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Blood-material Interactions With Degradable Shape Memory Polymer Foams

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Abstract

Shape memory polymer (SMP) foams are a class of smart materials that show promise for managing uncontrolled hemorrhage on the battlefield. The current standard treatments of gauze and tourniquets are not able to effectively control hemorrhage in up to 80% of combat injuries. Most hemostatic devices also require removal within 12 hours of application. Patient transportation to a facility for removal may not be possible within this time frame and the removal process itself can cause further damage to the wound. As an alternative hemostatic device, polyurethane SMP foams have shown to be effective and biocompatible hemostats; however, removal still remains a concern. Degradable SMP foams have been developed through modification of a previously tested polyurethane SMP system to overcome this limitation. In this work, *in vitro* hemocompatibility and overall blood interactions with these degradable SMP foams is assessed. The primary outcome of these studies shows that the polyurethane SMP foams maintain hemocompatibility with incorporation of specific degradable components. The data collected here will aid in further development of this degradable SMP system as well assist future *in vitro* and *in vivo* studies leading into clinical trials of this SMP system for hemostatic applications.

Blood-Material Interactions with Degradable Shape Memory Polymer Foams

By

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1. Introduction

1.1 Clinical Need: Uncontrolled Hemorrhage

Every year, approximately 1.5 million lives are lost due to uncontrolled hemorrhage. Of the casualties that occur on the battlefield, hemorrhage from traumatic injury accounts for half of combat fatalities as well as 80% of combat deaths that are potentially survivable [1]. The majority of these deaths occur before military personnel can be moved to a medical facility for treatment. Injured personnel can bleed out over the course of hours, though most deaths occur in the first 5-10 minutes after injury [2], [3]. Being able to quickly control bleeding in the combat zone is a critical part of reducing the number of soldiers KIA (killed in action before reaching a medical facility).

As the body loses large quantities of blood, there is no longer adequate oxygen delivery to critical organs in the body and a person undergoes hemorrhagic shock. Damage to kidneys, liver, intestines are among the initial effects of severe blood loss. As hemorrhage continues and there is inadequate oxygen delivery to the brain and myocardium, brain damage and fatal arrhythmias follow [4]. Being able to slow and control this bleeding before a person reaches a medical care facility will not only improve patient survival but decrease lasting damage in survivors [1].

1.2 Current Solutions: Advantages and Disadvantages

While there are multiple hemostatic devices and dressings in clinical use, a significant need remains for an effective hemostat that is easy to apply, does not require time sensitive/complicated removal, and is biocompatible.

Tourniquets are among the current standards of care on the battlefield for controlling hemorrhage. Tourniquets are commonly used in combat as they are relatively quick and easy way to slow bleeding. However, use of a tourniquet for longer than 4 hours can cause permanent nerve and tissue damage [5]. Additionally, confusion among military personnel on safe practices with application, loosening, and removal of the tourniquet can lead to improper use and unnecessary nerve paralysis and limb ischemia [6]. Despite the prevalence of tourniquets as a standard of care on the battlefield, they are only effective in 7% of cases [5]. Use of gauze for packing a wound is another commonly used treatment for hemorrhage control. In general, packing a wound with gauze controls hemorrhage by creating pressure within the wound cavity that is sufficient to stop bleeding from vessels [7]. QuikClot (QCCG) is a promising gauze dressing in clinical use that employs the clay material kaolin to activate the coagulation cascade and speed up clotting time as compared to traditional gauze [8], [9]. Overall, gauze is relatively inexpensive and easy to apply, however periodic dressing changes are needed which can be time consuming and the removal process can cause re-bleeds and further damage to the wound [5].

XStat is a hemostat currently in use that has shown to be effective at quickly slowing bleeding with the advantage of easier application than gauze and tourniquet. XStat comes in the form of a syringe applicator with 92 small sponges that are injected into a wound cavity. Upon entering the wound these sponges expand to fill the space, absorbing blood and applying pressure to the wound walls. Though effective, the sponges have the disadvantage of increasing removal time 22-fold compared to

traditional gauze [10]. The need to remove XStat sponges within 4 hours of application introduces a further complication, as transportation to a medical facility where this can be performed may not be possible in this amount of time.

1.3 Shape Memory Polymer Foams

Shape memory polymers (SMPs) are 'smart' materials that can be reversibly deformed and fixed into a temporary secondary shape, and later recovered to the original primary shape in the presence of a specific stimulus [11]. Thermal SMPs in particular are fabricated to have a permanent or primary shape and can be heated above the material's transition temperature, deformed, and cooled to fix in the secondary shape for storage or transportation (illustrated in **Figure 1**). Upon application of a thermal stimulus that is above the SMP's transition temperature, the material will return to its primary fixed shape [12].

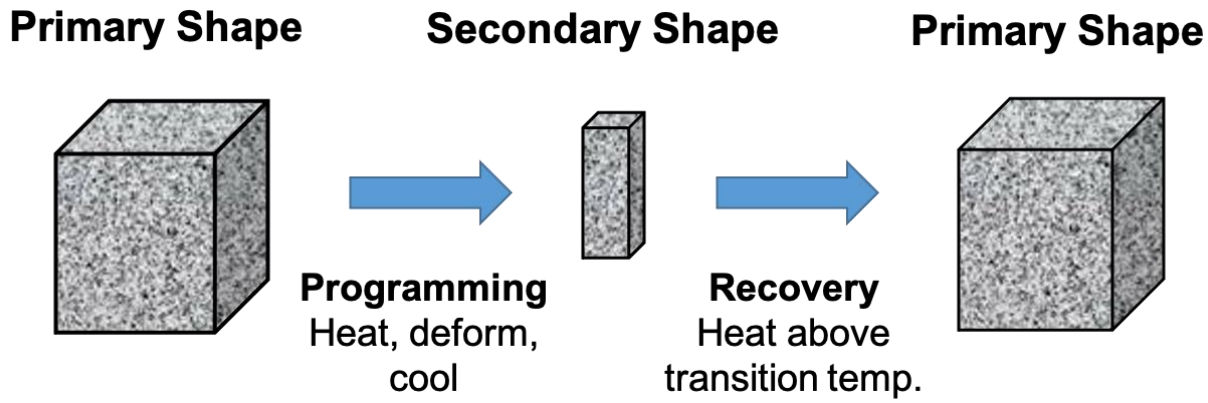

Figure 1. Illustration of SMP shape programming and recovery.

Our group has developed a temperature sensitive polyurethane SMP foam system for use as a hemostat. This SMP has a dry transition temperature above body

temperature (Tg > 40°C) and can remain in its secondary shape for storage and transportation in both very cold and hot environments. When wet, (e.g. after insertion into a bleeding wound) the transition temperature of this SMP is reduced to below body temperature (Tg < 37°C) due to disruption of hydrogen bonds and overall plasticization, and shape change occurs [13]. Polyurethane SMP foams to date have demonstrated great biocompatibility, rapid clotting ability due to high surface area and thrombogenic surface chemistry, high tunability of actuation temperature as well as unique shape retention properties [14]–[16].This SMP system shows promise for hemostatic applications, as it can be transported at a small volume, actuated upon exposure to body temperature blood in a bleed site, and expanded to promote hemostasis through application of pressure and thrombogenic properties.

One of the important features of this SMP system is the ability to tune the chemistry and adjust properties for specific applications. To address the limitations of hemostats currently in use, degradable linkages have been incorporated into the backbone of our polyurethane SMP system to create a degradable hemostatic device [17]. Specifically, 15% or 30% nitrilotriacetic acid-diethylene glycol (NTA-DEG), 30% triethylene glycol (TEG), or 30% diethylene glycol (DEG) monomers have been included into the control polyurethane foam composition of hexamethylene diisocyanate (HDI), triethanolamine (TEA), and N,N,N',N'-tetrakis(2-hydroxypropyl) ethylenediamine (HPED). A degradable hemostatic foam would not need to be removed and could be left in place during the healing process (**Figure 2**), decreasing the urgency for patient

transport to a medical facility and avoiding further damage and rebleeds due to wound dressing removal.

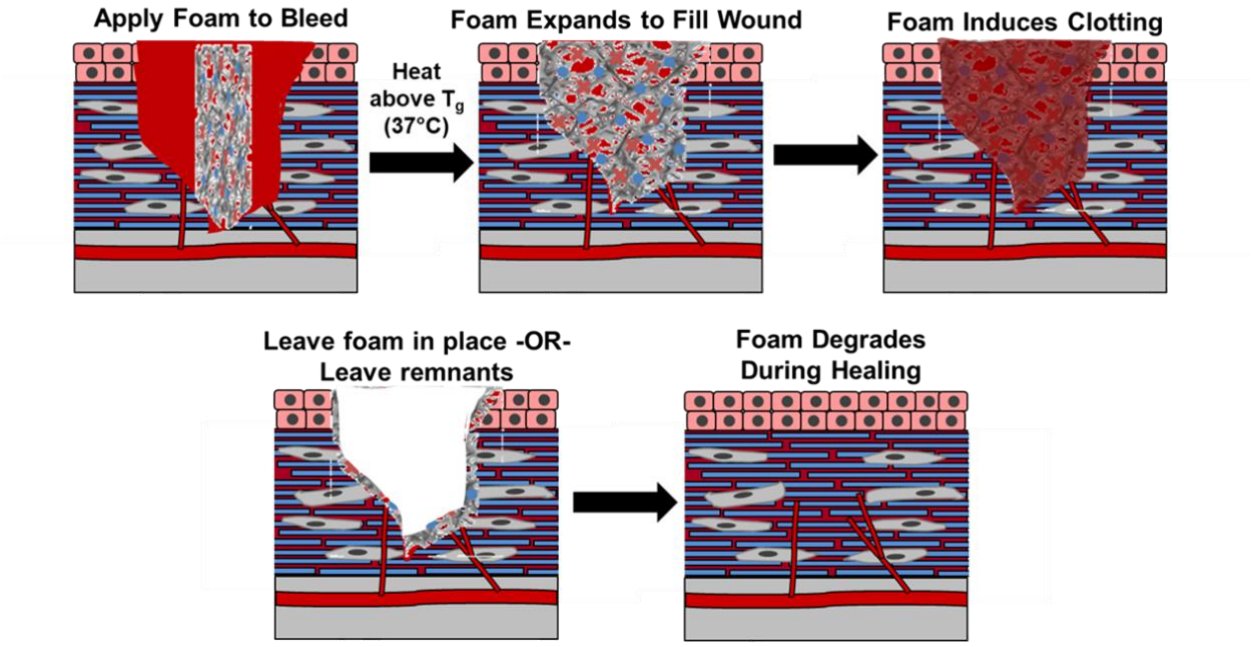

Figure 2. Expansion of degradable shape memory polymer in a bleed.

The original SMP foam formulation has shown consistently rapid blood clotting in pre-clinical models; however, blood interactions must be evaluated in new formulations to ensure that these desirable clotting properties are maintained.

1.4 Hemocompatibility

Contained in the blood are various components, including red blood cells (RBCs) that are responsible for oxygen transport (44%), plasma (55%), platelets that play a role in recognizing foreign substances and initiate clotting, and leukocytes responsible for immune response (~1%) [18]. Together these components play a crucial role in the

human body. As such, hemocompatibility is one of the most important criteria for a material that is to be used *in vivo*. International guidance has been developed for any blood-contacting medical devices, requiring hemocompatibility evaluation according to International Organization for Standardization (ISO) 10993-4, “Selection of Tests for Interactions with Blood” [19], [20].

While the specific tests required by ISO differ based on the application of the material or device, there is a list of main test categories recommended for evaluation, including: thrombosis, coagulation, platelet function, hematology, and immunology. Hemocompatibility is defined by the lack of adverse interactions or destruction of any blood components. For the application of our polyurethane SMP, we sought to evaluate our system’s ability to induce clotting by looking at the time for stable clot formation to occur as well as the recruitment and activation of platelets. This material differs from “traditional” hemocompatible materials as it is intended to function as a hemostat, and thrombogenic (clot inducing) properties are desirable. Further, the hematology of the material was assessed through hemolysis measurement.

1.4.1 Coagulation

Hemostasis following injury occurs by two main mechanisms in the body, platelet plug formation (primary hemostasis) and the coagulation cascade (secondary hemostasis). These two independent processes work together to result in a strong clot at the damaged region of the vessel that is impermeable to blood and as a result, stops bleeding [21]. This clot is primarily composed of activated platelets, fibrin, and red blood cells that are entrapped within the network at the site of vessel damage. The

coagulation cascade consists of two separate pathways that converge to ultimately lead to the formation of the insoluble protein fibrin that assembles into a highly branched network [22]. The intrinsic pathway of coagulation is initiated when the first factor in the pathway, Factor XII or serine protease, is exposed to endothelial collagen due to trauma within the vascular system. From here a cascade of activations of other factors leads to the conversion of prothrombin into thrombin, which then cleaves fibrinogen into the fibrin monomer that makes up the essential “mesh” network of a clot [23]. The extrinsic pathway, also called the tissue factor (TF) pathway, is initiated by external trauma to the vascular system that results in TF coming into contact with blood. A cascade similar to the intrinsic pathway results in the formation of thrombin that again serves to convert fibrinogen into fibrin. By having two different pathways of coagulation activation, the initiation, growth and maintenance of fibrin can be better controlled and regulated throughout the clot formation process [24].

1.4.2 Platelet Adhesion and Activation

Alternatively, circulating platelets play an important role in hemostasis by forming a plug through activation and adhesion to the site of injury. Most studies for hemocompatible materials focus on minimizing or avoiding platelet activation, though for the application of a hemostat, platelet activation and adhesion are desirable [25]. When a vessel is damaged and endothelial collagen is exposed, this collagen as well as von Willebrand factor (vWF) will bind the platelets to the site of damage. This binding process and the thrombin formed during the coagulation cascade lead to the activation of platelets, and the platelets in turn release their cellular components (granules).

Release of granule contents functions to further recruit, aggregate and adhere circulating platelets to the damaged vessel [26], [27].

1.4.3 Hemolysis

Hemolysis, defined as the rupture of RBCs and the release of their contents, is a crucial indicator of the fragility of the RBC membrane when in contact with a particular material. Hemolysis can occur due to mechanical forces or as a result of material mediated interactions, which is what we primarily focus on here. In general, hemolysis is undesirable as it can impact the body's ability to transport oxygen to tissues, and the presence of free hemoglobin can cause inflammation and vascular dysfunction [28].

Overview

In this study I look at the interactions of degradable shape memory polymer foams with porcine blood to provide a preliminary assessment of the hemocompatibility of this material. This data will aid in future *in vitro* and *in vivo* studies leading into clinical trials of this SMP system as a hemostatic device. The tests performed in this work are adapted from other studies in literature to assess coagulation, hemolysis, and platelet attachment and activation responses of our degradable SMP foams when in contact with blood.

2. Methods

2.1 Test Samples

Polyurethane foams were prepared in a 2-step reaction between isocyanates (NCO) and hydroxyls (OH) as previously described by Singhal et al [16]. Foam formulations included a control composed of hexamethylene diisocyanate (HDI), triethanolamine (TEA), and N,N,N',N'-tetrakis(2-hydroxypropyl) ethylenediamine (HPED). Biostable foams included 30% diethylene glycol (DEG) or 30% triethylene glycol (TEG) in place of TEA. Biodegradable foams included 15% or 30% nitrilotriacetic acid-DEG (NTA)-DEG. Foam compositions are shown in **Table 1**. QuikClot Combat Gauze (QCCG) and XStat were utilized as clinical controls.

Table 1. Foam formulations tested in blood interaction studies.

Sample	**HDI (mol% NCO)**	**TEA (mol% OH)**	**HPED (mol% OH)**	**DEG (mol% OH)**	**TEG (mol% OH)**	**NTA-DEG (mol% OH)**
Control	100%	30%	70%	--	--	--
30% DEG	100%	--	70%	30%	--	--
30% TEG	100%	--	70%	--	30%	--
15% NTA-DEG	100%	15%	70%	--	--	15%
30% NTA-DEG	100%	--	70%	--	--	30%

Foam density and pore size specific to each foam formulation (previously measured) were used to calculate the mass required so that all foam samples had equal surface

area in each study. The surface areas used to calculate sample size and the foam masses for each study are shown in **Table 2**.

Table 2. Surface areas and foam masses for each blood interaction study.

Study	**Surface Area (cm^2)**	**Control (mg)**	**30% DEG (mg)**	**30% TEG (mg)**	**15% NTA-DEG (mg)**	**30% NTA-DEG (mg)**
Coagulation Time	32.60	3.54	3.70	2.0	3.74	1.96
Hemolysis	143.8	78.0	81.4	44.1	82.6	43.1
LDH Assay	34.81	18.9	19.72	10.68	20.0	10.43

2.2 Blood Absorption

For initial measurement of blood interactions with samples, blood absorption was analyzed after incubation of SMPs and clinical controls (gauze and XStat®) in blood. Porcine blood (Lampire Biological Laboratories, Pipersville, PA, USA) anticoagulated with Na-Citrate upon collection was stored at 4°C in a refrigerator for up to 3 weeks from the bleed date until ready to be used. Approval for the use of porcine blood was obtained from the Syracuse University Biosafety Committee prior to experimentation. Dry samples (n=3, ~50 mg) were weighed and incubated in 2 mL of porcine blood at 37°C. Two sets of absorption measurements were collected, one from a 1-hour incubation period and the other from a 24-hour incubation period. Samples were weighed at the end of the incubation time, and blood absorption was calculated as:

$$\%\ Absorbed\ = \frac{Wb\ -\ Wd}{Wd}\ x\ 100\%$$

Where W_b is the sample's mass in blood and W_d is the dry mass.

2.3 Coagulation Time Study

The relative time for complete clot formation was determined by incubating samples with porcine blood followed by measuring lysate absorbance at six-minute lysing intervals for 30 minutes. Samples (n=4) were analyzed at 0, 6, 12, 18 and 30 minutes. Previously weighed foam samples were placed in 1.5 mL centrifuge tubes arranged in 4x5 formation prior to testing. Empty polypropylene tubes served as negative (non-clotting) controls, and QCCG (gauze) and XStat were used as clinical controls.

Porcine blood anticoagulated with Na-Citrate upon collection was stored at 4°C in a refrigerator for up to 3 weeks from the bleed date until ready to be used. Ten mL of blood was pipetted from the primary blood container into a separate tube and allowed to rest at room temperature for 15 minutes. Then, 100 µL of 1 M $CaCl_2$ was added to the blood to obtain a final $CaCl_2$ concentration of 0.01 M to reverse the anticoagulant. Using a multichannel pipette, 50 µL of blood was immediately added to each centrifuge tube, a stopwatch was started, and 1 mL of DI water was added to the 0 minute sample tubes to stop the clotting progress and lyse free (not clotted) red blood cells. This lysing step was repeated at each time point until the 30-minute time point was reached.

When all samples had been lysed, each tube was centrifuged at 2300 rpm for 15 minutes. Before imaging, 200 µL of lysate was taken from each tube and pipetted into a 96 well-plate. Then, tubes were inverted and imaged using a digital camera.

Absorbance of the lysate was measured at 540 nm in a Biotek Synergy 2 Multi-Mode Microplate Reader (Winooski, VT, USA) to determine the relative amount of hemoglobin released at each time point, indicating the progress of clot formation at that time. Data points for each 6-minute lyse point were averaged and plotted with respect to time.

2.4 Hemolysis Assay

To calculate percent hemolysis upon incubation with SMP foams, samples (n=6) were weighed according to masses in **Table 2** and placed in 15 mL centrifuge tubes. Gauze and XStat were utilized as clinical controls. Prior to the experiment, all samples were incubated in 1X phosphate buffered saline (PBS, Fisher Bioreagents, Fair Lawn, NJ, USA) for 5 hours at 37°C. Positive (hemolytic) controls were empty polypropylene tubes with 5 mL of DI water, and negative (non-hemolytic) controls were empty tubes with 5 mL of PBS. Control tubes (n = 6) were incubated for 1 hour at 37°C. While foams were incubating, a separate solution of 1X PBS was prepared and warmed to 37°C. After incubation, samples were removed from the PBS and placed into a fresh, 15 mL centrifuge tube containing 5 mL of the warmed 1X PBS.

Porcine blood was anticoagulated with Na-Citrate upon collection and was stored at 4°C in a refrigerator until ready to be used. With all centrifuge tubes set up, 5 mL of the blood was removed from the primary blood container, transferred to a separate test tube and placed in a water bath at 37°C for 15 minutes. The tube of blood was then gently inverted 5 times to mix. Then, 100 µL of whole blood was added to each sample and control tube. All tubes were then incubated in a water bath at 37°C for 1 hour. To

separate lysate from whole blood components, samples were centrifuged at 1000 rpm for 10 minutes. Following centrifugation, 200 µL of supernatant was taken from each tube and pipetted into a 96 well-plate. A Biotek Synergy 2 Multi-Mode Microplate Reader (Winooski, VT, USA) was used to detect the absorbance of the released hemoglobin for each well at 545 nm. Using collected absorbance (OD) values, hemolysis was calculated as:

$$Hemolysis\ (\%) = \frac{OD(sample) - OD(negative\ control)}{OD(positive\ control) - OD(negative\ control)}\ \ x\ 100.$$

2.4 Platelet Attachment (LDH Assay)

For quantifying the attachment of platelets to samples, a Cayman Chemical LDH Cytotoxicity assay kit (Ann Arbor, MI, USA) was used after incubating SMP foams and controls in whole blood, stored as described previously. Plasma was obtained through centrifugation of whole blood at 3000 RPM for 15 minutes. A standard was generated by diluting plasma with PBS to concentrations of: 100%, 50%, 25%, 12.5% and 6.25%. Hemocytometer counts at each plasma concentration (n=4) were acquired and used for standard values.

SMP foams (n = 4) were cut to have the same surface areas (**Table 2**) and placed in the wells of a 24 well-plate. Then, 1 mL of whole was added to each sample well, and the plate was incubated at 37°C for 30 minutes.

Non-attached platelets were washed away with PBS by first dipping samples in a solution of 1X PBS then rinsing with a squirt bottle of PBS. Samples were transferred to another plate containing 1 mL of fresh PBS in each well, and 100 µL 10% Triton X-100

(Acros Organics, Fair Lawn, NJ, USA) was added to wells and incubated at 37°C for 1 hour to lyse the attached platelets. Then, 100 µL of supernatant was taken from each sample well and transferred to a 96 well-plate. The LDH Reaction solution (100 µL, included in kit) was added to each well, and the plate was incubated for 30 minutes at 37°C on an orbital shaker. Following incubation, absorbances were read on the microplate reader at 490 nm.

In addition to LDH assay measurements, platelet attachment was quantified through brightfield imaging with a Leica Inverted Fluorescent Microscope (Wetzlar, Germany) Three random regions of interest on each sample were imaged at 40X magnification and platelets counted manually.

2.5 Platelet Activation

SMP foams (approximately 0.5 cm^3) were incubated in whole blood and rinsed of non-attached platelets, according to the above procedure for the LDH assay. In order to observe activity states and activation of the attached platelets, samples were prepared for scanning electron microscope (SEM) imaging. Samples were fixed in a solution of 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) overnight at 4°C. Following fixation, samples were dehydrated in solutions with increasing concentrations of ethanol: 1) 30 minutes in 50% ethanol, 2) 30 minutes in 70% ethanol, 3) 30 minutes in 95% ethanol, 4) 30 minutes in 100% ethanol. Final dehydration was accomplished through drying overnight in a vacuum oven at room temperature, and samples were sputter coated with 5-10 nm of gold.

SEM analysis was performed using a Joel NeoScope JCM-5000 Scanning Electron Microscope (Peabody, MA, USA) at an operating voltage of 15 kV. Random regions of interest were imaged at 5000X, 1000X and 500X. Images were analyzed qualitatively for signs of platelet aggregation and morphology change.

3. Results and Discussion

3.1 Blood Absorption

Degradation of the polyurethane foams primarily occurs through oxidation, where the presence of reactive oxygen species breaks the bonds of tertiary amines present in the monomers [29]. In the control foam, this oxidative degradation occurs relatively slowly. The incorporation of either TEG or DEG slows down the foam degradation process. The ester formulations with NTA-DEG degrade considerably faster than the control foams due to the introduction of hydrolytically degradable ester bonds. Thus, this selection of SMP foams (Control, 30% DEG, 30% TEG, 15% NTA-DEG, and 30% NTA-DEG) provide a spectrum of biodegradation rates. While the polyurethane SMP system is hydrophobic overall, the inclusion of more hydrophilic DEG, TEG, or NTA-DEG to tune degradation speed has the potential to affect interactions with blood. Generally, proteins preferentially adsorb to hydrophobic surfaces [30]. Additionally, general changes in the chemistry (e.g., introduction of new dipole-dipole bonds between ester linkages in NTA-DEG foams) and charge (e.g., electronegative O in ether linkages in DEG and TEG) can alter protein interactions with materials. In the clotting process, the adsorption of fibrin as well as other blood proteins to a surface plays a crucial role in thrombus formation [31]. Thus, it is important to characterize the effects of chemical changes on blood interactions.

As an initial measure of blood interactions with these foams, blood adsorption was measured at 1 and 24 hours.

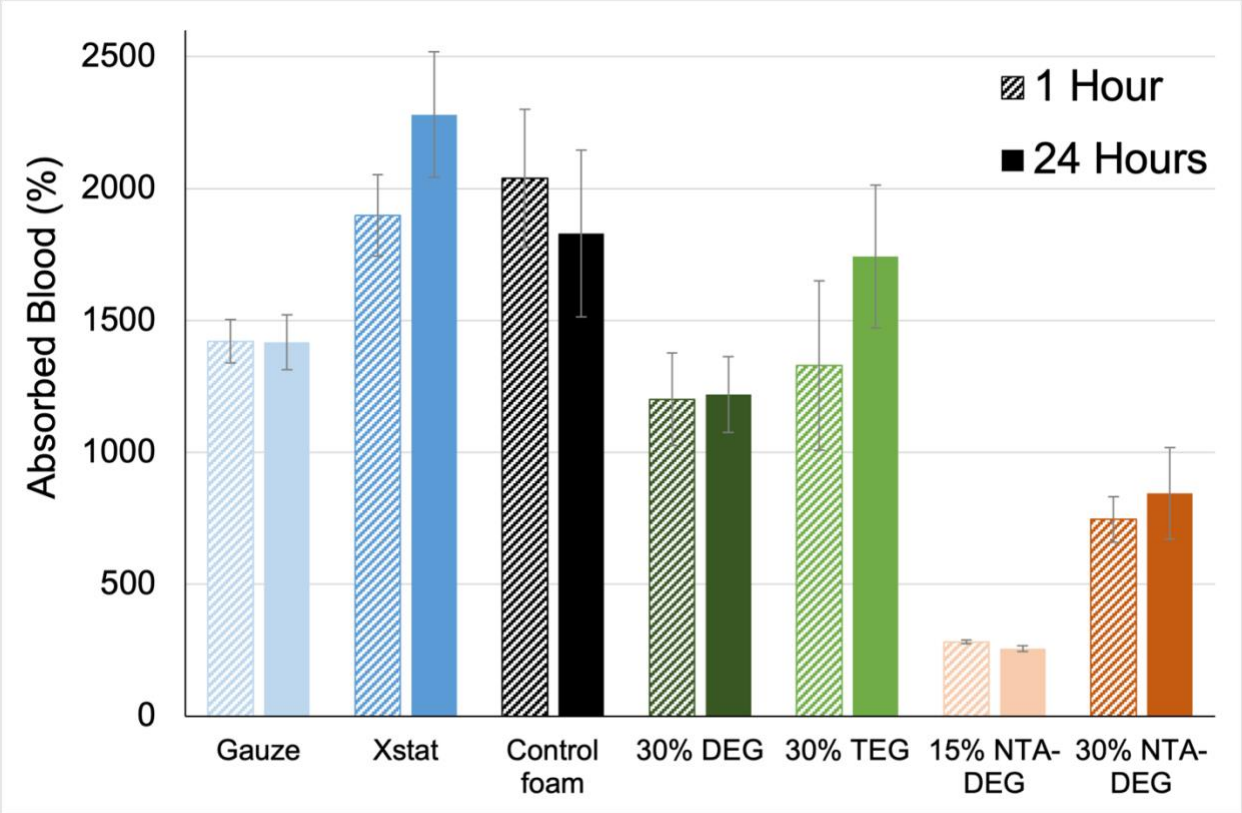

Figure 3. Blood absorbed over 1 hour and 24 hours at 37°C (n=3). Average ± standard deviation displayed.

Blood absorption volumes (**Figure 3**) were found to be the highest in the control foam, and lowest in 15% NTA-DEG. Dipole-dipole bonds in 15% NTA-DEG restrict water access within chains and may be responsible for the comparatively low absorption volume. When NTA-DEG content is increased to 30%, the hydrophilicity of DEG is sufficient enough to surmount dipole-dipole interactions and allow for increased water interactions within the network, and increased overall blood absorption. Blood absorption for 15% NTA-DEG decreases slightly relative to the control foam after a 24-hour incubation period, which could be due to a more open pore structure in 15% NTA-DEG that reduces the volume of blood that is retained after the sample is removed. Looking at DEG and TEG foams absorption volumes, the open pore structure may have

a similar effect on blood retention and explain why these foams absorbed less than the control foam. DEG and TEG foams also introduce charges from dipoles that could additionally impact interactions with proteins and overall blood absorption volume.

3.2 Coagulation Time

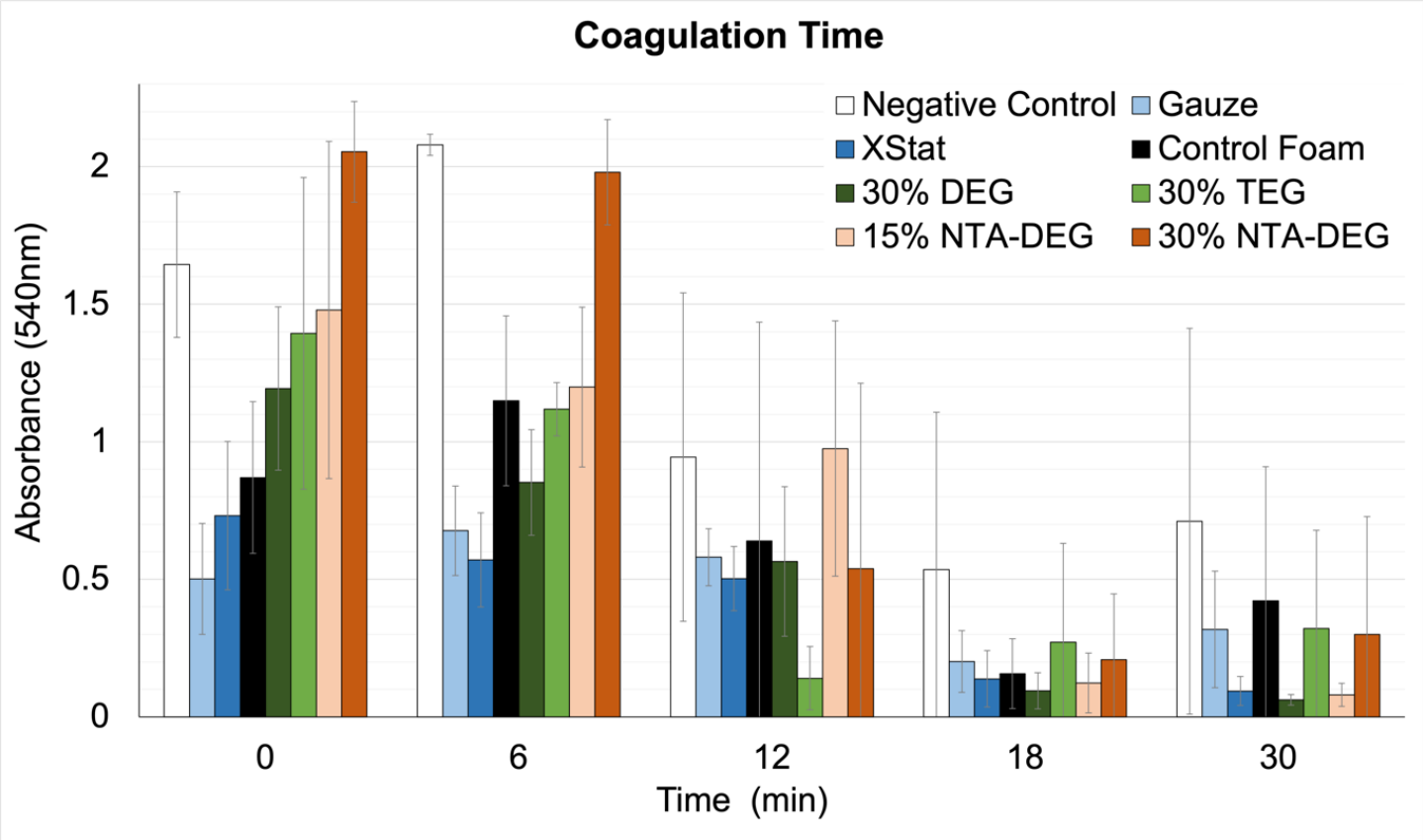

Figure 4. Blood clotting times as a function of absorbance at 540nm (n=3). Average ± standard deviation displayed.

The coagulation time test used absorbance values of lysed blood as an indicator of the clot formation progress. When DI water is added at each time point, free RBCs (not in a stable clot) lyse and release hemoglobin into the supernatant solution. The relative amount of hemoglobin released is determined through the absorbance reading,

with higher absorbance values corresponding to a higher concentration of free hemoglobin in the supernatant. As a stable blood clot forms, RBCs become trapped in an impermeable network of platelets and fibrinogen and do not lyse upon the addition of DI water [32]. Progress of clot formation is indicated by changes in the amount of hemoglobin released at consecutive time points, with absorbance decreasing as a stable clot forms and less RBCs are lysed when DI water is added. See **Figure 5** for representative images of lysates at each time point.

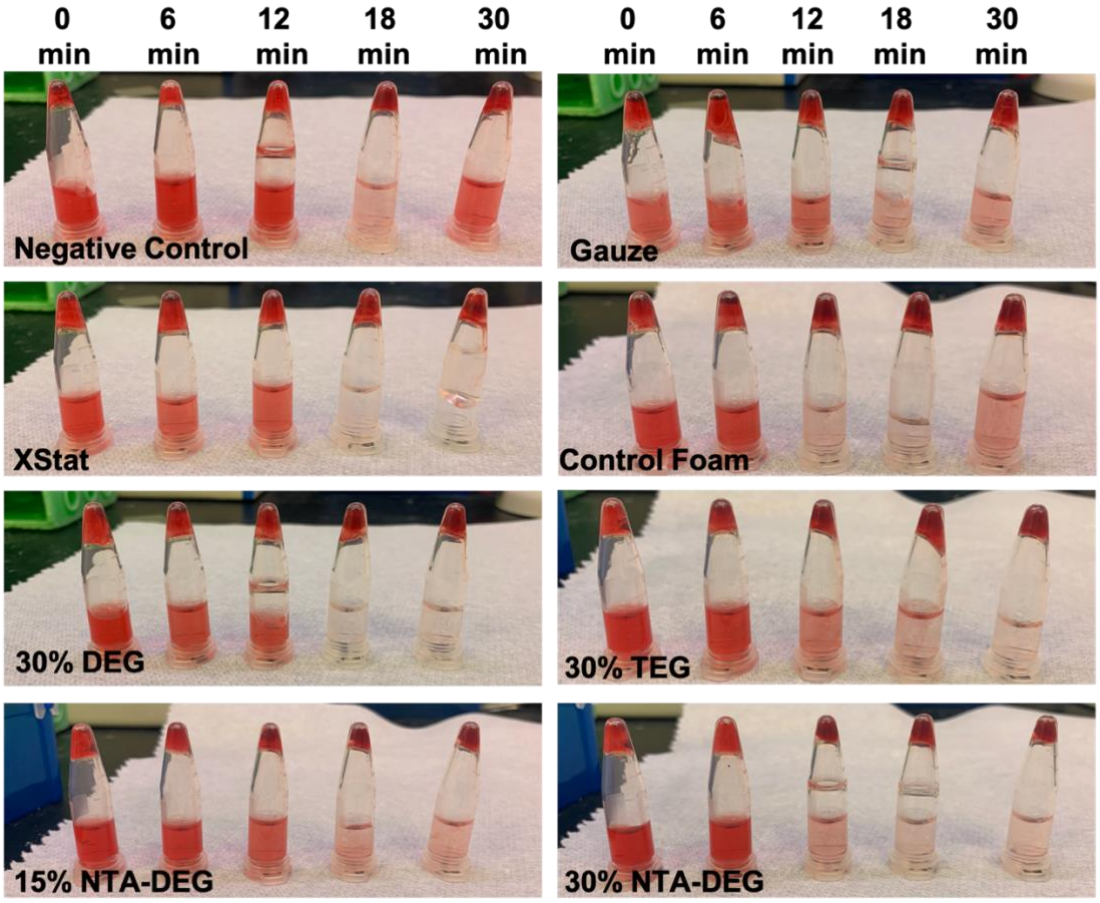

Figure 5. Representative images of lysates from coagulation time assay. Negative control: empty tube.

Results (**Figure 4**) showed that at 6 minutes, all of the SMP foams tested had higher free hemoglobin concentrations than clinical controls (gauze and XStat), suggesting less stable clots in the SMP foams. By 12 minutes, absorbance values for SMPs and clinical controls reached comparable values, with slightly higher values measured for 15% NTA-DEG. At 18 minutes, clot formation was stabilized, and all SMP foam absorbances were within range of the clinical control values and lower than the negative control. This result indicates that the incorporation of degradable linkages from NTA-DEG slows down clot formation but does not adversely affect the overall clot forming abilities of the SMP system. The inclusion of hydrophilic DEG and TEG does not affect the overall hydrophobicity and/or charge of the polymer enough to impact the interactions of platelets and blood proteins with the material surface, and ultimately does not impact the material's clotting abilities.

3.3 Hemolysis

Table 3. Percent Hemolysis measurements (n=3). Displayed as average ± standard deviation.

Sample	Hemolysis (%)
DI Water	100.0 ± 10.5
PBS	0.0 ± 0.3
Gauze	0.0 ± 0.2
XStat®	15.5 ± 25.8
Control Foam	0.0 ± 0.2
30% DEG	0.0 ± 0.4
30% TEG	0.0 ± 0.2
15% NTA-DEG	0.0 ± 0.3
30% NTA-DEG	0.1 ± 0.5

Percent hemolysis was determined following the incubation of samples with blood via measurement of supernatant absorbance following centrifugation. Similar to coagulation testing, the absorbance was an indicator of the concentration of free hemoglobin present in the supernatant as a result of lysing. Under the testing conditions performed here, any hemolysis that occurred can be assumed to be due to surface-material specific interactions rather than direct mechanical forces. When RBCs come into contact with any synthetic material, the cell membrane is subject to stresses that are dependent on the properties of the material such as: surface charge, the presence of substrates, and surface curvature or topography, which may affect the extent of interaction [33]. Results from this hemolysis assay (**Table 3**) indicate that all of the SMP formulations induced approximately 0% hemolysis and were comparable to the negative control (PBS) and the clinical control (gauze). Interestingly, the XStat clinical control induced increased hemolysis, with high variability between samples.

This result demonstrates that the interfacial interactions between SMPs and blood cells do not result in stresses large enough to disrupt the cell membrane and lead to cell lysis. There is no established value of allowable hemolytic potential for blood contacting materials, though any hemolysis is generally undesirable as it can lead to impaired oxygen transport to tissues over time and other unwanted side effects. As these SMPs caused approximately zero hemolysis upon incubation, we can conclude that the hemolytic potential of these materials is minimal, which supports the overall hemocompatibility of these SMP foams.

3.4 Platelet Attachment

3.4.1 LDH Assay

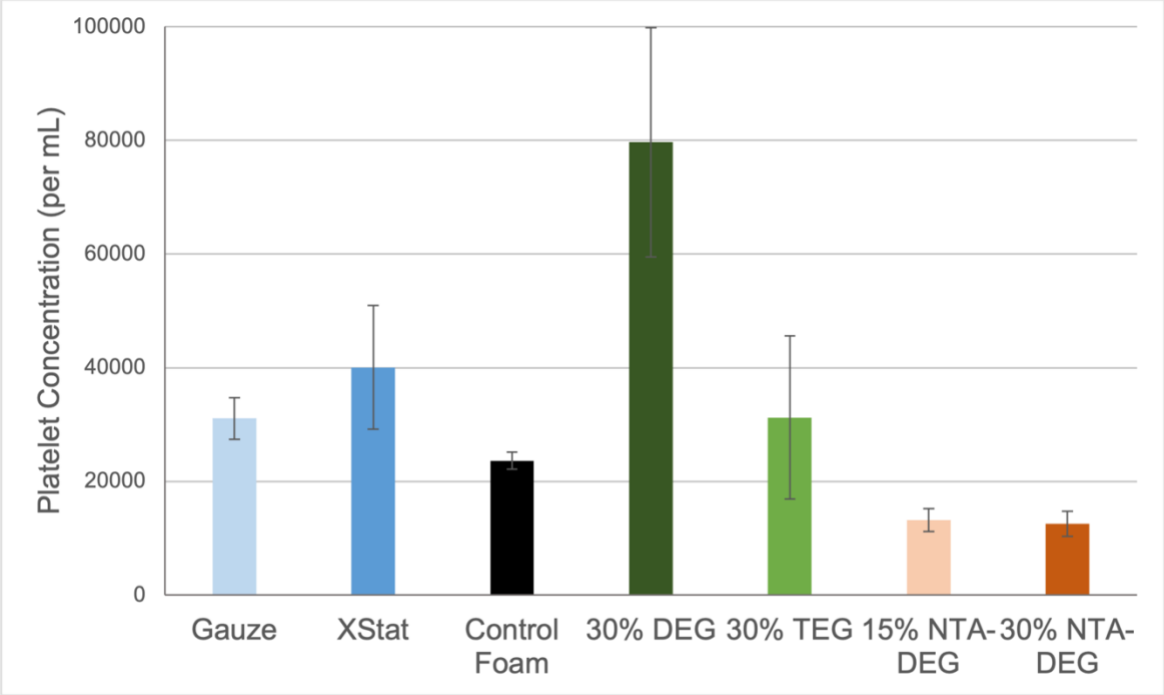

Figure 6. Average concentration (platelets/mL) of attached platelets on each sample (n=3). Average ± standard deviation displayed.

LDH is an enzyme that is found in the cytosol of most cell types of the body, including platelets. This assay utilized the presence of LDH in platelets as a means of quantifying how many platelets attached to each sample type. After lysing samples that had been rinsed of non-attached platelets and adding an LDH reaction solution, absorbance readings were fitted to a standard curve to determine the number of attached platelets. In creating a standard curve (using hemocytometer counts plotted against absorbance readings), decreasing concentrations of plasma resulted in a linear decrease in platelet numbers that could be used to quantify the concentrations of attached platelets on samples. Results can be found in **Figure 6**. The 30% DEG foams

showed the highest levels of platelet attachment by far, with the next highest being XStat®, having approximately half the number of attached platelets as compared to DEG. The 30% TEG and control foams had comparable platelet attachment values to the clinical controls, gauze and XStat®. Ester-containing foams, 15% NTA-DEG and 30% NTA-DEG, displayed slightly lower quantities of adhered platelets compared to controls, which correlates with the blood absorption and coagulation time assay results.

Platelet attachment is a critical part of hemostasis and is essential in the formation of a platelet plug at the site of vessel damage. As platelet attachment is mediated by interactions between the platelet membrane and plasma proteins (such as vWF) adsorbed onto a surface, results from this assay indicate the ability of plasma proteins to adsorb to tested sample surfaces and induce platelet interactions.
As described previously, various surface characteristics, such as charge and relative hydrophobicity, can impact protein adsorption to biomaterials. In the case of TEG and DEG, results from this assay indicate that their incorporation into SMP foams did not negatively impact the ability of platelets and plasma proteins to interact with the surface of these materials. Specifically for 30% DEG, it appears that its presence greatly improved surface interactions for platelets and proteins. This result requires further investigation to better understand the mechanism.

Ester-containing (NTA-DEG) SMP formulations had lower platelet attachment numbers, which is generally attributed to alterations in chemistry and secondary interactions (i.e., between ester bonds), hydrophilicity, and foam morphology, all of

which could affect how platelets interact with the surface. In general, platelets were found attached to all samples, showing that platelet interactions and overall plug formation are possible to varying degrees. This data interpreted in combination with clotting time observations suggests that the while the relative number of platelets attached to each sample and the relative rates of clotting differ, all foams are still capable of inducing stable clot formation.

3.4.2 Brightfield Imaging

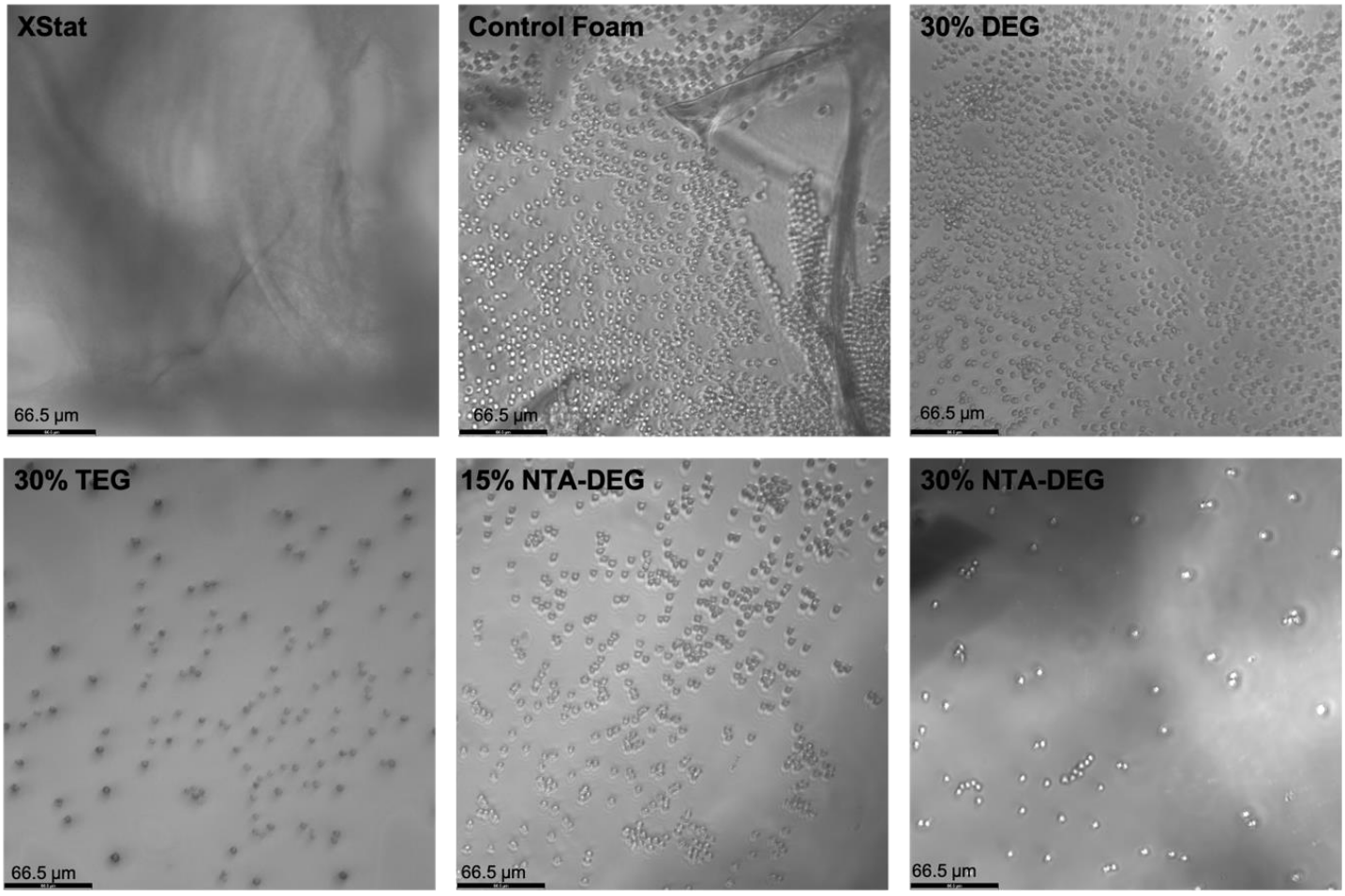

Figure 7. Representative brightfield images of attached platelets at 40X magnification. Area for each image is approximately 0.110mm^2.

Table 4. Average platelet counts determined from brightfield images (n=3). Average ± standard deviation displayed.

Sample	Average Platelet Count
Gauze	-
XStat®	-
Control Foam	754 ± 451
30% DEG	1230 ± 239
30% TEG	125 ± 42
15% NTA-DEG	371 ± 130
30% NTA-DEG	134 ± 52

Brightfield imaging was performed in supplement to the LDH assay to give a more detailed view of platelet interactions with each SMP formulation. Platelets were manually counted and averaged to provide an additional reference for attachment numbers (**Table 4**). Supporting results from the LDH assay, 30% DEG displayed a much higher number of platelets on to the surface than all other samples, suggesting favorable surface characteristics for platelet interactions. In this assay, the control SMP foam performed relatively better and had higher platelet numbers than in the LDH assay. The quantity of attached platelets on NTA-DEG and TEG foams were comparable. Due to the fibrous nature of gauze, bright field images could not sufficiently capture a large enough surface area of fibers in one focal plane to allow for platelet quantification, and the image is omitted here. XStat® samples did not have enough attached platelets to find and focus on. A representative image is included in **Figure 7** (XStat); however, no platelet number is included because counting was not possible with the obtained images.

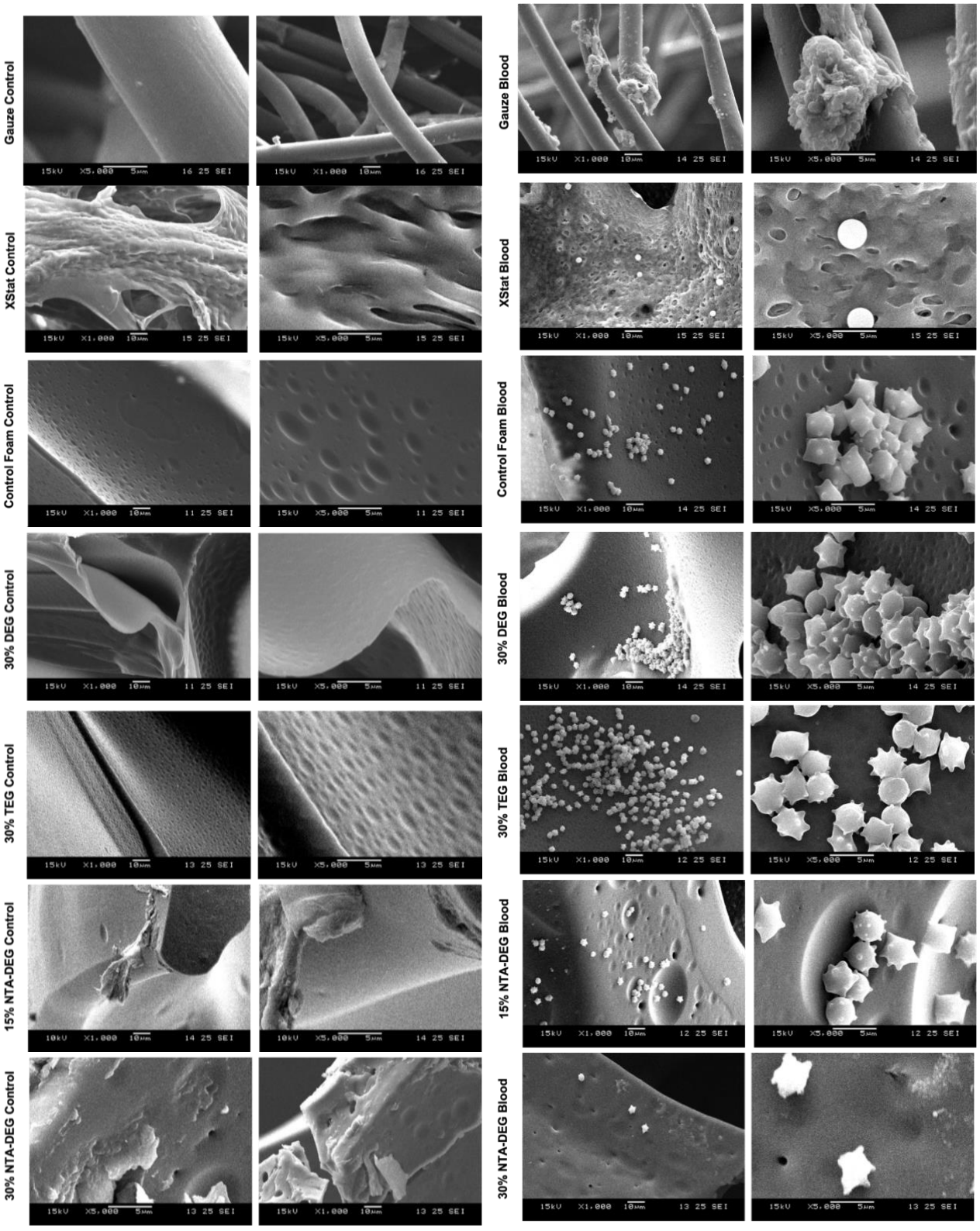

Figure 8. SEM images of samples before (control) and after (blood) incubation in whole blood.

3.5 Platelet Activation – SEM

SMP foams imaged by SEM gave a better idea of platelet activation and aggregation behavior on each material. As seen in **Figure 8**, platelets were found on all materials, in varying quantities and states. Generally, a circulating platelet that is not activated is shaped like a biconcave disk as seen in Figure 7 (XStat). Activation can be caused by multiple stimuli, including contact with von Willebrand factor that coincides with attachment and exposure to thrombin produced through the coagulation cascade. Once activation occurs and a platelet is adhered, the platelet will release granules from its cytoplasm, which release various chemical mediators that play a role in coagulation and inflammation [34]. This degranulation process can be seen in SEM images in **Figure 8** (blood samples) and is denoted by the small protrusions coming from the platelets. All SMP samples imaged showed that platelets were activated and had aggregated in clusters of varying sizes on the surfaces of the materials. Clinical controls had a lower degree of platelet adhesion, with gauze appearing to have more platelet activity and aggregation than on XStat®, which showed very few platelets and little to no observable activation. As stable clot formation depends on the entrapment of RBCs and activated platelets within a fibrin network, this SEM evidence of activation and aggregation of platelets on SMP foams suggests that all tested foams support platelet adhesion and activation.

When combined, these platelet studies indicate that the new foam formulations with tunable degradation rates are able to support platelet adhesion and activation, which is crucial to hemostasis and clot formation.

4. Conclusions

The blood interaction tests performed in this work provide a preliminary assessment of the hemocompatibility of degradable SMP foams. As polyurethane SMPs have previously shown good biocompatibility and overall hemostatic performance, we focused on determining how the inclusion of DEG, TEG, and NTA-DEG to tune degradation rates impacts the material interactions with blood. Understanding how these degradable SMPs interact with whole blood and blood components is a crucial aspect of assessing the potential of these materials to be used clinically as hemostatic devices in the future.

Degradable SMP foams demonstrated capability of stable clot formation, platelet attachment, and overall hemocompatibility comparable to clinical controls. From the data gathered here, it is evident that the chemical modifications do not affect the overall clotting abilities of the SMPs, as all degradable foams were found to support protein interactions and the formation of stable clots *in vitro*. However, inclusion of the ester-containing NTA-DEG does not appear to reduce platelet interactions and clotting speeds. Future work to further investigate specific effects of surface charge and relative hydrophobicity would be useful in understanding the mechanism of protein and platelet interactions observed in this work.

Overall, the data obtained in this study provides important insight to the performance of these materials as hemostatic devices. The methods established here can be used for future studies to evaluate the performance of blood contacting materials for hemostatic applications. Information gathered for each degradable SMP formulation

will be useful in optimization of these materials and for future *in vitro* and *in vivo* pre-clinical trials.

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