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Characterizing the modification of surface proteins with poly(ethylene glycol) to interrupt platelet adhesion

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Abstract

Surface protein modification with poly(ethylene glycol) (PEG) can inhibit acute thrombosis on damaged vascular and biomaterial surfaces by blocking surface protein–platelet interactions. However, the feasibility of employing protein reactive PEGs to limit intravascular and biomaterial thrombosis in vivo is contingent upon rapid and extensive surface protein modification. To characterize the factors controlling this potential therapeutic approach, the model protein bovine serum albumin was adsorbed onto polyurethane surfaces and modified with PEG-carboxymethyl succinimidyl ester (PEG-NHS), PEG-isocyanate (PEG-ISO), or PEG-diisocyanate (PEG-DISO) in aqueous buffer at varying concentrations and contact times. It was found that up to 5 PEGs could be attached per albumin molecule within one min and that adsorbed albumin PEGylation approached maximal levels by 6 min. The lability of reactive PEGs in aqueous buffer reduced total protein modification by 50% when the PEG solution was incubated for 7 min prior to application. For fibrinogen PEGylation (performed in the solution phase), PEG-NHS was more reactive than PEG-ISO or PEG-DISO. The γ peptide of fibrinogen, which contains several key platelet-binding motifs, was highly modified. A marked reduction in platelet adhesion was observed on fibrinogen-adsorbed polyurethane treated with PEG-NHS or PEG-DISO. Relative differences in platelet adhesion on PEG-NHS and PEG-DISO modified surfaces could be attributed to differences in reactivity towards fibrinogen and the size of the polymer backbone. Taken together, these findings provide insight and guidance for applying protein reactive PEGs for the interruption of acute thrombotic deposition.

Keywords

Platelet adhesion; Thrombosis; Protein adsorption; Fibrinogen; Poly(ethylene glycol); Protein modification

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1. Introduction

Thrombotic deposition onto damaged vascular surfaces and blood-contacting biomaterials remains a significant source of patient morbidity and mortality and limits the application of biomaterials in many settings. Damage to vascular surfaces can be initiated by common procedures such as angioplasty, anastomoses, and endarterectomy, where subendothelial adhesive proteins are commonly exposed as a matter of course. For blood contacting synthetic materials, the rapid adsorption of plasma adhesive proteins, fibrinogen in particular, can endow the surface with the means to specifically bind and activate platelets [1]. Blocking the recognition event between circulating platelets and adhesive proteins on susceptible surfaces has been the subject of substantial research wherein the most abundant platelet adhesion receptor, $\alpha_{IIb}\beta_3$, has been the target for monoclonal antibody fragments (e.g. the clinically utilized abciximab) and agents designed to contain or mimic the $\alpha_{IIb}\beta_3$ binding sequence arginine–glycine–aspartic acid [2,3]. Although this approach has met with clinical success, the targeting of the platelet adhesion receptor end of the platelet receptor–adhesive ligand pairing necessitates careful management of potential bleeding complications. These complications are not unexpected since essentially all of a patient's platelets are being targeted to prevent the adhesion event at a specific site.

A more logical approach might be to target the adhesive ligand for masking instead of the platelet receptor, leaving systemic hemostatic mechanisms intact for needed action to prevent bleeding complications. This targeted approach might be possible following a vascular intervention when either the surgical field was still open or when catheter access was still in place. For a synthetic material, it could be achieved following initial material contact with patient blood or plasma, but prior to extended blood contact.

To investigate this approach we have previously reported on the use of protein-reactive poly(ethylene glycol) (PEG) to modify and thus mask subendothelial adhesive proteins exposed on damaged placental arteries in vitro [4], adsorbed fibrinogen on biomaterial surfaces [5], pre-clotted Dacron [5], and balloon-injured rabbit femoral arteries in vivo [6]. In these studies the protein reactive PEG was PEG-diisocyanate (PEG-DISO) and the primary experimental endpoint was the inhibition of platelet deposition onto the modified surfaces. It was shown that acute platelet deposition, measured over periods varying between minutes to one hour, could be dramatically inhibited and that the reaction between the surface proteins and PEG-DISO could be achieved in time periods as short as 1 min.

While these reports have demonstrated that molecularly masking adhesive ligands with PEG on thrombogenic surfaces is possible in short time frames and using an aqueous solution at near physiologic pH, an examination of the basic factors controlling this reaction has not been performed. The broader literature addressing the solution-based PEGylation of therapeutic proteins for improved stability [7–12] has generally not focused on the constraints of extremely short reaction times and aqueous systems at physiologic pH that may be required with, for instance, an intracoronary application of reactive PEG following an angioplasty procedure.

In this report, we have examined the reactivity of three commercially available protein-reactive PEGs, PEG-carboxymethyl succinimidyl ester (PEG-NHS, M_w 5000), PEG-isocyanate (PEG-ISO, M_w 5000), and PEG-DISO (M_w 3400), over a range of reaction times and PEG concentrations. A model protein, bovine serum albumin (BSA), adsorbed onto polyurethane was utilized to quantify the number of PEGs attached per albumin molecule and the overall albumin modification in each reaction with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Additionally, the hydrolytic lability of the reactive PEGs was determined by measuring surface BSA modification

following suspension of the reactive PEG in aqueous buffer for various time periods. Fibrinogen PEGylation was also studied, however due to its high molecular weight, MALDI-TOF could not be performed from a modified fibrinogen adsorbed on a surface, thus solution-modified fibrinogen was denatured and the degree of PEGylation of the individual chains was quantified. Finally, platelet adhesion onto PEG-modified fibrinogen surfaces was quantified to compare the concentration effect of two of the reactive PEGs studied. Overall, the results of this report provide guidance for further pre-clinical testing of PEG masking of adhesive ligands to reduce acute thrombotic deposition.

2. Materials and methods

2.1. Materials

BSA and bovine fibrinogen were purchased from Sigma-Aldrich (St. Louis, MO). Both proteins were used without further purification. PEG-NHS (5000 M_w), PEG-ISO (5000 M_w), and PEG-DISO (3400 M_w) were purchased from Shearwater Polymers Inc. (Huntsville, AL; now Nektar Therapeutics (San Carlos, CA)). Tecoflex solution grade (SG)-80A, an aliphatic thermoplastic polyether-based polyurethane, was obtained from Thermedics Polymer Products (Wilmington, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available.

2.2. PEG-modification of surface adsorbed BSA

Polyurethane was dissolved in tetrahydrofuran to a final concentration of 1% (w/v) and 5 μ L was spotted onto a MALDI sample plate, which was left at room temperature until dry. The resulting polyurethane surface was smooth and clear. BSA (5 μ L, 6.9 mg/mL in water) was then spotted on top of the polyurethane surface and allowed to dry at room temperature.

The modification reaction was initiated by adding PEG-NHS, PEG-ISO, or PEG-DISO dissolved in phosphate buffer saline (PBS: 136.9 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM sodium phosphate dibasic, 1.8 mM potassium phosphate monobasic, pH 7.4; 5 μ L) onto the BSA adsorbed surface. The concentration of reactive PEG in the reactions was varied (0.0–7.8 mM). The reaction was terminated after a specified time (0–10 min) by removing the reaction solution from the polyurethane surface and immediately rinsing with PBS (5 μ L).

In experiments involving partial hydrolysis of reactive PEGs, each of the three PEG species (3.0 mM) was incubated in PBS for a specified time (0–11 min) prior to being introduced onto the adsorbed BSA. PEGylation of the protein-adsorbed surface was allowed to proceed for 1 min in all hydrolysis experiments.

2.3. Synthesis and characterization of PEG-modified fibrinogen

Typically, the molecular weight of monodisperse molecules up to 100–200 kDa can be determined with high accuracy using MALDI-TOF. Fibrinogen, which has a molecular weight of 340 kDa [13], was thus initially modified in a buffer solution with PEG and subsequently denatured into its $A\alpha$, $B\beta$, and γ polypeptide chains. The denatured PEG-fibrinogen was then adsorbed onto a polyurethane surface. By denaturing the PEG-protein conjugate, the extent to which each polypeptide was modified could be determined by MALDI-TOF analysis.

PEG-NHS, PEG-ISO, or PEG-DISO was added to PBS containing fibrinogen (8.8 mg/mL) with different molar ratios of PEG-to-protein examined (10–92). Since the solution reaction could not be readily terminated, the reaction was allowed to proceed with mixing for 2 h at room temperature to ensure near completion and loss of remaining PEG reactivity. After

modification, the PEGylated fibrinogen was denatured in a reducing environment containing urea (8 M) and dithiothreitol (10 mM) for 4 h in a 37 °C water bath. For control experiments involving unmodified fibrinogen, the protein was denatured under the same conditions.

Denatured modified or native fibrinogen was adsorbed onto a polyurethane-coated MALDI sample plate. The polyurethane surface was prepared as previously described in the surface adsorption of BSA. Protein (5 µL) was spotted on the polyurethane surface and dried by incubation at room temperature. The protein-adsorbed surface was subsequently rinsed three times.

2.4. MALDI-TOF analysis of protein modification

MALDI-TOF analysis of surface adsorbed protein was carried out using an Applied Biosystems PerSeptive STR Mass Spectrometer (Foster City, CA). A saturated sinapinic acid matrix solution (0.4 mL water, 0.3 mL acetonitrile, and 1 µL trifluoroacetic acid) was added (2 µL) to the polyurethane surface and allowed to co-crystallize with the adsorbed protein at room temperature. After evaporation of the matrix solution, the protein spectrum was measured and recorded. For each reaction condition studied two to three independent spectra were obtained for subsequent analysis. The instrument was operated in a linear mode using a 2.5 kV accelerating voltage.

A series of noise filtering and Gaussian smoothing steps were performed to enable clear differentiation of individual peaks in the spectra. Smoothing of the data inevitably alters peak shape to some degree, which can introduce significant error in the analysis of relative peak areas. Therefore, we analyzed total protein modification and the relative amount of each modified protein species using peak height ratios [14,15]. Total protein modification was defined as the ratio of the sum of peak heights generated by all PEG-protein conjugates to the sum of peak heights generated by remaining native protein and all modified conjugates (Eq. (1)). The relative amount of each PEG-protein conjugate was similarly defined as the ratio of the peak height of the corresponding modified species to the sum of peak heights generated by the remaining native protein and all modified conjugates (Eq. (2)). P_0 and P_i represent the height of the peak corresponding to unmodified protein and protein modified with “i” PEGs respectively. The heights of all peaks were measured from the baseline of the spectra.

$$\text{Total protein modification (\%)} = \frac{\sum P_i}{(P_0 + \sum P_i)} \times 100\%, \quad (1)$$

$$\text{Relative modification (\%)} = \frac{P_i}{(P_0 + \sum P_i)} \times 100\%. \quad (2)$$

2.5. Measurement of platelet adhesion onto fibrinogen-adsorbed surfaces

Glass coverslips were coated with 1% (w/v) polyurethane in tetrahydrofuran and allowed to dry completely at room temperature. Fibrinogen, (1.5 mg/mL in PBS) was introduced (1 mL) onto the polyurethane coated coverslip and allowed to incubate at room temperature for 35 min to allow for adsorption. Surface adsorbed fibrinogen was then treated with PEG-NHS or PEG-DISO (3–16% (w/v)) in PBS for 5 min at room temperature.

Immediately following modification, the treated coverslips were loaded into a parallel plate perfusion chamber [16] after which the chamber was flushed with PBS. Whole blood was

collected by antecubital venipuncture after obtaining informed consent from donors (IRB #000766) who had not taken any platelet active drugs in the previous week. The blood was anticoagulated with heparin (4 units/mL) and the platelets were fluorescently labeled by quinacrine dihydrochloride (10 μM final concentration) addition [17]. Using a Harvard Apparatus syringe pump (South Natick, MA) blood was perfused over the coverslip at a wall shear rate of 1000 s^{-1} for 5 min. The coverslip was then rinsed with HEPES-tyrode buffer (pH 7.4) and platelet adhesion on the surface was analyzed using an inverted stage epi-fluorescent video microscope with a Princeton Instruments RTE/CCD-1300-YHS camera (Trenton, NJ). The percent surface coverage of adhered platelets was quantified in captured images using Scanalytics IPLab version 3.2 (Fairfax, VA) scientific imaging software and a 2-way ANOVA was used to test for the impact of polymer type (NHS or DISO) and polymer dose on platelet adhesion.

3. Results and discussion

3.1. Modification of adsorbed BSA—effect of time

BSA adsorbed surfaces in this study were prepared by depositing the protein onto a polyurethane coating. It is reasonable to assume that only a fraction of the deposited BSA was tightly adsorbed and that the remainder of the protein had dried and deposited on top of this adsorbed layer. PEGylation of the surface protein was initiated by introducing a solution of protein reactive PEG onto the dry protein deposit. The upper layers of the protein deposit would be expected to re-solubilize in the bulk solution and compete with the adsorbed BSA for the PEG. This scenario is not unlike the *in vivo* application of reactive PEGs on the surface of a damaged vessel in that there will be competing surface-associated proteins, from residual blood, for instance, that will potentially react with the PEG excess. Thus, the PEGylation results reported in this study may underestimate the extent of PEG-attachment to surface adsorbed proteins. The surface protein PEGylation reaction was terminated via rinsing with an excess of PBS at which point any solubilized protein should have been washed away. For the purposes of this study, the protein remaining on the surface after rinsing is defined as adsorbed. The degree of adsorbed protein PEGylation was determined by MALDI-TOF analysis.

A typical MALDI-TOF spectrum for adsorbed BSA modified with PEG-NHS (3.1 mM) for 10 s (0.17 min) is shown in Fig. 1. The peak generated by unmodified BSA (at 66,000 Da) is apparent as are the peaks representing BSA modified with increasing numbers of PEG molecules. The offset between peaks is approximately the 5000 Da added to the native BSA from each attached PEG. Relative amounts of unmodified BSA remaining and of each PEGylated species were quantified using ratios of peak heights. There are several examples of previous reports involving protein PEGylation that employ analogous means of determining relative amounts of native and modified protein [18–21]. It is possible, however, that PEGylation may alter the degree of protein ionization as well as the path of flight of the protein to the detector, which could have an impact on peak height. To date, there are no detailed reports describing the effects of attached PEGs on MALDI-TOF analysis of proteins. For this study, we have assumed this impact to be minimal.

As seen in Figs. 2a and b, adsorbed BSA was modified with up to 5 and 3 PEGs per BSA molecule when reacted with PEG-NHS (3.1 mM) and PEG-ISO (3.1 mM) respectively, after only 10 s. Because PEG-DISO contains two protein reactive groups, a lower concentration of PEG-DISO (1.0 mM) relative to the other reactive PEG species was used in the modification of surface BSA. This concentration slightly overcompensates for the fact that the PEG-DISO has twice the number of reactive groups per mole of PEG-ISO. Modification with PEG-DISO resulted in the attachment of as many as 4 PEGs per BSA molecule in the initial 10 s (Fig. 2c). The total amount of BSA modified with at least one PEG after 10 s

exceeded 85, 62, and 62% using PEG-NHS, PEG-ISO, and PEG-DISO respectively. In all three PEGylation reactions, the number of PEGs attached per BSA molecule and total BSA modification approached near maximal levels by 6 min. Differences in the relative reactivities of the PEGs towards adsorbed BSA were apparent when longer reaction times used. When modifying the adsorbed BSA for between 4 and 10 min, the reactions with PEG-NHS and PEG-DISO yielded a greater mean number of conjugated PEGs per BSA molecule relative to modification with PEG-ISO (Figs. 2a–c).

These results demonstrate that for all of the reactive PEGs studied, substantial PEGylation of the BSA model protein could be achieved within the one min reaction window. In fact, brief incubations of only 10 and 30 s resulted in a majority of BSA molecules being modified. For application of a reactive PEG solution in conjunction with a balloon drug delivery catheter or other device designed to deliver a fluid to the vessel wall, the data indicates that if interruption of blood flow is needed to achieve the fluid delivery, a period of 1 min or possibly less would be adequate. The 1 min delivery time would likely be reasonable for transient coronary occlusion in an angioplasty setting. For delivery of a reactive PEG solution to an anastomotic site or a freshly endarterectomized carotid artery surface in an open surgical field, the constraints on reaction time are not as stringent. For these applications one could envision an incubation period of 5–6 min being reasonable, where the results show that near maximal protein modification might be achieved. In the biomaterial setting, the time constraints of several min for reaction would not seem to be an issue. Here, one might consider a period where the blood-contacting surface of a device is incubated with patient blood or plasma, followed by rinsing and incubation with the reactive PEG solution. Finally, the results demonstrate an advantage in using *N*-hydroxysuccinimide (NHS) over isocyanate terminated PEG to achieve more rapid PEGylation under these conditions.

3.2. Modification of adsorbed BSA-effect of reactive PEG concentration

The effect of concentration of reactive PEG on adsorbed BSA PEGylation was examined for a one min reaction time with the results shown in Figs. 3a–c. As one would expect, the distribution in the number of attached PEGs shifted towards increased BSA modification as the concentration of reactive PEG was increased. Moreover, the extent of BSA modification was greatest when modified with PEG-DISO at all concentrations. Treatment with 2 mM PEG-DISO resulted in modification of approximately 90% of the surface adsorbed BSA whereas 3 mM PEG-NHS and 8 mM PEG-ISO were required to obtain an equal extent of modification (Fig. 4). As the extent of BSA modification approached 100%, the impact of the concentration of the reactive PEGs progressively lessened, suggesting that the PEGylation reaction becomes sterically hindered or that the available lysines are exhausted.

The increased sensitivity of the extent of PEGylation on PEG-DISO concentration, versus on the concentrations of PEG-NHS or PEG-ISO, most likely is due to each PEG-DISO molecule containing two protein reactive groups. Hence, the stoichiometric ratio of PEG-to-protein is effectively doubled when using equivalent molar concentrations of PEG-DISO. Comparison between the extent of BSA PEGylation using equivalent concentrations of PEG-NHS and PEG-ISO indicates PEG-NHS is more reactive with surface proteins, consistent with our previous results. The concentration results shown in Figs. 3 and 4 generally provide guidance for the selection of a reactive PEG type and concentration for rapid surface protein modification, with the caveats that BSA reactivity may vary to some extent from the adhesive proteins of interest and that hydrolysis of the reactive PEG solution prior to application may lead to a higher concentration requirement. These limitations will be at least partially examined below.

3.3. Lability of protein reactive PEGs in aqueous buffer

To quantify the reduction in reactivity associated with time spent by the protein reactive PEGs in aqueous buffer, adsorbed BSA was contacted for one min with PEG-NHS, PEG-ISO, or PEG-DISO (at 3 mM) after the PEG solution had been previously incubated for up to 11 min. Although favoring reaction with amines, both of the reactive groups are subject to a competitive reaction with the water in the vehicle. The distribution in number of attached PEGs per BSA molecule are shown in Fig. 5a–c and the extent of protein modification as a function of incubation time in Fig. 6. As anticipated, the mean number of PEGs conjugated to BSA dropped as the time in which the reactive PEGs were incubated prior to initiating PEGylation was lengthened. The maximum number of attached PEGs decreased 3-fold upon incubation of the PEGs for 9 min, the maximum incubation time studied for all three polymers. Total BSA modification with PEG-NHS, PEG-ISO, and PEG-DISO was reduced by 50% following incubation in buffer for 6 min.

The rapid loss of reactivity of the functionalized PEGs will impact the design of further pre-clinical testing approaches. Employing organic solvents or modifying the pH of the buffer solution, which might be feasible for therapeutic protein modification *in vitro*, are not attractive options for this application. Clearly, the PEG solution needs to be made in the procedure room. For application through a catheter, time spent in buffer prior to site delivery should be minimized, or concentrations adjusted to provide some appropriate degree of compensation. For application in an open surgical field or to a pre-adsorbed biomaterial surface, generating the solution should be reasonably easy with a simple mixing device for the lyophilized PEG and aqueous buffer components.

3.4. Modifying fibrinogen with PEG

Fibrinogen is a homodimer with each monomeric subunit comprised of A α , B β , and γ chains that are tightly associated by several disulfide bridges [13]. As a result of the high-molecular weight of native fibrinogen (340 kDa), characterization of fibrinogen PEGylation using MALDI-TOF was not possible. It was for this reason that BSA was used as a model protein to characterize the kinetics of PEG-attachment to adsorbed proteins. It was possible, however, to characterize fibrinogen PEGylation by modifying native fibrinogen in the solution phase followed by denaturation and reduction of the PEG-protein conjugate to component A α , B β , and γ chains, which possessed molecular weights amenable to MALDI-TOF analysis. MALDI-TOF results for unmodified and denatured fibrinogen, seen in Fig. 7, show A α , B β , and γ chains to have molecular weights of 63,093, 54,902, and 48,228 Da, respectively. These results were consistent with reported individual fibrinogen chain molecular weights [22,23]. Differences in the MALDI-TOF peak heights generated by A α , B β , and γ chains as is evident in the spectra is most likely a result of variations with which the individual chains ionize and travel down the time-of-flight tube.

In the MALDI-TOF spectra of denatured PEG-fibrinogen that had been modified with PEG-NHS, peaks representing the A α , B β , and γ chains with up to two attached PEGs could be identified based on an offset of 5000 Da molecular weight of the PEG-NHS (Fig. 8). Given the partial overlap of peaks from different chains that were unmodified, once or twice modified, this analysis was necessarily more complex and limited than with BSA. The extent of B β chain modification, defined as the ratio of the sum of peak heights representing all PEG–B β chain conjugates to the sum of the peak height of remaining native B β chain and all PEG–B β chain conjugates, was quantified as a measure indicative of total fibrinogen PEGylation. Modification of the B β chain with all three protein reactive PEGs increased over the range of PEG-to-protein molar ratios (Fig. 9). At all commonly measured PEG-to-protein ratios, the extent of B β chain PEGylation was greatest when modified with PEG-NHS.

In Fig. 10 the extent of $A\alpha$, $B\beta$, and γ chain modification was compared for PEG-DISO modified fibrinogen. For each chain, the ratio of the peak height of the chain modified with a single PEG to the unmodified chain height was determined (i.e. $A\alpha_1/A\alpha_0$, $B\beta_1/B\beta_0$, γ_1/γ_0). By normalizing the peak height of the PEGylated chains to that of each respective unmodified chain, variations in peak heights between the unmodified fibrinogen chains (Fig. 7) should not impact the analysis. Chain species modified with more than one PEG were not included in the analysis due to overlapping of peaks in the MALDI-TOF spectra, which interfered with peak height measurements. The extent of $A\alpha$, $B\beta$, and γ chain PEGylation was linearly proportional to the molar ratio of PEG-DISO to fibrinogen. However, modification of the γ peptide was markedly greater than that of the $A\alpha$ and $B\beta$ peptides at all PEG-to-protein ratios. Of note, the fibrinogen γ chain possesses several $\alpha_{IIb}\beta_3$ recognition motifs to which activated platelets can bind, including the sequence HHLGGAKQAGDV located at the carboxyl terminus of the chain, $\gamma 400-411$, which has been identified as a primary site of platelet-fibrinogen interactions [24,25]. Modification of the lysine in this sequence, K406, or other proximal lysines on the γ chain would be expected to impact platelet binding, as might modifications that block integrin recognition sites on the other chains.

The results from the characterization of PEG-modified fibrinogen agree with the adsorbed BSA results in suggesting that PEG-NHS provides comparatively more reactivity than ISO terminated PEG, and that PEG-NHS might be expected to be more effective in interrupting platelet adhesion to adsorbed fibrinogen surfaces. A couple of significant limitations to these studies were that the fibrinogen was modified in solution, where we examined ratios of reactive PEG to fibrinogen as a compromise from being able to study the treatment of adsorbed fibrinogen. With conformational changes that would occur with fibrinogen adsorption, dependent upon the surface studied, not only would fibrinogen reactivity for platelets be altered [1], but also reactivity of the adsorbed protein for PEG would likely vary. In the final experiments of this study we examined the relative ability of PEG-NHS and PEG-DISO treatment to interrupt acute platelet adhesion onto polyurethane surfaces pre-adsorbed with fibrinogen. The results from the fibrinogen modification studies would suggest that PEG-NHS should be more effective than PEG-DISO.

3.5. Platelet adhesion to fibrinogen adsorbed polyurethane treated with reactive PEGs

Fig. 11 presents platelet adhesion results following 5 min of blood perfusion over polyurethane surfaces pre-adsorbed with fibrinogen and then modified with buffered solutions of 3, 10, or 16% PEG-NHS or PEG-DISO. For control purposes platelet adhesion is also shown for polyurethane adsorbed with fibrinogen that was not treated with reactive PEG and polyurethane not pre-contacted with fibrinogen or reactive PEG. Weight percents of reactive PEGs are reported to provide results comparable to earlier studies using 20% PEG-DISO [4,5]. The 3, 10 and 16% solutions corresponded to molar concentrations of 6, 20, and 32 mM, respectively for PEG-NHS and 9, 29, and 47 mM for PEG-DISO. Treatment with PEG-NHS reduced platelet adhesion over 7-fold compared to unmodified fibrinogen adsorbed surfaces. Modification with PEG-DISO resulted in a two- to three-fold decrease in platelet adhesion. Although there was a trend toward decreased platelet adhesion with increasing reactive PEG concentrations, the concentration effect was not significant.

PEG-NHS modified fibrinogen surfaces bound significantly fewer platelets relative to surfaces treated with PEG-DISO ($p < 0.01$). This might be a result of the difference in reactivity between PEG-NHS and PEG-DISO towards fibrinogen demonstrated above. The difference in molecular weights of the reactive PEGs (3400 Da for DISO versus 5000 Da for NHS) might also be a determining factor in the difference since a larger attached PEG molecule could provide more steric hindrance for platelet receptor–ligand interactions for an equivalent number of attached PEGs.

The temporal trend in platelet adhesion on PEG-NHS and PEG-DISO modified-fibrinogen surfaces was also explored (Fig. 12a,b). Platelet adhesion increased in a comparable manner on polyurethane regardless of whether it was pre-adsorbed with fibrinogen or not; the latter due to the putative rapid adsorption of adhesive proteins. PEG-NHS and PEG-DISO treated surfaces had reduced rates of platelet adhesion with PEG-NHS treatment showing very low-platelet adhesion levels for the entire period and PEG-DISO treatment slowly increasing in platelet adhesion in a manner dependent upon the contacting PEG concentration.

The concentrations employed for the reactive PEGs in the platelet adhesion studies fell between the 20% solutions previously reported to have a substantial impact on interrupting acute thrombotic deposition and the lower concentrations of approximately 3 mM that were shown to provide near maximal modification of adsorbed BSA (Fig. 4) [4–6]. The BSA results would suggest that further reductions of contacting reactive PEG concentrations would have been possible for both PEG-DISO and PEG-NHS, but the platelet adhesion results indicate that platelet adhesion would likely have increased for PEG-DISO if this were to be done. The PEGylation of fibrinogen in solution demonstrated a greater reactivity for PEG-NHS versus PEG-DISO for fibrinogen, which would explain the differences seen between the two PEGs at concentrations that would be expected to maximally modify adsorbed BSA.

The platelet adhesion studies were acute in nature and did not investigate the potential of the PEGylated fibrinogen surfaces to be displaced by adhesive proteins. Earlier work by our group indicated that there was not evidence of this effect after one hour of plasma perfusion over a treated surface [5], but with reduced concentrations of reactive PEG used this may not be the case. It is also notable that platelet adhesion was quantified here, not platelet deposition. The results thus do not consider the important build up of platelets in the flow field. From qualitative observation of the blood perfusion studies, it was apparent that the modified surfaces had very low deposition as well as adhesion. In other words, the adhered platelets were not forming the base of thrombi growing in the third dimension.

4. Conclusions

The objective of this study was to examine critical variables controlling PEG masking of adhesive ligands to interrupt acute thrombotic deposition. It was found that adsorbed BSA (as a model protein) could be modified with multiple PEGs in as little as 10 s when reacted with PEG-NHS, PEG-ISO, or PEG-DISO, and that modification with each of these protein reactive PEGs approached completion within 6 min. PEG-NHS and PEG-DISO were found to be more reactive with adsorbed BSA relative to PEG-ISO at equivalent times and concentrations, which can be at least partially explained by the dual functionality of PEG-DISO versus PEG-ISO. Incubation of the reactive PEGs in aqueous buffer prior to surface protein modification resulted in a marked reduction in PEGylation efficiency with a half-life of approximately 7 min, indicating that reactive PEG solutions must be delivered to the treatment site rapidly. PEG-attachment to fibrinogen in solution found more modification with PEG-NHS relative to both PEG-ISO and PEG-DISO, whereas comparative analysis of individual fibrinogen chain modification found relatively greater modification of the γ chain, which might result in modifications affecting key adhesive peptide sequences. Treatment of adsorbed fibrinogen on polyurethane with varying concentrations of PEG-NHS or PEG-DISO resulted in a significant reductions in platelet adhesion compared to control surfaces and a relatively greater inhibitory effect by PEG-NHS.

Overall these results provide insight and guidance for applying protein reactive PEGs for the interruption of acute thrombotic deposition. Studies utilizing in vivo models of thrombotic

deposition will be required to further characterize the relationships between the extent of surface protein PEGylation and platelet deposition.

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Fig. 1.

A typical MALDI-TOF spectrum of PEGylated adsorbed BSA. BSA adsorbed onto a polyurethane surface was modified with PEG-NHS (3.1 mM) for 10 s. The molecular weight difference between adjacent peaks is approximately 5000 Da, corresponding to the average molecular weight of PEG-NHS.

Fig. 2.

Extent of surface adsorbed BSA PEGylation as a function of reaction time. Surface adsorbed BSA was reacted with (a) PEG-NHS (3.1 mM), (b) PEG-ISO (3.1 mM), and (c) PEG-DISO (1.0 mM) for a specified time after which the relative amount of each PEG-BSA conjugate was determined via MALDI-TOF analysis. The first two sets of bars are for 10 s (0.17 min) and 30 s, respectively. Alternating black and white sets of bars are employed to enhance the contrast between the data and the overall clarity of the figure.

Fig. 3. Extent of surface adsorbed BSA PEGylation as a function of (a) PEG-NHS, (b) PEG-ISO, and (c) PEG-DISO concentration. Each reaction was terminated after 1 min and MALDI-TOF analysis was used to determine the relative amount of each PEG-BSA conjugate. Alternating black and white sets of bars are employed to enhance the contrast between the data and the overall clarity of the figure.

Fig. 4. Impact of PEG-NHS ($\diamond$), PEG-ISO ($\square$), and PEG-DISO (Δ) concentration on total modification of surface adsorbed BSA. Error bars are not presented for clarity. The average standard deviation was 14%.

Fig. 5. Extent of surface adsorbed BSA PEGylation as a function of partial hydrolysis of the protein reactive PEGs. Surface adsorbed BSA was modified with (a) PEG-NHS, (b) PEG-ISO, and (c) PEG-DISO that had been incubated in buffer for a specified time period. The relative amount of each PEG–BSA conjugate was determined via MALDI-TOF analysis. Alternating black and white sets of bars are employed to enhance the contrast between the data and the overall clarity of the figure.

Fig. 6. Impact of partial hydrolysis of PEG-NHS ($\diamond$), PEG-ISO ($\square$), and PEG-DISO ($\triangle$) on total modification of surface adsorbed BSA.

Fig. 7. MALDI-TOF spectrum for denatured fibrinogen. Peaks representing the A α , B β , and γ chains are labeled.

Fig. 8.

A typical MALDI-TOF spectrum of adsorbed denatured PEG-fibrinogen. Fibrinogen was modified with PEG-NHS using a 14:1 molar ratio of PEG to protein. The PEG-fibrinogen was subsequently denatured and adsorbed onto a polyurethane surface. Peaks representing unmodified A α , B β , and γ chains (A α ₀, B β ₀, γ ₀) and A α , B β , and γ chains modified with one (A α ₁, B β ₁, γ ₁) or two (B β ₂) PEGs are labeled. The molecular weight difference between each modified peptide species is approximately 5000 Da, which corresponds to the average molecular weight of PEG-NHS.

Fig. 9. Impact of the PEG-to-protein molar ratio on total modification of the fibrinogen B β chain using PEG-NHS ($\diamond$), PEG-ISO ($\square$), and PEG-DISO (Δ).

Fig. 10. Relative modification of fibrinogen A α ($A\alpha_1/A\alpha_0$ (◇), B β ($B\beta_1/B\beta_0$ (□), and γ (γ_1/γ_0 (Δ)) chains with PEG-DISO as a function of the PEG-to-protein molar ratio. The relative amounts of unmodified chains ($A\alpha_0$, $B\beta_0$, γ_0) and chains modified with one PEG ($A\alpha_1$, $B\beta_1$, γ_1) were determined by MALDI-TOF analysis.

Fig. 11. Platelet adhesion on fibrinogen-adsorbed polyurethane surfaces modified with varying weight percentages of PEG-NHS (white) and PEG-DISO (black) solutions. Whole human blood was perfused over the surfaces for 5 min after which surface coverage by platelets was measured via fluorescence microscopy. Fibrinogen-adsorbed polyurethane surfaces (PU/fib) and untreated polyurethane (PU) were employed as control surfaces (gray).

Fig. 12. Accumulation of platelets on fibrinogen-adsorbed surfaces modified with 3% (◇), 10% (□), and 16% (Δ) PEG-NHS (a) or PEG-DISO (b) when perfused with blood for up to 5 min. Surface coverage by platelets was measured via fluorescence microscopy. Polyurethane (●) and fibrinogen-adsorbed polyurethane (■) surfaces were used as control surfaces.