Ex vivo evaluation of blood coagulation on endothelial glycocalyx-inspired surfaces using thromboelastography

Yanyi Zang^{1,2} · Jessi R. Vlcek¹ · Jamie Cuchiaro³ · Ketul C. Popat^{1,4,5} · Christine S. Olver⁶ · Matt J. Kipper^{1,5,7} · Melissa M. Reynolds^{1,3,5}

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Abstract

Purpose Present blood-contacting materials have not yet demonstrated to be effective in reducing blood coagulation without causing additional side effects clinically. We have developed an endothelial glycocalyx-inspired biomimetic surface that combines nanotopography, heparin presentation, and nitric oxide (NO)-releasing features. The resulting modified surfaces have already shown promise in reducing unfavorable blood-material interactions using platelet-rich plasma. In this study, the efficacy of modified surfaces for reducing coagulation of human whole blood was measured. In addition, the effects of leached polysaccharides and chemical modification of the modified surfaces were evaluated.

Methods Leached polysaccharides in the incubation solution were detected by a refractive index method to determine the potential influences of these modified surfaces on the blood coagulation observation. Chemical modifications by the nitrosation process on the polysaccharides in the modified surfaces were detected using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). Clot formation parameters were measured using thromboelastography (TEG), a clinically relevant technique to evaluate whole blood coagulation.

Results No polysaccharides were detected in the heparinized polyelectrolyte multilayer-coated titania nanotube array surface (TiO₂NT + PEM) incubation solution; however, polysaccharides were detected from NO-releasing TiO₂NT + PEM surface (TiO₂NT + PEM + NO) incubation solution both after the nitrosation process and after all NO was released. The structures of thiolated chitosan and heparin were altered by *t*-butyl nitrite. All heparin-containing surfaces were shown to slow or inhibit clot formation.

Conclusion This study is the first to evaluate these endothelial glycocalyx-inspired surfaces using clinically relevant parameters, as well as proposing potential influences of these modified surfaces on the inhibition of clot formation.

Keywords Nanotexture · Polysaccharides · Nitric oxide · Glycocalyx · Thromboelastography · Refractive index

Introduction

Since the first attempt of using the “Lane plate” for bone fracture repair in 1895, metal implants have become increasingly popular because of their advantages, such as excellent

corrosion resistance, ease of sterilization, and others [1–4]. Currently, metal implants have been used in a variety of blood-contacting medical applications, for example, biosensors, vascular stents, and pacemakers. However, coagulation at the interface between human whole blood and the

✉ Melissa M. Reynolds
Melissa.Reynolds@colostate.edu

¹ School of Biomedical Engineering, Colorado State University, Fort Collins, CO, USA

² Present Address: Autonomous Reanimation and Evacuation Research Program, The Geneva Foundation, Brook City-Base, San Antonio, TX, USA

³ Department of Chemistry, Colorado State University, Fort Collins, CO, USA

⁴ Department of Mechanical Engineering, Colorado State University, Fort Collins, CO, USA

⁵ School of Advanced Materials Discovery, Colorado State University, Fort Collins, CO, USA

⁶ Veterinary Clinical Pathology Section, Colorado State University, Fort Collins, CO, USA

⁷ Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, CO, USA

surface of metal implants is still an unsolved limitation that impedes their performance [5]. The coagulation cascade starts when human whole blood interacts with implant surfaces, resulting in the deposition of blood plasma proteins such as fibrinogen. The adsorbed fibrinogen on the material surface is then converted into a fibrin clot in the presence of thrombin. The fibrin clots further induce platelet activation and aggregation, and lead to thrombus formation [6]. Without proper anticoagulant intervention, continuous blood clot formation places patients at high risk of medical device-induced thrombosis and may require an additional surgery to replace the implanted medical devices [7].

Current strategies involved in solving medical device-induced thrombosis include surface modification, anticoagulant administration, polymer coatings, and drug release [7–15]. Nanoscale and microscale surface features such as nanotextured surfaces have been shown to effectively reduce platelet activities and further inhibit thrombus formation on nitinol and titanium surfaces [8–10]. Heparin is a systemic anticoagulant that is commonly used in clinics either intravenously or subcutaneously to prevent thrombus formation. However, heparin can induce thrombocytopenia or even hemorrhagic complication, especially for patients with bleeding wounds [11, 12]. Immobilized heparin on material surfaces or heparinized surfaces is another approach to improve the biocompatibility of medical devices, such as Hepacoat™ on Bx Velocity Coronary Stents and Carmeda® BioActive surface on Cordis® stents [13–15]. The limitation of heparinized surfaces is that their inhibitory effects may not be enough to eliminate coagulation activation alone, and a supplemental anticoagulant therapy is required [16]. Drug-eluting surfaces also improve medical device performance and are commercially available. However, the duration of drug release may not be tuned to individual patient needs [17]. These approaches still have limitations of both performance and patient risk; therefore, new strategies are needed.

Endothelial glycocalyx-inspired multifunctional surfaces combine several surface features, including nanotextured and microtextured surfaces, heparin and chitosan polyelectrolyte multilayer (PEM) coatings, and the release of nitric oxide (NO) [18]. First, a bare titanium (Ti) surface was fabricated into a titania nanotube surface (TiO₂NT) using an electrochemical method to form TiO₂NT. Second, positively charged chitosan and negatively charged heparin were coated onto the nanotube surface to produce TiO₂NT + PEM. Finally, the surface coating was nitrosated to confer the ability to release NO molecules, yielding TiO₂NT + PEM + NO. The nanotextured surface has been shown to deactivate plasma components [10]. The polyelectrolyte multilayer coating was designed to mimic the glycosaminoglycan presentation in the endothelial glycocalyx, which may prevent blood protein adsorption and fibrin clot formation [19–21]. The combination of nanotextured surfaces with PEM coating

has been shown to reduce platelet adhesion and activation [22, 23]. Nitric oxide, an endogenous signaling molecule, is well-known for its antiplatelet and anticoagulation properties [24]. Multiple NO-releasing surfaces have been shown to mitigate blood protein deposition, and inhibit platelet adhesion and activation [25–30]. This three-in-one promising surface has been previously proven to significantly inhibit platelet adhesion and activation in contact with human platelet-rich plasma [18]. However, this coating has not been evaluated in a real-time manner using human whole blood.

Herein, we report the first real-time clot formation evaluation on an NO-releasing PEM coating on a titania nanotube array surface using human whole blood. In this study, thromboelastography (TEG) was performed. TEG has been widely used in various applications, ranging from coagulation management in clinical testing to the evaluation of newly designed biomaterials [31–33]. In addition, TEG allows the localized measurement of whole blood coagulation on a material surface, including initiation of clot formation, rate of clot formation, and clot strength. This is an advancement compared to plasma-based coagulation assays which only allow evaluation of the initiation of clot formation, and therefore may not be sensitive nor specific for a surface's effect on coagulation. Blood clot formation was not detected after blood was incubated with TiO₂NT + PEM surfaces for either 15 min or 60 min. Blood clotting was delayed after blood was incubated with TiO₂NT + PEM + NO surfaces for either 15 min or 60 min; however, clots still formed in the blood. Based on these results, we further conducted analyses on the incubation solution to detect the presence of released polysaccharides using a refractive index (RI) method. We also identified chemical modification of chitosan thioglycolic acid (chitosan-TGA) and heparin by the nitrosation process using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) [34]. This work reveals which features of these novel glycocalyx-inspired surfaces influence whole blood clotting kinetics, and provides insights into the design of biomimetic blood-compatible surfaces.

Materials and methods

Materials

All chemicals and solvents were purchased from commercial vendors and used without further purification unless otherwise noted. CP Grade 1 Surplus Titanium sheet (0.025" thickness) was purchased from Titanium Joe (Kingston, Ontario, Canada). Platinum foil was purchased from Surepure Chemetals (Florham Park, NJ, USA). Diethylene glycol (DEG; 99%) and hydrofluoric acid (HF; 48–51%) were purchased from Alfa Aesar (Tewksbury, MA, USA).

Low molecular weight chitosan (96.1% deacetylated, 50–190 kDa), thioglycolic acid ($\geq 98\%$), tris(2-carboxyethyl)phosphine hydrochloride ($\geq 98\%$), and *tert*-butyl nitrite (90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride ($\geq 99\%$) was purchased from Chem-Impex International (Wood Lawn, IL, USA). Heparin sodium was purchased from Celsus Laboratories, Inc. (from porcine intestinal mucosa, 14.4 kDa, 12.5% sulfur; Cincinnati, OH, USA). Acetic acid (99 + %) was purchased from ACROS organics (Geel, Belgium). Corning™ 0.22- μm PES vacuum filter/storage systems were purchased from Corning (Corning, NY, USA). Spectra/Por™ 8000 MWCO membrane tubing, methanol, ethanol, and 50-mL conical tubes were purchased from Fisher Scientific (Hampton, NH, USA). Diethyl ether was purchased from EMD Millipore (Burlington, MA, USA). Millipore water (18.2 M Ω -cm) was used for all experiments.

Surface preparation and characterization

Surfaces were prepared based on a previously reported method [10, 18, 35]. Briefly, TiO₂NT was fabricated through a 55-V 22-h DEG/HF/water-based electrochemical anodization process with titanium serving the anode and platinum serving the cathode. Chitosan-TGA was synthesized by adding 1.5 mL of thioglycolic acid and 3.8 g of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride into the mixture of 100 mL chitosan solution (1.0 g of chitosan in 100 mL of water) and 1.0 mL of acetic acid. The resulting solution was dialyzed against 4 L of water with 0.25 M HCl in 8000 MWCO tubing at pH 4–5 for 24 h in the absence of light. After dialysis, 0.1 g of tris(2-carboxyethyl)phosphine hydrochloride was added into the solution. The solution was further dialyzed again in 8000 MWCO tubing against 4 L of water with 0.25 M HCl at pH 4–5 for 7 days (50 g of NaCl was added into water for the first 3 days) without light. After dialysis, the resulting contents in the tubing were freeze-dried for 4 days in the absence of light. Lyophilized chitosan-TGA and heparin were dissolved into 0.2 M acetate buffer (pH = 4.5) to form a 0.01 M chitosan-TGA polymer solution and 0.01 M heparin polymer solution, respectively. Both polymer solutions were filtered through Corning™ 0.22- μm PES vacuum filter/storage systems. Acidified rinse solution was prepared by adjusting Millipore water pH to 4 using glacial acetic acid. Positively charged chitosan-TGA and negatively charged heparin were coated for 10 bilayers onto a TiO₂NT surface using a layer-by-layer coating process. The TiO₂NT samples (1.0 cm × 1.0 cm) were each placed in the wells of a 24-well plate. For each bilayer, 1 mL of solution was added into each well that contained TiO₂NT surface in the following order: acidified rinse solution, chitosan-TGA polymer solution,

acidified rinse solution, and heparin solution. Between each solution, the well plate was shaken for 5 min and the solution was removed by aspiration. After 10 bilayers were applied, the surface in each well was rinsed with 1 mL of acidified rinse solution and dehydrated using a series of ethanol concentrations (50%, 70%, 90%, 100%, and 100%) with 5 min for each concentration. The surfaces were then dried in a chemical fume hood before nitrosation. For the nitrosation process, all steps were performed in the dark. Each surface was soaked in 300 μL of *tert*-butyl nitrite overnight on a shaker. The next day, surfaces were rinsed with 300 μL of methanol followed by 300 μL of diethyl ether before being dried under vacuum for 30 min. Nitrosated surfaces were then covered with aluminum foil and used within 1 h. Surfaces were characterized using scanning electron microscopy (SEM) for surface morphology ($n = 3$) and nitric oxide analyzer (NOA) for NO-releasing measurement ($n = 3$).

Detection of released polysaccharides in the soaking solution via a refractive index (RI) method

Refractive index detection was carried out on an Agilent 1260 Infinity II HPLC equipped with RI detector via direct sample injection. Data were analyzed using Agilent OpenLab CDS (version 3.4.0) software. The mobile phase used was water. A flow rate of 0.3 mL/min and a 10- μL injection volume ($n = 5$, each analyzed in triplicate) were used. The detector temperature was set to 30 °C and in the positive polarity mode. Ti, TiO₂NT, TiO₂NT + PEM, and one set of TiO₂NT + PEM + NO surfaces were soaked into 0.5 mL of water for 60 min at 37 °C with gentle shaking. Another set of TiO₂NT + PEM + NO surfaces was incubated overnight to ensure NO was fully released from the surfaces. These surfaces were then submerged in 0.5 mL of water for 60 min at 37 °C with gentle shaking before performing the RI measurement. To distinguish if the RI method is specific to leached heparin, 200 $\mu\text{g/mL}$ working solutions of heparin, nitrosated heparin, chitosan-TGA, and nitrosated chitosan-TGA were analyzed and the instrument responses were compared. These 200 $\mu\text{g/mL}$ working solutions were prepared in DI water.

Detection of chemical modification by nitrosation process via attenuated total reflectance-Fourier transform infrared (ATR-FTIR)

Nitrosated samples were submerged in *tert*-butyl nitrite overnight, dried, and rinsed with methanol and ethyl ether. The samples were then air-dried shielded from light for at least 24 h before analysis. ATR-FTIR spectra were collected using a Thermo Nicolet 6700 spectrometer equipped with a diamond ATR crystal between 650 and 4000 cm^{-1} . Solid

samples of heparin, nitrosated heparin, chitosan-TGA, and nitrosated chitosan-TGA were analyzed and their spectra compared. Representative spectra were reported.

Human whole blood collection

This research was approved by the Colorado State University Institutional Review Board for the protection of human subjects with protocol # 19-9039H and the Colorado State University Institutional Biosafety Committee for the utilization of biohazardous materials with protocol # 14-070B. In total, three individuals donated blood for this study. Human whole blood was collected at Colorado State University Health and Medical Center from healthy donors who were lacking coagulopathies and had not used thromboxane inhibitors for at least 2 weeks. Blood was drawn into 4.5-mL citrated BD Vacutainers® venous blood collection tubes (12.35 mg of sodium citrate, 2.21 mg of citric acid). After blood collection, human whole blood in each tube was transferred into a 50-mL conical tube and allowed to rest for 20 min prior to use. All blood was used within 4 h following collection time.

Thromboelastography (TEG)

One milliliter of citrated human whole blood was incubated with modified surfaces in microcentrifuge tubes for 15 min and 60 min at room temperature, and unmodified Ti surfaces were used as controls ($n \geq 5$ batches of surfaces). After incubation, 340 μ L of citrated human whole blood was transferred into TEG disposable cups with 20 μ L of 0.2 M calcium chloride. TEG was then performed without activating reagents at 37 °C until clot maximum amplitude (MA)

was detected or until 60 min after TEG was started. Several clinically relevant clot formation parameters were recorded (Supporting Information Figure S1) including reaction time (R) which is the amount of time required for the first detection of a 2-mm amplitude clot in blood, initial clot formation time (K) which is the amount of time required to progress to a 20-mm amplitude clot, the angle between baseline and the tangential line of R (α -angle) which shows the speed of fibrin formation and cross-linking comparing to the “thrombin burst” in the cell-based model, and maximal amplitude (MA) which is the detected clot strength and relates to clot stability.

Statistical analysis

One-way analysis of variance (ANOVA) followed by post hoc Tukey–Kramer multiple comparison test at a 95% confidence interval ($p < 0.05$) was used to evaluate statistical differences. All reported values were expressed as mean $\pm$ standard deviation.

Results and discussion

Surface characterization

SEM images at two different magnifications of Ti, TiO₂NT, TiO₂NT + PEM, and TiO₂NT + PEM + NO surfaces are shown in Fig. 1. The nanotubular structures on the TiO₂NT, TiO₂NT + PEM, and TiO₂NT + PEM + NO surfaces were identical since they were all fabricated from Ti surfaces. Anodized titanium nanotubular surfaces are known for their bioactivity such as the promotion of cellular responses, cell

Fig. 1 Representative SEM images of Ti, TiO₂NT, TiO₂NT + PEM, and TiO₂NT + PEM + NO surfaces. Scale bars in the upper row of images are all 10 μ m. Scale bars in the lower row of images are all 1 μ m

adhesion, cell differentiation, and other properties in various implant applications. [36, 37] In addition, anodized titanium nanotubes have been shown to significantly decrease platelet adhesion and bacterial adhesion independently or in combination with other substances. [10, 18, 38] The performance and function of anodized titanium greatly depend on the diameter and size of the nanotube structure [39, 40]. In this study, the size and diameter of the nanotube array were selected based on a published literature which showed promising results in the inhibition of platelet activity. [18] The PEM coating on both TiO₂NT + PEM and TiO₂NT + PEM + NO surfaces covered individual nanotubes and blocked the pores; however, the coating did not change the nanotube structure. There was no distinct difference between TiO₂NT + PEM and TiO₂NT + PEM + NO surfaces, which indicated that the nitrosation process did not visually affect the PEM coating.

The representative real-time and cumulative NO release from TiO₂NT + PEM + NO surfaces is shown in Supporting Information Figure S2. Both real-time and cumulative NO release showed that the majority of NO was released from the surface during the first 25 min and nearly no NO was released after 40 min. Nitric oxide release from healthy blood vessel endothelial cells exhibits $0.5\text{--}4 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ or $1.25\text{--}10 \text{ nmol cm}^{-2}$ if released for 25 min [41]. In this study, TiO₂NT + PEM + NO surfaces were able to release approximately 4 nmol cm^{-2} of NO during the first 25 min, which is within the range of NO release from endothelial cells. The amount of NO delivery from this study has been increased tenfold compared to our previously published work [18]. A previous study has showed that a $13.7 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ of NO release or $42.5 \text{ nmol cm}^{-2}$ if released for 25 min is expected to actively inhibit the coagulation cascade in extracorporeal life support in a rabbit model [42]. The amount of optimal NO release from different NO-donor platforms for human in vivo applications is still unknown. Changes on PEM coating thickness, NO-donor selection, and nitrosation process for optimal NO release require further investigation.

Detection of released polysaccharides in soaking solution via refractive index (RI) method

The suitability of the RI detection method was calibrated using serial dilutions of a heparin stock solution. The detector signal was linear between 3000 and 120,000 nRI*min ($R^2 = 0.9998$), corresponding to a heparin concentration of 15 µg/mL–10 mg/mL (Supporting Information Figure S3). The limit of detection (LOD) was defined as three times the standard deviation of the blank and the limit of quantitation was defined as ten times the standard deviation of the blank. The method LOD and LOQ were 900 nRI*min and 3000 nRI*min, respectively.

The detection method was not specific to heparin when the RI signals from 200 µg/mL solutions of heparin, nitrosated heparin, chitosan-TGA, and nitrosated chitosan-TGA were compared. The RI signals detected from the 200 µg/mL nitrosated heparin, chitosan-TGA, and nitrosated chitosan-TGA solutions were 25%, 46%, and 105% higher, respectively, than that from the heparin solution of the same concentration. For this reason, RI data of leached polysaccharides in the soaking solutions is reported as detector signal (nRI*min) rather than concentration (µg/mL).

Blood coagulation may be affected by direct contact of polysaccharides that are released into the soaking solution, bound onto surfaces, or both. Polysaccharides released from the modified surfaces, which were present in the soaking solution, were detected by the RI method. Ti and TiO₂NT surfaces were included as negative controls, in addition to the heparin-containing surfaces. As shown in Table 1, signals from TiO₂NT + PEM soaking samples, as well as both Ti and TiO₂NT controls, were below the method limit of detection. The TiO₂NT + PEM + NO incubation samples produced signals of $18,000 \pm 2000 \text{ nRI*min}$ and $17,000 \pm 2000 \text{ nRI*min}$, after nitrosation and after all NO was released, respectively.

To determine whether the nitrosation process or nitrogen species in the solution after all NO was released affects the release of polysaccharides, two sets of TiO₂NT + PEM + NO surfaces were evaluated: TiO₂NT + PEM + NO surfaces immediately after nitrosation and TiO₂NT + PEM + NO surfaces after all NO was released. The signals of both sets of TiO₂NT + PEM + NO soaking solution were observed, which indicated the presence of polysaccharides (Table 1). Since signal height of both TiO₂NT + PEM + NO soaking solutions was similar, it can be inferred that nitrogen species in the solution after all NO was released does not influence polysaccharide release. There was an increasing signal from the soaking solution of nitrosated TiO₂NT + PEM + NO surfaces compared to the soaking solution of TiO₂NT + PEM surfaces, establishing that the nitrosation process is altering the degree of polysaccharide release. However, as shown in Table 2, the RI method was sensitive to all polysaccharides

Table 1 Refractive index signal (nRI*min) of modified surface soaking samples and controls

Soaking samples	Refractive index signal (nRI*min)
Ti (control)	<LOD
TiO ₂ NT (control)	<LOD
TiO ₂ NT + PEM	<LOD
TiO ₂ NT + PEM + NO (after nitrosation)	$18,000 \pm 2000$
TiO ₂ NT + PEM + NO (after all NO was released)	$17,000 \pm 2000$

Table 2 Refractive index signal (nRI*min) corresponding to 200 µg/mL solutions of heparin, nitrosated heparin, chitosan-TGA, and nitrosated chitosan-TGA

Sample (200 µg/mL solution)	Refractive index signal (nRI*min)	% Relative heparin signal
Heparin	49,900 ± 300	-
Nitrosated heparin	62,100 ± 200	125%
Chitosan-TGA	72,900 ± 300	146%
Nitrosated chitosan-TGA	102,200 ± 100	205%

potentially present in the soaking solution such as heparin, chitosan, nitrosated heparin, and nitrosated chitosan. Further experimentation is required to quantify individual polysaccharides present in the soaking solution such as anion-exchange chromatography or size-exclusion chromatography, as well as to identify the exact mechanism that is causing the increased release of polysaccharides after nitrosation.

Detection of chemical modification by the nitrosation process via attenuated total reflectance-Fourier transform infrared (ATR-FTIR)

The effects of *tert*-butyl nitrite on chitosan-TGA and heparin structures were independently evaluated by ATR-FTIR. As shown in Fig. 2, both the structures of chitosan-TGA and heparin were altered by *tert*-butyl nitrite. Chitosan-TGA has been developed and used as an NO-releasing agent [18, 43]. Nitrous acid has been shown to modify the structures of heparin and chitosan at low pH [44, 45].

The hydrolysis of *tert*-butyl nitrite may generate nitrous acid which can impact the structure of heparin. However, structural modification of heparin is only observed when the pH is lower than 2.5 [44, 46]. The disappearance of the vibrational mode at 1610 cm⁻¹ in the heparin spectrum and the appearance of a weak vibrational mode at 1734 cm⁻¹ in the nitrosated heparin spectrum indicate a structural change caused by the *tert*-butyl nitrite. Similarly, the appearance of the vibrational mode at 1311 cm⁻¹ in the nitrosated chitosan-TGA spectrum indicates a structural change after exposure

Fig. 2 ATR-FTIR spectra of heparin (black), nitrosated heparin (purple), chitosan-TGA (green), and nitrosated chitosan-TGA (yellow). **A** Full spectra of heparin and nitrosated heparin with fingerprint region.

B Full spectra of chitosan-TGA and nitrosated chitosan-TGA with fingerprint region

to *tert*-butyl nitrite. The sharp spike occurring at 650 cm^{-1} is an instrument artifact. It is possible that the structural change observed in heparin will impede its ability to activate antithrombin and further inhibit the coagulation cascade. Additionally, the nitrosation process may disturb the surface layer of heparin and cause polysaccharides to disperse into solution. This physically changes the surface roughness and might further affect the coagulation cascade [47].

Evaluation of real-time blood clot formation on modified surfaces by thromboelastography

In the thromboelastogram (TEG), the reaction time (R) is the time needed for initial fibrin formation by activating the intrinsic pathway and is dependent on enzymatic factor activity. In healthy blood that has not been exposed to anticoagulants, reaction times can range between 5 and 10 min in non-activated TEG. A 2-mm amplitude of blood clot formation was reached on each surface type after 15- and 60-min incubation in human whole blood, except for the $\text{TiO}_2\text{NT} + \text{PEM}$ surfaces (Fig. 3). The time until clot formation was significantly prolonged on $\text{TiO}_2\text{NT} + \text{PEM} + \text{NO}$ surfaces after 15-min incubation (13.1 ± 2.8 min) compared to Ti (7.7 ± 1.1 min) and TiO_2NT (6.8 ± 1.8 min) surfaces (Fig. 3A). For the 60-min incubation samples, the time to initial clot formation (R) was significantly prolonged

on $\text{TiO}_2\text{NT} + \text{PEM} + \text{NO}$ surfaces after 60-min incubation (17.5 ± 8.6 min) compared to Ti (5.6 ± 1.0 min) and TiO_2NT (3.8 ± 0.0 min) surfaces (Fig. 3B). From the reaction time values, nanotexturing of the surfaces does not seem to influence initial clot formation. It is unclear why the $\text{TiO}_2\text{NT} + \text{PEM}$ surfaces inhibit coagulation while the $\text{TiO}_2\text{NT} + \text{PEM} + \text{NO}$ surfaces allow for coagulation. The addition of the NO should further inhibit clot formation, but this was not observed in this study. Several factors may contribute to this observation, such as inactivated heparin after nitrosation, resumption of platelet activities after all NO was released, NO alteration of fibrinogen or thrombin changing their susceptibility to heparin, or other effects.

Heparin has been widely used clinically as an anticoagulant. [48–50] It exhibits anticoagulant activities, either bound on top of the coating or released from the coating. In addition to the heparin enriched at the surface, heparin was possibly released from the PEMs and directly interacted with the blood in the solution. While both $\text{TiO}_2\text{NT} + \text{PEM}$ and $\text{TiO}_2\text{NT} + \text{PEM} + \text{NO}$ surfaces leached polysaccharides, the concentration of polysaccharides released into the $\text{TiO}_2\text{NT} + \text{PEM} + \text{NO}$ surface soaking solution was approximately 200-fold more than that released into the $\text{TiO}_2\text{NT} + \text{PEM}$ surface soaking solution. Therefore, the $\text{TiO}_2\text{NT} + \text{PEM} + \text{NO}$ surface soaking solution should have a higher heparin concentration than the $\text{TiO}_2\text{NT} + \text{PEM}$

Fig. 3 Reaction time (R) of modified surfaces after 15-min and 60-min incubation in human whole blood ($n \geq 5$ batches of surfaces, * indicates $p < 0.05$, > 60 min indicates no endpoint value was provided by TEG during 60-min test). **A** Reaction time (R): 15 min incubation. **B** Reaction time (R): 60 min incubation

Fig. 4 Initial clot formation time (*K*) of modified surfaces after 15-min and 60-min incubation in human whole blood ($n \geq 5$ batches of surfaces, * indicates $p < 0.05$, N/A indicates no endpoint value was provided by TEG during 60-min test). **A** Initial clot formation time (*K*): 15 min incubation. **B** Initial clot formation time (*K*): 60 min incubation

surface soaking solution. The additional heparin in solution should have further inhibited coagulation. On the contrary, the TiO₂NT + PEM surfaces that released less heparin were found to inhibit coagulation. However, from the ATR-FTIR data shown above, the nitrosation process itself structurally modified heparin on the TiO₂NT + PEM + NO surfaces and/or in the incubation solution, which could inactivate heparin and further inhibit its anticoagulation activity on blood.

Chitosan and its derivatives are effective hemostatic agents that enhance the blood coagulation cascade to accelerate the wound healing process [51–57]. At low pH, the positively charged ammonium groups on chitosan and its derivatives interact with negatively charged proteins and glycolipids in the red blood cell membrane, inducing the aggregation of red blood cells [52, 58]. Previous studies also show that chitosan and its derivatives are able to trigger platelet adhesion and aggregation, through the interaction between positively charged chitosan and negatively charged phosphatidyl serine in the platelet membrane [59–61]. The complete hemostasis mechanism of chitosan and its derivatives is still unclear [62]. In this study, both TiO₂NT + PEM and TiO₂NT + PEM + NO surfaces have chitosan layers, but TiO₂NT + PEM did not induce coagulation. For both surface groups, heparin is the top coating layer for the 10-bilayer PEMs used here, so heparin is enriched at the surface and can more readily interact with blood components

compared to chitosan. However, from the RI study, significantly more polysaccharides were released from the NO-releasing surfaces. This may have caused more chitosan in the lower layers of the PEMs to be exposed, resulting in coagulation in the NO-releasing surfaces.

NO is an inhibitor of platelet activation, which is a key step in the coagulation cascade. [63–65] The antiplatelet effects of NO are reversible, and since NO has a short half-life, these effects can be surpassed by pro-coagulation signals quickly. [66, 67] Even though NO can act to inhibit coagulation, it is not surprising that these surfaces did eventually coagulate after all NO was released. In addition, NO has been shown to mediate fibrinogen deposition which may further affect the formation of thrombin and change their susceptibility to heparin [25, 30]. More studies are required to fully understand the mechanisms of why TiO₂NT + PEM surfaces completely inhibited coagulation while TiO₂NT + PEM + NO surfaces did not.

Evaluation of clot development and strengthening

The initial clot formation time (*K*) reflects the time it takes for the clot to develop and strengthen, which is dependent on the factors such as thrombin and platelets. This typically occurs in 1–3 min [68]. TiO₂NT + PEM + NO

Fig. 5 α -angle of modified surfaces after 15-min and 60-min incubation in human whole blood ($n \geq 5$ batches of surfaces, * indicates $p < 0.05$, N/A indicates no endpoint value was provided by TEG during 60-min test). **A** α -angle: 15 min incubation. **B** α -angle: 60 min incubation

surface (3.9 ± 0.8 min) showed an increase in initial clot formation time (K) compared to Ti (2.5 ± 0.6 min) and TiO₂NT surfaces (2.6 ± 0.9 min) after blood was exposed to the surfaces for 15 min (Fig. 4A). At 60 min, TiO₂NT + PEM + NO surfaces (5.1 ± 3.2 min) showed an increase in initial clot formation time (K) compared to TiO₂NT surfaces (1.4 ± 0.2 min), but not Ti surfaces (1.9 ± 0.4 min) (Fig. 4B).

The α -angle also helps characterize the propagation and strengthening of the clot. The α -angle is dependent on fibrin deposition and cross-linking [69]. In healthy blood, α -angle ranges from 47 to 78° [68]. The lower α -angle indicates reduced clot formation. The measured α -angle of TiO₂NT + PEM + NO surfaces ($40.9 \pm 7.0^\circ$) was lower compared to Ti surfaces ($54.3 \pm 7.5^\circ$), but not significantly different from TiO₂NT surfaces ($51.4 \pm 10.6^\circ$) (Fig. 5A); the α -angle of TiO₂NT + PEM + NO surface ($40.4 \pm 12.8^\circ$) was lower compared to Ti surfaces ($63.3 \pm 5.5^\circ$) and TiO₂NT surfaces ($69.4 \pm 2.5^\circ$) after 60-min incubation (Fig. 5B).

Reduced values of K and the α -angle were observed for TiO₂NT + PEM + NO surfaces compared to Ti and TiO₂NT surfaces. For these values at both 15 min and 60 min, TiO₂NT + PEM + NO surfaces also were just outside the normal range for healthy, untreated blood. Ti and TiO₂NT surfaces were not significantly different from one another

and were within the healthy range for all values of K and α -angle. Thus, nanotexturing alone was not enough to slow the rate of clot development.

Clot development and strengthening are dependent on the enzymatic factors that produce thrombin which leads to the conversion of fibrinogen to fibrin clot, and the deposition/cross-linking of fibrin on the surface of the growing clot [70–73]. NO is well-known for antiplatelet properties, which could help explain the reduced values of K and α -angle [63–65]. However, since NO has a short half-life (millisecond range) [66, 67], blood will not contain NO after the transfer of human whole blood from microcentrifuge tubes containing incubated surfaces into TEG cups. Instead, the blood will contain NO oxidation products and probably methemoglobin within red blood cells. Blood incubated with TiO₂NT + PEM + NO surfaces for 15 min should be exposed to less NO than blood incubated for 60 min, containing less NO oxidation products and methemoglobin, which probably leads to the different results between these two groups.

The heparin-enriched surface and the polysaccharides in the incubation solution would also influence clot development. Heparin works to inhibit coagulation by reversibly binding to antithrombin, thus inhibiting thrombin activity [74]. Heparin acts as a classically recycling catalyst. This

Fig. 6 MA of modified surfaces after 15-min and 60-min incubation in human whole blood ($n \geq 5$ batches of surfaces, * indicates $p < 0.05$, N/A indicates no endpoint value was provided by TEG during 60-min test). **A** Maximal amplitude (MA): 15 min incubation. **B** Maximal amplitude (MA): 60 min incubation

makes it an efficient anticoagulant because a single heparin molecule can continually bind to new antithrombin, thus activating it more. Total inhibition of clot propagation is not observed on these surfaces. This may be a result of several factors as discussed with RI and ATR-FTIR results.

Evaluation of maximal amplitude

The MA refers to the maximum clot strength and is proportional to fibrinogen concentration [69]. Clot strength values typically range from approximately 49 to 73 mm [68]. After 15-min incubation in human whole blood, there was no significant difference in the clot strength between Ti (57.4 ± 1.5 mm), TiO₂NT (55.4 ± 7.5 mm), and TiO₂NT + PEM + NO (54.1 ± 1.9 mm) sample conditions (Fig. 6A). The TiO₂NT + PEM + NO surfaces (53.1 ± 3.3 mm) had a significantly lower clot strength (MA) compared to Ti surfaces (57.8 ± 2.1 mm) and TiO₂NT (59.2 ± 1.0 mm) after 60-min incubation in human whole blood (Fig. 6B).

At this stage, the clot is already developed and the overall clot strength relies on the number of platelets in the clot that are interacting with the fibrin clot deposited on the surfaces. As stated previously, NO is a negative regulator

of platelet activation. This may slow down clot development. Once the platelets are activated, the short NO anti-platelet signal from TiO₂NT + PEM + NO surfaces may not be enough to overpower the procoagulant signals during 15-min incubation period compared to 60-min incubation period. Thus, the short NO release does not then have an effect on the activities of the platelets once they become fully activated and reduce clot strength. Additionally, the heparin from the TiO₂NT + PEM + NO surfaces was not able to completely inhibit thrombin production. Once enough thrombin is produced, and fibrin is deposited on the clot, heparin would not have significant influence on clot strength in a short period of time, but would reduce the clot strength if the surfaces were incubated for 60 min.

Conclusions

In this work, we have shown the ex vivo real-time blood clotting thromboelastographic performance of modified surfaces using human whole blood. Heparin-containing surfaces effectively inhibited or slowed blood clot formation with and without nitrosation. In addition, RI and

ATR-FTIR results indicated that polysaccharides released into solution during the incubation period and nitrosation process modified both chitosan-TGA and heparin, which would be factors that affect coagulation on modified surface.

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Data availability Data available on request from the authors.

Declarations

Conflict of interest The authors declare no competing interests.

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