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Anti-thrombogenic properties of a nitric oxide-releasing dextran derivative: evaluation of platelet activation and whole blood clotting kinetics

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Abstract

Controlling platelet activation and clotting initiated by cardiovascular interventions remains a major challenge in clinical practice. In this work, the anti-thrombotic properties of a polysaccharide-based nitric oxide (NO)-releasing dextran derivative are presented. Total platelet adhesion, platelet morphology and whole blood clotting kinetics were used as indicators to evaluate the anti-clotting properties of this material. With a total NO delivery of 0.203 ± 0.003 μmol , the NO-releasing dextran derivative (Dex-SNO) mixed with blood plasma demonstrated a significantly lower amount of platelet adhesion and activation onto a surface and reduced whole blood clotting kinetics. Nearly 75% reduction in platelet adhesion and a significant retention of platelet morphology were observed with blood plasma treated with Dex-SNO, suggesting this to be a potential anti-platelet therapeutic agent for preventing thrombosis that does not have an adverse effect on circulating platelets.

Keywords

Nitric oxide; S-nitrosothiol; anti-thrombogenic; platelet adhesion; platelet activation; platelet morphology; whole blood clotting kinetics

1. Introduction

Platelet activation plays a key role in atherothrombotic processes, such as inflammation and fibroproliferation^{1,2} and is an underlying cause of many cardiovascular disorders. Furthermore, platelet activation on the surface of medical implants leads to device failure. Consequently, anti-platelet therapy is an important strategy for treating acute coronary diseases³⁻⁵ and preventing clot formation during post-percutaneous coronary interventions (PCIs)⁶⁻⁹. For instance, without appropriate anticoagulation regimens, late stent thrombosis can lead to serious complications with mortality rates as high as 20 – 45%⁹.

Current approaches to control platelet-initiated intravenous clotting include administering systemic anti-coagulants and chemically or physically modifying the biomaterial used to

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fabricate the device. Various anti-platelet agents routinely administered to control clotting include aspirin (thromboxane inhibitor), clopidogrel (adenosine diphosphate antagonists), abciximab (glycoprotein-IIb/IIIa antagonists), warfarin (vitamin K epoxide reductase inhibitor), and heparin (inhibitor of factor IIa). However, all of these treatments are systemic and irreversibly compromise platelet function. As a result, the management of clotting remains a challenge for clinical care personnel¹⁰.

Intravenous clot formation during PCIs can be reduced by a non-fouling surface coating or by suitable surface modification to inhibit platelet activation and the subsequent coagulation cascade. Non-fouling surfaces prepared with poly(ethylene glycol) (PEG) and zwitterionic polymeric materials have proved to be very effective in preventing blood coagulation around implants¹¹. Similarly, heparin-coated dialysis catheters and cardiopulmonary bypass (CPB) surfaces showed a greater reduction in thrombosis and infection, and superior cumulative survival compared to non-coated controls^{12, 13}. However, implant failures due to coating delamination have been observed in several clinical trials and the irreversible platelet activation events remain problematic¹⁴. Moreover, even though heparin is extensively used as an anti-coagulant, it has many disadvantages. For example, heparin's mode of action is based on its binding capability to anti-thrombin (AT)¹⁵. In addition to AT, heparin has the affinity for binding to various proteins such as histidine-rich glycoprotein, platelet factor 4 (PF4), vitronectin, fibronectin, von Willebrand factor (vWF), tissue plasminogen activator (tPA), and thrombospondin. This non-specific binding limits the availability of heparin for binding to AT, limiting its overall therapeutic effectiveness¹⁶. Furthermore, heparin inhibits fibrin-bound thrombin and Factor Xa in the prothrombinase complex¹⁷. Finally and most importantly, heparin increases the risk of hemorrhage, thrombocytopenia and osteoporosis, making the management of critically-ill patients more difficult¹⁸. Taken together, although used as a standard care, more innovative approaches and treatment strategies are needed to overcome the limitations of the current anti-thrombogenic treatments, such as heparin, that are well-documented in the literature.^{19, 20} In this manuscript, we demonstrate the use of a naturally occurring dextran derivative which can prevent platelet activation on surfaces, thus providing an alternative to heparin.

Others have conducted preliminary studies on the influence of NO on platelet adhesion and activation on material surfaces. As a natural regulator of platelet function, NO plays a significant role in controlling thrombotic responses without compromising platelet function^{21–25}. Specifically, Bohl and West²⁶ reported nearly 80% reduction in the adherent platelets on glass slides coated with NO-producing PEG hydrogels. Meyerhoff et al. showed that NO-releasing poly(vinyl chloride) (PVC) coatings resulted in ~20% reduction in platelet adhesion²⁷ and NO-releasing Elast-eon E2As polymers reduced thrombus formation by 33%²⁸. Similarly, NO-releasing catheters with surface-bound heparin were shown to inhibit Factor Xa and thrombin (Factor IIa)²⁹. Comparable improvements in the hemocompatibility of NO releasing materials have been reported by others^{30–35}. Furthermore, a number of small molecule NO-donors, such as organic nitrites, have been evaluated as anti-platelet agents various *in vitro* studies^{36, 37}. However, nitrite tolerance, short half-lives, and lack of therapeutically relevant and controlled release kinetics are some of the drawbacks associated with these small molecule NO-donors.

Despite the prevalence of anti-platelet studies resulting from surface released NO, little has been done to explore the anti-platelet effects of water soluble NO donors on surface clotting. Given NO's well-established reversibility on platelet function, finite loading NO donor capabilities within polymers, and the limited diffusion kinetics of surface released NO, such studies examining the anti-coagulation effects of NO donor are needed. Herein we report for the first time, a systematic and detailed evaluation of a naturally occurring polysaccharide based NO-releasing dextran derivative as a potential anti-thrombogenic agent (Fig. 1). Due

to excellent water solubility, known bio-distribution, and enzymatic degradability³⁸, this material provides an exceptional strategy for designing anti-thrombogenic dextran derivatives suitable for intravenous administration that can prevent platelet activation on surfaces. More importantly, NO-bioavailability from this material can be conveniently modulated by adjusting the therapeutic dosage or frequencies. In this study, the responses of platelets and red blood cells to the dextran-derivative were evaluated using multiple analytical techniques to comprehensively understand the influence of the material on various aspects of clot formation.

2. Materials and Methods

2.1 Materials

Dextran (from *Leuconostoc* spp., $M_r \sim 40,000$) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Spectra/Por[®] dialysis membrane (molecular weight cut off (MWCO) 8000) was from Spectrum labs (Rancho Domingues, CA, USA). *N*-Hydroxysuccinimide (NHS) and *t*-butyl nitrite were purchased from Acros Organics (Morris Plains, NJ, USA), disodium ethylenediamine tetraacetate dihydrate (Na-EDTA), phosphate buffered saline (PBS) tablets were from EMD Chemicals (Gibbstown, NJ, USA), and calcein-AM was from Invitrogen (Eugene, OR, USA). All other chemicals were procured from Sigma-Aldrich and used as received.

2.2 Synthesis and characterization of S-nitrosated dextran-cysteamine

S-nitrosated dextran-cysteamine (Dex-SNO) was prepared following our early reported method^{38, 39}. In brief, dextran was carboxymethylated using monochloroacetic acid followed by conjugation with the amine group of cysteamine using carbodiimide chemistry. The thiolated dextran derivative (Dex-SH) was *S*-nitrosated using *t*-butyl nitrite in anhydrous methanol protected from direct exposure to light. All intermediates and the final products were extensively characterized using ¹H NMR (using a Varian Inova 400 MHz FT-NMR, Varian NMR Systems, Palo Alto, CA, USA), FTIR- ATR (using a Nicolet 6700 spectrometer, Thermo Electron Corporation, Madison, WI, USA), Ellman's assay (for thiol quantification, using a BioTek Synergy 2 plate reader (Biotek, Winooski, VT, USA), UV (for *S*-nitrosation reaction monitoring and quantification, using a Nicolet Evolution 300 spectrophotometer, Thermo Electron Corporation), and GPC (for molecular weight profile and monitoring degradation kinetics, using a Viscotek GPC, Houston, TX).

2.3 Platelet adhesion and activation

Platelet adhesion and activation studies were performed by following a modified procedure reported previously^{40–42}. In brief, whole blood from the healthy donor was drawn into standard 6 mL vacuum tubes coated with the anti-coagulant, EDTA. Initial drawn blood (about 6 mL) was discarded to avoid any skin plug and to eliminate the locally activated platelets caused by the needle insertion. The subsequent blood vials were centrifuged at 150 g for 15 min to separate the plasma from the red blood cells. The plasma was then pooled together into fresh tubes and used immediately for adhesion and activation studies. Four sets of experiments were performed: (i). plasma mixed with Dex-SNO (active), (ii) and plasma mixed with Dex-SH (reagent control), (iii) unmodified dextran (reagent control), and (iv) plasma without any additives (experimental control). Additives were added at a concentration of 1.15 mg/mL. All data collection was done in multiples with and $n(\text{min}) = 5$ and each experiment was repeated at least with 3 different platelet population. For SEM, $n(\text{min}) = 24$, at least 3 images per sample and minimum 8 samples were analyzed.

Platelet adhesion and activation experiments were performed using tissue culture polystyrene (PS) discs (Greiner Bio-One GmbH, Frickenhausen, Germany). All PS discs

were sterilized prior to experiment by sonicating in 70% ethanol for 30 min (two times), washed two times with PBS buffer, air dried and finally exposed to UV for 30 min. Each sterilized tissue culture disc was incubated with 1 mL freshly pooled plasma in a 24-well plate at 37 °C and 5% CO₂ on a horizontal shaker plate (100 rpm) for 2 h. After 2 h, un-adhered cells were removed by carefully aspirating the supernatant plasma from the well plate followed by rinsing the discs gently with PBS buffer (2x). The discs were transferred to a new 24-well plate and incubated with 1 mL of 5 µM calcein-AM solution in PBS buffer for 20 min at room temperature. The discs were then rinsed with PBS and immediately imaged using a fluorescence microscope with high efficiency filter set 62 HE BP 474/28 (green) (Zeiss, Jena, Germany). Cell adhesion was determined using the fluorescence microscope images and quantified using ImageJ software (NIH, Bethesda, MD, USA).

The platelet-leukocyte morphology was investigated using scanning electron microscopy (SEM) (JEOL JSM-6500F, JEOL USA, Peabody, MA, USA) to visualize the platelet-leukocyte interaction with the tissue culture disc surfaces in the presence and absence of NO-releasing Dex-SNO. The un-adhered cells were removed carefully by aspirating the plasma from the well plate followed by gently rinsing the discs with PBS buffer (2x). The discs were then transferred to a petri-dish and the adhered cells were fixed by incubation in primary fixative solution made from 6% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1 M sucrose at room temperature for 45 min followed by incubating in a secondary fixative solution (primary fixative without glutaraldehyde) in a separate petri-dish for 10 min. The discs were then dehydrated by incubating in consecutive solutions of ethanol (35, 50, 70, and 100%) for 10 min each and finally in hexamethyldisilazane (HMDS) for another 10 min. The discs were then air dried overnight and stored in a desiccator until further imaging by SEM at 15 kV after coating with 10 nm layer of gold. The SEM images were then used to determine the percentage of the adhered platelets that were unactivated, or had a short-dendritic or a long-dendritic morphology. The following scheme was used to determine the morphology of the adhered platelets: (i) unactivated: Platelets that are normal and with compact central body, (ii) short-dendritic: Platelets with smaller dendrites and partially activated and (iii) long-dendritic: Platelets with many long dendrites and completely activated. Platelets with a round, compact body were classified as non-activated, those with smaller dendrites and partially activated were classified as short dendritic, and those with long dendrites exhibiting complete activation were classified as long dendritic.

2.4 Whole blood clotting kinetics

Influence of the anti-thrombogenic properties of the Dex-SNO on the whole blood clotting kinetics was investigated using whole human blood obtained from the same non-smoking healthy volunteer acquired through venepuncture. Initial drawn blood (about 6 mL) was discarded and subsequently drawn blood was collected in 10 mL sterile centrifuge tubes without any anti-coagulants. 2 mL of this blood was mixed with a freshly prepared solution of Dex-SNO in deoxygenated DI water (net concentration of 1.15 mg/mL blood) and 5 µL of this blood was immediately dropped onto the surface of sterilized tissue culture discs placed in a 24-well plate. Similarly blood mixed with the same concentration of non-nitrosated dextran-cysteamine (Dex-SH) and unmodified dextran were used as reagent controls and blood without any of these additives used as the experimental control.

The blood was allowed to clot on sterilized tissue culture discs for up to 2 h and the free haemoglobin concentration, as a measure of the extent of clotting, was measured at regular intervals. After the specific clotting periods, the discs were transferred into a different 24-well plate and immersed in 1 mL DI water. The discs were gently agitated for 30 s and left in the DI water for another 5 min to release the free haemoglobin from red blood cells. The free haemoglobin content was measured at a wavelength of 540 nm using a plate reader. The value of absorbance is directly proportional to the concentration of free haemoglobin in DI

water and provides a direct correlation to the extent of blood clotting on the surface. Experiments were repeated on multiple days using the blood drawn from the same donor to check the reproducibility and reliability of the method and results.

2.5 Real-time NO release kinetics using blood plasma

Real-time NO release from the blood plasma mixed with Dex-SNO was evaluated using Sievers chemiluminescence NO analyzers[®] (NOA 280i, GE Analytical, Boulder, CO, USA) following a modified procedure reported previously⁴³. Experiments were performed using ultra high purity N₂ gas containing 5% CO₂ as the carrier gas. The instrument was calibrated before each experiment using this carrier gas as the zero gas and a 45 ppm NO gas. 2 mL freshly pooled blood plasma sample mixed with Dex-SNO (concentration 1.15 mg/mL) was introduced into a 4 mL NOA measuring cell and the NO release was measured at 37 °C while shielding the sample from direct exposure to light. Experiments were repeated in triplicate and measurements were recorded at a data interval of 5 s at a flow rate of 200 mL/min with a cell pressure of 9.7 Torr and an oxygen pressure of 6 psig. Real-time NO release measurements using blood plasma were recorded for the initial 1 h and the results were extrapolated to 2 h using the standard curve fitting trend-line equation.

3. Results and Discussion

3.1 S-nitrosated dextran-cysteamine synthesis and real-time NO release under blood plasma conditions

NO-releasing S-nitrosated dextran-cysteamine derivative was synthesized and characterized using a method previously reported in our lab. Because of the characteristic stabilization of the S-nitrosothiol moiety, as a function of the conjugation chemistry we used, the resultant NO-donor presented controlled and specific NO-release capabilities under arterial blood conditions³⁸ making it suitable for dextran derivative applications. In a previous study, the material demonstrated its capability to undergo enzymatic degradation by dextranase (from *Penicillium* sp.) and also exhibited a significant inhibition of the growth of both human breast (MCF-7) and canine mammary carcinoma (CMT-12) cells *in vitro*⁴⁴. In this study we present a detailed evaluation of the anti-thrombogenic capabilities of this water soluble NO-releasing dextran derivative, targeting as a suitable dextran derivative for delaying thrombosis. Several complementary techniques were employed to elucidate the impact of the dextran derivative on clotting over a two hour period. The two hour time point was chosen for optimal selectin expression, platelet aggregation, and leukocyte recruitment as identified in other studies⁴⁵.

Real-time NO release kinetics from the Dex-SNO mixed with freshly separated blood plasma was evaluated using NO analyzers[®] at 37°C and protected from direct exposure to light. Experiments were performed with a Dex-SNO concentration of 1.15 mg/mL, which has a total storage capacity of 0.236±0.003 µmol NO. This NO dosage was previously reported to induce various proliferative and protective effects, including vasodilation, angiogenesis, and cancer treatments^{44, 46}. In plasma, the cumulative NO-release profile of Dex-SNO in blood plasma exhibits a steady rate of NO release over 2 h (Fig. 2) with a total NO delivery of 0.203±0.003 µmol.

3.2 Platelet adhesion and activation

Platelet adhesion initiates blood coagulation, thus the level of platelet adhesion onto a surface can be used as an indicator of thrombogenicity. In this study, freshly pooled plasma, isolated from whole human blood, was treated with same concentrations of NO-releasing Dex-SNO, parent non-nitrosated Dex-SH, and unmodified dextran. The adhesion of platelets within the treated or untreated plasma onto polystyrene surfaces was investigated after 2 h of

contact time. Platelets were stained with calcein-AM and platelet adhesion was evaluated by examining surfaces under a fluorescence microscope. High magnification (50x) fluorescence microscopy images were analysed to determine the quantity of adhered platelets on surfaces using ImageJ software.

The results indicated a significant reduction in platelet adhesion on surfaces with dextran treated plasma samples compared to untreated sample (Fig. 3). The cell coverage analysed using the ImageJ software from fluorescence images (Fig. 4) showed nearly 45% decrease in cell coverage with plasma mixed with dextran and nearly 75% reduction with plasma mixed with Dex-SNO and Dex-SH. This decrease in cell coverage and platelet adhesion onto the tissue culture slides with all dextran mixed plasma samples can be mainly attributed to the capability of dextran in preventing platelet aggregation and adhesion.^{47–49} In these studies, the extent of platelet aggregation was increased on the dextran surface due to increased fibrin formation; however, the Dex-SNO material overall had less propensity to form clots. Consequently we explored additional methods to confirm the anti-thrombogenic capabilities of the NO-releasing dextran derivative.

Upon close comparison between the fluorescence microscopy images (Fig. 3), we can see that the platelets treated with unmodified dextran showed some swelling compared to untreated and other samples treated with modified dextran derivatives. This swelling phenomenon can be attributed to the absorption of unmodified dextran by the platelets⁵⁰, which interestingly is not observed with the chemically modified analogues under similar experimental conditions.

In order to evaluate the specific influence of the NO-releasing Dex-SNO on cellular functionality, morphological changes of the platelets were studied in detail using SEM imaging. Platelet activation triggers various blood coagulation factors resulting in a massive acceleration of thrombin generation⁵¹, which results in the formation and broadening of atherosclerotic plaques through the release of adhesive ligands such as P-selectin, and ultimately leads to arterial thrombosis and acute coronary syndromes^{2, 52}. Moreover, activated platelets are an important source of inflammatory mediators, which can induce inflammatory responses in the healthy endothelium and can alter the normal endothelial-cell functions^{53, 54}. Consequently dextran derivatives that can prevent this initial platelet activation will have potential therapeutic implications in preventing the subsequent thrombotic events.

Previous studies using various exogenous NO-donors^{55, 56} have shown that the intracellular generation of NO significantly inhibits platelet activation by both cGMP-dependent protein kinase (cGKs) and cAMP-dependent protein kinase (PKA) mechanisms^{57, 58}. However, these studies were performed by measuring groups of platelets using flow cytometry techniques. This technique is incapable of measuring cell morphologies. Therefore the methodology we reported herein provides a very convenient technique to evaluate the influence of the NO-releasing dextran derivative on platelet activation directly by evaluating individual platelet morphologies attached on to the surface of a tissue culture disc.

SEM images (Fig. 5) were taken to investigate platelet activation after 2 h of surface contact time. The lower magnification images (Fig. 5(A), (C), (E), and (G)) show the extent of platelet attachment to the surface. The platelets mixed with NO-releasing Dex-SNO exhibited lower amounts of platelet adhesion than both untreated platelets and platelets mixed with dextran or Dex-SH. The higher magnification images (Fig. 5(B), (D), (F), and (H)) more clearly show the morphology of the attached platelets. The control platelets, platelets with dextran, and platelets with Dex-SH exhibited high activation with long dendritic extensions adhered to the tissue culture disc surface. The platelets with NO-

releasing Dex-SNO, however, exhibited a more rounded morphology with only small dendritic extensions in some cases and fewer platelets in general. Further, the higher magnification SEM images were used to determine the percent of adhered platelets exhibiting one of the three morphologies (non-activated, short dendritic, or long dendritic). These results indicate that while all the samples exhibited some degree of platelet activation, the platelets in NO-releasing Dex-SNO remained significantly less activated, with fewer long dendritic and more short dendritic platelet morphologies (Fig. 6) than the control and other dextran polymers. This data clearly suggests that NO-released from the Dex-SNO prevented platelet activation and maintained the platelet morphology during the contact period.

In previous studies, a nearly 50% reduction in platelet adhesion has been reported using polymeric materials with a total loading capacity of 0.7 to 1.3 $\mu\text{mol NO/mg}$ ³⁵. In contrast, the use of the water soluble NO-releasing dextran derivative presented herein achieved ~75% reduction in platelet adhesion (Fig. 4) with a significantly smaller loading of NO ($0.236 \pm 0.003 \mu\text{mol NO}$). This data clearly demonstrates the significance of the increased bioavailability of NO from Dex-SNO for inhibiting platelet activation and adhesion on surfaces as a result of the favourable dextran derivative solubility and enhanced, but controlled NO-release kinetics. This extend of platelet control has not been observed before with systemic anti-platelet agents.

3.3 Whole Blood clotting kinetics

Immediately after platelet adhesion and activation, the blood coagulation cascade progresses into the development of a fibrin matrix. Whole blood clotting kinetics is vital in the success or rejection of a blood-dilating dextran derivative when evaluating it as a potential precursor for anti-thrombogenic therapies. Free haemoglobin concentration values were measured in terms of absorbance for control, dextran, Dex-SH and Dex-SNO treatments for 2 h. The results indicate that blood clotting was delayed significantly with the Dex-SNO treatment compared to control treatments (Fig. 7). With whole blood, a nearly 80% reduction in the free haemoglobin was observed within the initial 30 minutes indicating a significant amount of clotting within that period, which is in accordance with reported literature⁵⁹. However at the same period, only 10% reduction in the initial haemoglobin content was observed with the presence of NO-releasing Dex-SNO material. Even after a 2 h clotting period only 70% reduction in the free haemoglobin was observed, which was significantly higher than that observed with all experimental controls. Experiments were repeated on multiple days using blood from the same donor. Each day, the same trend was observed for each group of materials confirming the reproducibility and reliability of the results. These results also showed delayed clotting kinetics with both dextran and Dex-SH derivatives; however, as we can conclude from the Fig 7, the influence of NO-releasing material on blood clotting is very prominent.

4. Conclusions

In this study, we presented a detailed and systematic evaluation of a systemic anti-thrombogenic NO-releasing dextran derivative as a potential material suitable preventing thrombosis formation on surfaces during post-percutaneous coronary interventions. Contrary to the previously reported surface coated NO-releasing polymeric materials for improving thromboresitivity; we evaluated this water soluble NO-releasing Dex-SNO as a substitute for currently available systemic anti-coagulants. The anti-thrombogenic characteristics were evaluated *in vitro* by studying the extent of platelet activation and the whole blood clotting kinetics on sterilized tissue culture discs for up to 2 h in presence of various experimental and reagent controls. Our results suggest that with a delivery of $0.203 \pm 0.003 \mu\text{mol NO}$, Dex-SNO significantly decreased the amount of platelet adhesion and activation as well as

whole blood clotting kinetics. Moreover, in this study we evaluated detailed morphological changes of individual platelets using SEM, and found a significant reduction in platelet activation with Dex-SNO treated samples. Because platelet activation plays a key role in recurrent thrombotic events, capability of Dex-SNO to prevent these initial adverse bioresponses provides a very promising strategy for designing NO-releasing therapeutic agents with reversible platelet function, a challenge not yet achieved by other anti-coagulants.

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Fig. 1. Schematic illustration of the anti-thrombogenic properties of NO-releasing *S*-nitrosated dextran-cysteamine (Dex-SNO) in preventing platelet activation and adhesion.

Fig. 2. Representative initial real time (top) and cumulative (bottom) NO release profile from Dex-SNO mixed blood plasma (1.15 mg/mL) at 37 °C for 2 h (n = 3).

Fig. 3. Representative fluorescence microscopy images of adhered platelets stained with calcein-AM on polystyrene with Blank [(A) 10x, (B) 50x], Dex [(C) 10x, (D) 50x], Dex-SH [(E) 10x, (F) 50x] and Dex-SNO [(G) 10x, (H) 50x] treatments.

Fig. 4. Quantity of adhered platelets stained with calcein-AM on polystyrene with control, Dex, Dex-SH and Dex-SNO treatments. Quantification was performed using ImageJ software. Control treatments were significantly different than Dex, Dex-SH and Dex-SNO treatments. Dex treatments were significantly different than Dex-SH and Dex-SNO surfaces.

Fig. 5. Representative SEM images of adhered and activated platelets on polystyrene blank [(A), (B)], Dex [(C), (D)], Dex-SH [(E), (F)], and Dex-SNO [(G), (H)].

Fig. 6. Distribution of different shapes of adhered platelets after exposure to Dex-SNO and controls (measured from SEM).

Fig. 7.

Free haemoglobin concentration values were measured in terms of absorbance for control, Dex-SH and Dex-SNO treatments for 120min. At 15min there were significant differences between control and Dex-SH treatments and Dex-SH and Dex-SNO treatments. At 45min there were significant differences between control and Dex-SNO treatments and Dex-SH and Dex-SNO treatments. At 30min, 60min, 90min and 120min there were significant differences between all treatments ($p < 0.05$).