Surface Modification with PEG and Hirudin for Protein Resistance and Thrombin Neutralization in Blood Contact

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Introduction: Various methods have been used to improve the blood compatibility of biomaterials, including the immobilization of passive molecules such as polyethylene glycol (PEG) and bioactive molecules such as heparin.[1] Passive molecules provide a surface that is resistant towards further interactions (non-fouling). Bioactive molecules may promote the adsorption of specific proteins.[2] In this study, a novel approach is used to create "bifunctional" surfaces that are both protein resistant and bioactive. It is hypothesized that the dual effects of PEG and hirudin may provide a non-fouling, thrombin-neutralizing surface. Hirudin is a peptide of molecular weight 6.9 kDa, found in the salivary glands of medicinal leeches. It binds with high affinity to thrombin and inhibits thrombin.[3] To reach our objective, two surface modification methods were investigated: (1) A "direct" method wherein PEG was first conjugated to hirudin and the conjugate then grafted to the surface, (2) A "sequential" method wherein PEG was first grafted to the surface and then conjugated to hirudin.

Methods: For the direct method, a conjugate of PEG and hirudin was prepared by reaction of PEG-NHS ester disulfide (MW=1100) with hirudin through the amino groups. The conjugation was confirmed using MALDI-mass spectrometry. The conjugate was then attached to gold coated silicon by chemisorption through the disulfide group in the PEG moiety. For the sequential method, PEG-NHS ester disulfide (MW=1100) was first attached to the gold surface by chemisorption and hirudin was then conjugated to the chemisorbed PEG by reaction with the amino groups. Hirudin density on the surfaces was determined using ^{125}I-labeled hirudin. Protein resistance was investigated by exposure to plasma depleted of antithrombin and fibrinogen and to serum. Adsorbed proteins were detected by immunoblotting. Thrombin adsorption from these media was measured using the ^{125}I-labeled protein. The inhibitory activity of PEG-hirudin surfaces towards adsorbed thrombin was measured by chromogenic substrate assay.

Results and discussion: The products of the hirudin-PEG reaction showed MWs of 8, 9, 10 and 11 kDa (MALDI-MS), indicating varying numbers of PEGs from 1 to 4 per hirudin (corresponding to the four amino groups of hirudin).

Hirudin densities on the "direct" and "sequential" surfaces were 11±0.1 and 7.8±0.1 ng/cm^2 respectively. Hirudin density was thus significantly higher on surfaces prepared by the direct method. Immobilization of PEG prior to hirudin conjugation (sequential surfaces), apparently created a protein resistant layer resulting in a lower hirudin density.

As shown in Fig 1, protein adsorption from AT-Fg depleted plasma was similar on the PEG-hirudin and PEG-OH (control) surfaces. The only major differences were in the thrombin blots where the PEG-hirudin surfaces showed a band for thrombin at the expected MW of 37 kDa, whereas the PEG-OH surface showed no response for thrombin. The immunoblot data, along with labeled thrombin adsorption experiments (data not shown), confirmed the specific affinity of hirudin-modified surfaces for thrombin. Similar results were obtained for adsorption from serum.

Chromogenic substrate assay data showed that PEG-hirudin surfaces inhibited adsorbed thrombin to a greater extent than PEG-OH surfaces (Fig 2). The direct surfaces showed lower inhibitory activity than the sequential ones. This may be the result of reduced availability of hirudin molecules on the direct surface due to screening by conjugated PEG molecules.

Conclusions: The PEG-hirudin surfaces showed similar protein resistance to the PEG-OH surfaces. Both types of PEG-hirudin surface bound and inhibited thrombin specifically; the sequential surfaces were more effective than the direct ones.

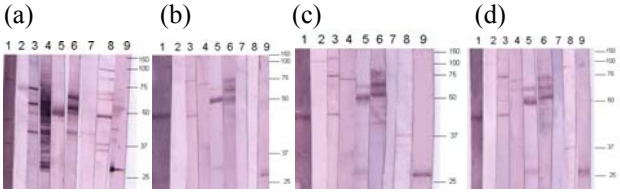

Figure 1: Immunoblots of plasma proteins eluted from surfaces exposed to AT-Fg depleted plasma: (a) AT-Fg depleted plasma, (b) PEG-OH (Control), (c) Sequential surface, (d) Direct surface. Lanes: 1. HMWK, 2. plasminogen, 3. C3, 4. albumin, 5. IgG, 6. vitronectin, 7. protein C, 8. thrombin, 9. apolipoprotein-AI.

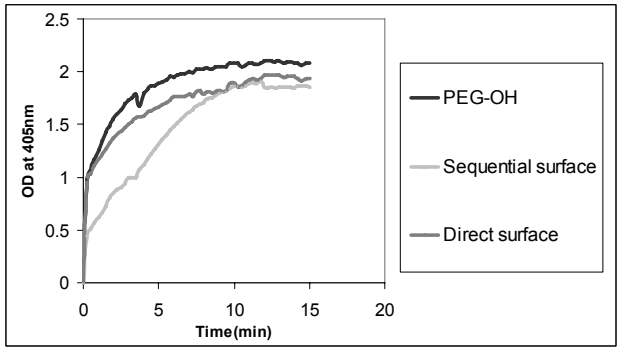

Figure 2: Thrombin chromogenic substrate assays. Rate of OD change (slope) gives a measure of thrombin activity.

References:

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