 50.3     | 12.1      | 37.6     |
| A2      | 37.1     | 17.7      | 45.2     |
| A3      | 38.9     | 20.1      | 41.0     |

“The values were rounded off to zero decimal places. There is a significant difference in surface energy ($p < 0.05$) between all surfaces except A1 and A3 and Ti and Ti3.

Carbon is present on the surface due to impurities in the XPS chamber or on the surface. Ti1, Ti2 and A1, A2 have lower C 1s peaks compared to Ti and A, respectively (Table 3),
Fibrinogen is an inflammatory protein that gets converted to fibrin in the presence of thrombin and directly influences the platelet adhesion and activation. It is excreted by the liver and is present in blood. It is a planar protein that has a trinodular structure linked by two coiled-coil regions with a molecular weight of 340 kDa. When factor XII zymogen interacts with an unfamiliar surface (e.g., implant surface), it converts prothrombin to thrombin and in the presence of thrombin, fibrinogen gets converted to fibrin and drives the kinetics of thrombin formation. Hence, it is important to understand the amount of protein adsorbed to the surface. Albumin is the most abundant protein in the blood. It is a passivating protein that prevents blood thrombosis. It is a globular protein with a molecular weight 66.5 kDa. However, more albumin does not directly correlate to less blood clotting as it is just one of many factors. In this study, all modified surfaces are more hydrophilic than unmodified surfaces. More hydrophilicity is equated with less protein adsorption on the surface due to the energy barrier created by the liquid adsorbed to the surface. The results for fibrinogen adsorption follow the following trend (Figure 5a): A3 > A1 > A2 > Ti3 > Ti2 > Ti > A > Ti2, which does not follow the apparent contact angle trend (Table 1). Ti2 has significantly lower fibrinogen adsorption compared to all of the other surfaces since it is the most hydrophilic and has the densest porous structure compared to other surfaces (Figure 1). However, A2 with almost a similar contact angle and less dense porous structure has significantly higher fibrinogen adsorption compared to Ti2.
Table 4. Statistical Comparison of Different Results on Unmodified and Modified Surfaces

| Surface | Fibrinogen adsorption - MicroBCA | Fibrinogen Binding - ELISA | Cell Adhesion - Calcein / F-Actin | Whole Blood Clotting - 45 mins |
|---------|---------------------------------|--------------------------|----------------------------------|-------------------------------|
| Ti      | 27.75                           |                          |                                  |                               |
| Ti1     | 57.56                           |                          |                                  |                               |
| Ti2     | 69.36                           |                          |                                  |                               |
| Ti3     | 34.70                           |                          |                                  |                               |
| A       | 25.85                           |                          |                                  |                               |
| A1      | 42.07                           |                          |                                  |                               |
| A2      | 63.07                           |                          |                                  |                               |
| A3      | 40.81                           |                          |                                  |                               |

Further, Ti1, Ti2, and Ti3 have lower protein adhesion compared to A1, A2, and A3, respectively, in spite of similar nanostructures but different feature sizes. This is because the protein adsorption is also not merely influenced by the contact angle but also majorly by surface topography.61 Other statistical differences between different surfaces are included in Table 4. In contrast, the results for albumin adsorption significantly differ from all the others. Further, there are no significant differences in albumin adsorption on other surfaces irrespective of changes in the contact angle and surface topography. The surface interaction with albumin is different than that of fibrinogen due to its globular shape and different charge.29

Cytotoxicity of different surfaces was evaluated using the lactate dehydrogenase (LDH) assay. The surface modification changes the topography and chemistry, and it is important to evaluate if these changes induce any toxicity to the cells in contact with the surface. Cells when influenced by any toxic element stop growing and eventually die. When a cell begins to breakdown, they lose membrane integrity and release components from the cytoplasm into the medium and one of the stable enzymes excreted during this process is LDH. Hence, the presence of this LDH enzyme is a marker for cytotoxicity.60 The host body should be able to tolerate the implant while maintaining stability, without any exclusion and destruction.61 The results indicate that all of the substrates have similar amount of LDH expression compared to the positive control (100% live cells), indicating that none of the surfaces is inducing toxicity to the cells present in the PRP (Figure 6). LDH expression from the negative control (100% dead cells) was significantly higher when compared to all of the surfaces and the positive control (Figure 6). Thus, the results indicate that none of the surfaces demonstrates short-term cytotoxic effects on the cells present in the PRP.

Fibrinogen binding from PRP on different surfaces was evaluated using an ELISA assay. Fibrinogen binding from PRP is a realistic environment since it is also influenced by other components that are present in PRP (other proteins, platelets, thrombin, leukocytes, etc.). Fibrinogen when in contact with thrombin gets converted to fibrin. This fibrin fibers form the blood clot on the surface. Hence, it is important to evaluate fibrinogen binding directly from the PRP on different surfaces. The surface-exposed PRP was assayed to measure the indirect fibrinogen binding from the PRP on different surfaces. The surface-exposed PRP was assayed to measure the indirect fibrinogen binding directly from the PRP on different surfaces. The surface-exposed PRP was assayed to measure the indirect fibrinogen binding from the PRP on different surfaces. The surface-exposed PRP was assayed to measure the indirect fibrinogen binding directly from the PRP on different surfaces. The surface-exposed PRP was assayed to measure the indirect fibrinogen binding directly from the PRP on different surfaces. The surface-exposed PRP was assayed to measure the indirect fibrinogen binding directly from the PRP on different surfaces.

Figure 6. Cell cytotoxicity for PRP exposed to different surfaces measured using the LDH assay. The results indicate no significant differences in the LDH activity on all of the surfaces and positive control (100% live cells), whereas the LDH activity for the negative control (100% dead cells) was significantly different than all of the other surfaces (*p < 0.05). The error bar represents the standard deviation.

ACS Omega 2020, 5, 8108–8120
Cell adhesion from PRP (platelet and leukocyte adhesion) on different surfaces was evaluated by fluorescence microscopy (Figure 8a). The surfaces with adhered cells were stained with calcein-AM. Platelet and leukocyte adhesion plays a vital role in the stimulation of the coagulation factors for hemostasis. However, nanotopography of surfaces also influences cell adhesion. In porous surfaces like Ti2, the dense porous structure majorly influences cell adhesion. The results indicate lower cell adhesion on Ti2 due to its dense porous structure that drastically reduces the area of contact with the cells. Cell adhesion through penetration into the porous structure is also not possible as the size of the pores is smaller than the size of the cells. Other statistical differences between different surfaces are included in Table 4.

Identification of different cell types (platelets and leukocytes) adhered on the surface from PRP was evaluated by fluorescence microscopy (Figure 9a). The surfaces with adhered cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and rhodamine–phalloidin. DAPI stains the nucleus of the cells, whereas rhodamine–phalloidin stains the cytoskeleton of the cells. Since platelets do not have a nucleus, they will not stain positive for DAPI, whereas both platelet and leukocyte nuclei stain positive for DAPI.

Cell adhesion from PRP (platelet and leukocyte adhesion) on different surfaces was evaluated by fluorescence microscopy (Figure 8a). The surfaces with adhered cells were stained with calcein-AM. Platelet and leukocyte adhesion plays a vital role in the stimulation of the coagulation factors for hemostasis. Leukocytes can impact coagulation directly by producing anticoagulant molecules or procoagulants or indirectly acting on platelets, other leukocytes, or endothelial cells. Hence, it is important to understand the influence of surface modification on cell adhesion. The fluorescence microscopy images show that Ti and A have higher cell adhesion when compared to all of the other surfaces (Figure 8a). The area covered by the cells adhered on the surface was calculated using ImageJ (Figure 8b). The results indicate a significant decrease in cell adhesion on the modified surfaces when compared to the Ti and A (Figure 8b), with Ti1 and Ti2 having the least cell adhesion. Further, Ti1, Ti2, and Ti3 showed less cell adhesion when compared to A1, A2, and A3, respectively. The surface area is a key factor that influences cell adhesion. In general, higher surface area results in higher cell adhesion. However, nanotopography of surfaces also influences cell adhesion. In porous surfaces like Ti2, the dense porous structure majorly influences cell adhesion. The results indicate lower cell adhesion on Ti2 due to its dense porous structure that drastically reduces the area of contact with the cells. Cell adhesion through penetration into the porous structure is also not possible as the size of the pores is smaller than the size of the cells. Other statistical differences between different surfaces are included in Table 4.
leukocytes will stain positive of rhodamine−phalloidin. Thus, by staining the cells with DAPI and rhodamine−phalloidin, leukocytes adhered on the surface can be identified. During thrombosis, once the platelets are activated, they interact with leukocytes and further enhance the platelet activation rate.71,72 Hence, it is important to identify and evaluate platelet and leukocyte adhesion on the surface. The fluorescence microscopy images show that Ti and A have higher leukocytes and platelets adhered on the surface when compared to all of the other surfaces (Figure 9a). The area covered by the leukocytes and platelets on the surface was calculated using ImageJ. The results from DAPI images indicate a significant decrease in leukocyte adhesion on all of the modified surfaces (Figure 9b), except Ti3, when compared to Ti and A. The results from rhodamine−phalloidin images indicate that platelet and leukocyte counts from rhodamine−phalloidin stains (Figure 9c) are significantly lower on all modified surfaces when compared to Ti and A, with Ti1 and Ti2 having least cell adhesion. Further, Ti1, Ti2, and Ti3 showed less cell adhesion when compared to A1, A2, and A3, respectively. Other statistical differences between different surfaces are included in Table 4. Similar trends as to that of the cell adhesion study were observed in this study.

Platelet activation and platelet−leukocyte complex formation on different surfaces were visualized using SEM. Platelets when activated change shapes, form dendrites, and start aggregating.26 The activated platelet may assist leukocyte localization during thrombosis and modulate their function.67 The leukocyte−platelet interaction thus initiates biosynthesis of cytokines and inflammatory reactions, which leads to several heart conditions.73 Therefore, it is crucial to evaluate if the surfaces induce platelet activation and platelet−leukocyte complex formation. The SEM images show activated platelets with dendrites on Ti and A (Figure 10). Further, leukocyte−platelet interactions are also observed on Ti and A (Figure 10, dotted circle). However, there is a drastic reduction in platelet activation and the absence of leukocyte−platelet interactions on all of the other surfaces. Further, platelets seem to aggregate on modified surfaces. However, the platelets are not activated as no dendrite extensions are present.

Whole blood clotting was evaluated on different surfaces by measuring the free hemoglobin present on the surface that was in contact with blood. The uncotted blood when diluted with DI water results in lysis of red blood cells and thus releasing hemoglobin. Hence, higher hemoglobin in the diluted solution indicates less blood clotting on the surface. The blood was allowed to clot for 45 min on all of the surfaces, and the amount of free hemoglobin was measured every 15 min (Figure 11). During the three-time points evaluated, the blood
clotting was significantly lower on all of the modified surfaces when compared to Ti and A. The amount of free hemoglobin on Ti and A decreased drastically from 15 to 45 min, indicating that the blood is clotting on the surface. However, Ti1, A1, Ti2, and A2 surfaces did not show a significant reduction in free hemoglobin from 15 to 45 min. Further, Ti3 and A3 did not show significant differences in free hemoglobin after 15 min, however showed a significant reduction in free hemoglobin after 30 and 45 min. After 45 min, Ti2 and A2 showed the highest free hemoglobin when compared to all surfaces, indicating minimum blood clotting. Other statistical differences between different surfaces are included in Table 4.

In summary, Ti1, Ti2, and Ti3 showed less blood clotting when compared to A1, A2, and A3, respectively, at all time points.

3. CONCLUSIONS

Various permutations and combinations of variables such as NaOH concentration, temperature, and time for treatment were used to determine the optimal conditions for fabrication that resulted in uniform and repeatable nanostructures on different surfaces. The results for cytotoxicity indicate that there were no short-term cytotoxic effects. Further, Ti3 and A3 did not show significant differences in free hemoglobin after 15 min, however showed a significant reduction in free hemoglobin after 30 and 45 min. After 45 min, Ti2 and A2 showed the highest free hemoglobin when compared to all surfaces, indicating minimum blood clotting. Other statistical differences between different surfaces are included in Table 4.

4. EXPERIMENTAL SECTION

4.1. Fabrication of Nanostructures on the Different Surfaces. The materials used in this study were sheets of 0.5 mm thick commercially pure titanium (grade 2) and the Ti–6Al–4V alloy. Square substrates of dimension 6 mm × 6 mm were cut from the sheets. The surfaces were polished using SiC abrasive sheets up to grade 1400. Polished substrates were then cleaned ultrasonically with acetone and deionized (DI) water for 10 min each. Three different fabrication processes were used to hydrothermally treat the substrates to form nanostructures on the surface and are explained below:

1. Substrates were immersed in 75 mL of 1 M NaOH solution inside an inert PTFE bottle and hydrothermally treated for 4 h at 200 °C inside an oven. The treated substrates were rinsed with DI water and were annealed for 1 h at 300 °C.

2. Substrates were immersed in 50 mL of 5 M NaOH solution inside an inert PTFE bottle and hydrothermally treated for 24 h at 60 °C inside an oven. The treated substrates were rinsed with DI water and were annealed for 1 h at 300 °C.

3. Substrates were immersed in 75 mL of 1 M NaOH solution inside an inert PTFE bottle and hydrothermally treated for 2.5 h at 200 °C inside an oven. The treated substrates were rinsed with DI water and were annealed for 1 h at 300 °C. After annealing, they were again rinsed with DI water and immersed in 50 mL of 0.6 M HCl solution inside an inert PTFE bottle for 1 h inside an oven. The treated substrates were rinsed with DI water and were annealed for 2 h at 600 °C.

After the treatments, all substrates were ultrasonically cleaned with DI water for 10 min and dried using nitrogen gas.

The following notation will be used in the manuscript for different surfaces: Ti for unmodified titanium, A for unmodified Ti–6Al–4V. The modified surfaces are abbreviated as Ti1, Ti2, Ti3, A1, A2, and A3, where the number corresponds to the hydrothermal treatment used.

4.2. Characterization of Different Surfaces. The modified surfaces were characterized using different techniques to evaluate their surface energy, morphology, chemistry, and crystal structure.

4.2.1. Surface Morphology. The surface morphology was characterized using a JEOL 6500 field emission scanning electron microscope (SEM), operated at an accelerating voltage of 15 kV. The surfaces were coated with 5 nm gold before imaging. The surfaces were imaged at magnifications of 5000× and 30 000×. The images were processed using the ImageJ software.

4.2.2. Contact Angle Measurements and Surface Energy Calculations. The surface hydrophobicity/hydrophilicity was characterized by measuring the apparent contact angle (θp) and advancing contact angle (θa) at room temperature using the sessile drop method (Ramé-hart 260F4 goniometer). Images were captured 3 s after 5 μL droplet of DI water (polar) and hexadecane (nonpolar) was placed on the substrate. The errors in apparent and advancing contact angles were ±4 and ±3°, respectively. The images were analyzed using the manufacturer-provided software to measure the contact angles. Apparent contact angles for blood and platelet-rich plasma (PRP) (see Section 4.5 for isolation of blood and PRP) were also measured using the same method.

The advancing contact angles for water and hexadecane were used to calculate the solid—vapor surface energy. The polar and dispersive components of the surface energy were calculated using Young’s equation (eq 3) and Owens–Wendt equation (eq 4).

\[
\gamma_d = \gamma_{sv} + \gamma_{lp} - 2\sqrt{\gamma_{sv}^2 + \gamma_{lp}^2} 
\]

\[
\gamma_d = \gamma_{sv} + \gamma_{lp} - 2\gamma_{sv} \sqrt{\frac{\gamma_{lp}}{\gamma_{sv}}} 
\]

where \(\gamma_{sv}\) is the liquid (water)—vapor interfacial tension (72.8 mN/m) and hexadecane (27.5 mN/m), \(\theta\) is the advancing contact angle, \(\gamma_{sv}\) is the solid surface free energy, \(\gamma_{lp}\) is the solid—liquid interfacial free energy, \(\gamma_{sv}^d\) and \(\gamma_{lp}^d\) are the dispersive components, and \(\gamma_{sv}^p\) and \(\gamma_{lp}^p\) are the polar components of the solid and liquid-free surface energy, respectively. The above calculations were performed using the ImageJ software.
two equations were solved to get the final equations to calculate the dispersive (eq 5) and polar (eq 6) components of the solid—vapor surface energy. The overall solid—vapor surface energy is the sum of the dispersive and polar components (eq 7).

\[
\gamma_{sv}^d = \gamma_{sv}^0 \left(1 + \cos^2 \theta^d \right) \left(1 + \cos^2 \theta^d \right) ^2 \\
\gamma_{sv}^p = \frac{1}{\gamma_{sv}^0} \left( \frac{\theta^p}{\cos \theta^p} \right) - \sqrt{\gamma_{sv}^d, \gamma_{sv}^d} \right) ^2 \\
\gamma_{sv} = \gamma_{sv}^d + \gamma_{sv}^p
\]

4.2.3. Surface Chemistry. The surface chemistry was characterized using a PE-5800 X-ray photoelectron spectrometer. Survey spectra were collected from 0 to 1100 eV with a pass energy of 187.85 eV. High-resolution spectra were collected for oxygen (O 1s) using a pass energy of 10 eV. The surface elemental composition was calculated using peak fit analysis in the Multipack and Origin software.

4.2.4. Surface Crystal Structure. The presence of anatase and rutile crystal phases was characterized by Bruker D8 glancing angle X-ray diffraction (GAXRD). XRD scans were collected at \( \theta = 1.5^\circ \), and 2\( \theta \) ranges were chosen based on significant peak intensities. Detector scans were run at a step size of 0.01 with a time per step of 1 s. Peaks were filtered and correlated to crystal structures using the DIFFRAC.T.EVA software.

4.3. Surface Preparation Prior to Biological Studies. Prior to biological studies, the surfaces were sonicated in acetone for 10 min followed by rinsing with DI water and phosphate-buffered saline (PBS) solution. The surfaces were sterilized by exposure to UV light for 15 min inside a biosafety cabinet.

4.4. Protein Adsorption on the Different Surfaces. Albumin and fibrinogen adsorptions were characterized using a micro-BCA assay. The surfaces were incubated for 2 h in a 48-well plate on a horizontal shaker (100 rpm) at 37 °C and 5% CO\(_2\). The protein solution was aspirated, and surfaces were rinsed two times with PBS. To measure the amount of protein adhered on different surfaces, the substrates were incubated for 1 h in a 48-well plate on a horizontal shaker (100 rpm) at 37 °C and 5% CO\(_2\). After the incubation, the PRP solution was aspirated and surfaces were rinsed three times with PBS to remove any nonadherent cells. One hundred microliter of surface-exposed PRP was pipetted to the 96-well plate, and the protocol provided by the manufacturer was followed. The absorbance of the resulting solution was measured at a wavelength of 450 nm using a plate reader.

4.5. Platelet-Rich Plasma (PRP) Isolation from Whole Blood. Whole blood was isolated through venipuncture from healthy donors, who refrained from having drugs that may have affected their blood. The isolation procedure was in accordance with the protocol approved by the Colorado State University Institutional Review Board. Procedures were performed in compliance with the National Institutes of Health’s “Guiding Principles for Ethical Research”. Informed consents were obtained from human participants prior to enrolling in this study. The blood was collected in 6 mL tubes coated with the anticoagulant, ethylenediaminetetraacetic acid (EDTA). The first tube of blood was discarded to account for the needle insertion. The PRP was isolated by centrifuging the blood tubes at 150g for 15 min. The centrifugation results into two layers, the top layer, which is PRP, and the bottom layer, which is the red blood cells. After centrifugation, the tubes were let to rest for another 15 min before the PRP was used. All of the biological studies were repeated at least three times with blood drawn from at least three different donors. However, for each experiment, the PRP was only pooled from the same donor. This is because there is donor-to-donor variability in the number of platelets and it is not possible to compare the absolute values from different donors.

4.6. Cytotoxicity of Different Surfaces. Cytotoxicity of different surfaces was evaluated using a commercially available lactate dehydrogenase (LDH) assay kit. The surfaces along with positive (provided with the assay) and negative controls (PRP treated with Triton-X provided with the assay) were incubated with 300 \( \mu \)L of PRP in a 48-well plate on a horizontal shaker (100 rpm) for 2 h at 37 °C and 5% CO\(_2\). After the incubation, the PRP solution was aspirated and surfaces were rinsed three times with PBS to remove any nonadherent cells. One hundred microliter of surface-exposed PRP was pipetted to the 96-well plate, and the protocol provided by the manufacturer was followed. The absorbance of the resulting solution was measured at a wavelength of 490 nm using a plate reader.

4.7. Fibrinogen Binding from PRP on Different Surfaces. Fibrinogen binding was measured using a commercially available ELISA kit for human fibrinogen. The surfaces were incubated in 300 \( \mu \)L of PRP in a 48-well plate on a horizontal shaker (100 rpm) for 2 h at 37 °C and 5% CO\(_2\). The surface-exposed PRP was then diluted to 1/10,000 with the diluent provided with the ELISA kit. This diluted PRP was pipetted to the 96-well plate, and the protocol provided by the manufacturer was followed. The absorbance of the resulting solution was measured at a wavelength of 450 nm using a plate reader.

4.8. Cell Adhesion on Different Surfaces. Cell adhesion on different surfaces was imaged by fluorescence microscopy. The surfaces were incubated in 300 \( \mu \)L of PRP in a 48-well plate on a horizontal shaker (100 rpm) for 2 h at 37 °C and 5% CO\(_2\). After the incubation, the PRP was aspirated and surfaces were rinsed three times with PBS to remove any unadhered cells. The surfaces were then incubated in dark with 1 mL of 5% calcine-AM solution in PBS for 20 min at room temperature. The stain solution was aspirated, and the surfaces were rinsed two times with PBS. The surfaces were imaged using a fluorescence microscope (Zeiss AxioVision) at 20x. All images were further processed using ImageJ to calculate the surface coverage of cells.

4.9. Identification of Platelets and Leukocytes Adhered on Different Surfaces. Identification of platelets and leukocytes adhered on different surfaces was imaged by fluorescence microscopy. The surfaces were incubated in 300 \( \mu \)L of PRP in a 48-well plate on a horizontal shaker (100 rpm) for 2 h at 37 °C and 5% CO\(_2\). After the incubation, the PRP was aspirated and surfaces were rinsed three times with PBS to remove any unadhered cells. The surfaces were fixed in 3.7% formaldehyde solution diluted with PBS for 15 min. The fixed surfaces were rinsed twice in PBS for 5 min each. Subsequently, the surfaces were incubated in a solution of
1% Triton-X diluted with PBS for 3 min. The surfaces were again rinsed twice in PBS and moved to a new 48-well plate. Two hundred microliter of 0.05% rhodamine−phalloidin (actin) solution in PBS was added to each well and incubated for 25 min. Twenty one microliter of 3% 4′,6-diamidino-2-phenylindole (DAPI) stain stock solution was added to each well and incubated for 5 more minutes. The surfaces were then rinsed twice in PBS and imaged using a fluorescence microscope. ImageJ was used to calculate the actin cell coverage and number of nuclei on the substrates. The DAPI will stain the nucleus of leukocytes blue, whereas the rhodamine−phalloidin will stain the cytoskeleton of both the platelets and leukocytes red.

4.10. Platelet Activation on Different Surfaces. Platelet activation on different surfaces was characterized using SEM. The surfaces were incubated in 300 μL of PRP in a 48-well plate on a horizontal shaker (100 rpm) for 2 h at 37 °C and 5% CO₂. After the incubation, the PRP was aspirated and surfaces were rinsed three times with PBS to remove any unadhered cells. The cells adhered to the surface were fixed by incubating the surfaces in a solution containing 6% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1 M sucrose in DI water for 45 min. The surfaces were then incubated in a buffer solution containing 0.1 M sodium cacodylate and 0.1 M sucrose for 10 min. This was followed by incubating the surfaces in 35, 50, 70 and 100% ethanol for 10 min each. The surfaces were air-dried and imaged using a SEM, as discussed in Section 4.2.1.

4.11. Whole Blood Clotting on Different Surfaces. Whole blood clotting on different surfaces was characterized by indirectly measuring the amount of free hemoglobin in unclotted blood after exposure of surfaces to whole human blood using a plate reader. For this study, blood was drawn in a vacuum tube without any anticoagulant and was used immediately after drawing. Five microliter of blood was pipetted on top of different surfaces in a 24-well plate, and the blood was allowed to clot for up to 45 min. After every 15 min, the surfaces were evaluated for the presence of free hemoglobin. Five hundred microliter of DI water was added to the surfaces and gently shaken for 30 s to lyse red blood cells that were not trapped in the clot on the surface. The absorbance of free hemoglobin released by the lysed red blood cells was measured at a 540 nm wavelength using a plate reader.

4.12. Statistical Analysis. Surface characterization was repeated for at least three different samples of each surface. SEM images and contact angle measurements were taken at three different locations on each sample (n_min = 9). Protein adsorption was carried out on at least three different samples of each surface and was repeated at least three times (n_min = 9). The LDH assay, fluorescence microscopy, platelet activation, and whole blood clotting were repeated at least two times (with PRP from different donors) with at least three different samples of each surface (n_min = 6). The quantitative results were analyzed using a two-way analysis of variance (ANOVA) test using the R software. The results were considered statistically significant with a p-value <0.05. The data presented (i.e., the arithmetic mean and standard deviation) is only from one donor as it is not appropriate to compare values between different donors due to the variability in the platelet counts for each donor. However, similar trends were observed for blood from different donors for all of the results presented, indicating the reproducibility of the data.

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**Notes**

The authors declare no competing financial interest.

### Acknowledgments

Research reported in this publication was supported by National Heart, Lung, and Blood Institute of the National Institutes of Health under award number R01HL135505 and R21HL139208. The authors acknowledge Patrick McCurdy from CIC CSU for his training with SEM and XPS, Roberta Maia Sabino from CSU for her training with biological experiments, Paulo Soares from PUCPR, Brazil for his assistance with XRD, and all of the people who donated blood for these experiments.

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