



Immobilization of an antithrombin–heparin complex on gold: Anticoagulant properties and platelet interactions

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ABSTRACT

The anticoagulant properties and platelet interactions of gold surfaces modified with an antithrombin–heparin (ATH) complex are reported. ATH was attached to gold through either a short disulfide (linker) or a thiol-terminated polyethylene oxide (PEO) (linker, spacer). Analogous surfaces were prepared with uncomplexed heparin. Antithrombin (AT) uptake was measured before and after selectively destroying the active pentasaccharide sequence of the heparin moiety, and was found to be predominantly through the active sequence on all of the surfaces. AT binding was higher on the ATH surfaces than on the corresponding heparin surfaces. Heparin activity was assessed by an anti-factor Xa assay. The ratio of active heparin density (from the anti-FXa assay) to total heparin density was taken as a measure of heparin bioactivity. The ratio was greater on the ATH- than on the heparin-modified surfaces, i.e. the PEO–ATH surfaces showed the greater proportion of active heparin. Platelet adhesion from flowing whole blood was found to be reduced on PEO- and ATH-modified surfaces compared to bare gold. The PEO–ATH modified surfaces, but not the heparinized surfaces, were shown to prolong the clotting time of recalcified plasma.

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1. Introduction

When a foreign material is exposed to blood, rapid adsorption of plasma proteins takes place. The properties of the protein layer determine subsequent surface interactions, including coagulation, complement activation, platelet adhesion and activation, and other blood cell responses [1,2]. These phenomena are severely limiting on the use of blood contacting medical devices, including stents, vascular grafts, oxygenators and catheters.

A popular strategy to reduce protein and platelet interactions on biomaterials is the incorporation of hydrophilic polymers such as polyethylene oxide (PEO) [3–5]. Due to its hydrophilic character, PEO acts as a barrier to protein attachment. It can also function as a spacer for attaching bioactive molecules. For example, the anticoagulant heparin has been immobilized to surfaces through PEO spacers [6,7]. The immobilization of heparin, on its own and in combination with other molecules such as PEO, has been extensively investigated [8–10], leading to a number of commercialized technologies [11]. Heparinized materials are, however, not without drawbacks. A significant disadvantage of heparin as a surface modifier, indeed for any application, is its variable activity and propensity to bind a large number of plasma proteins as well as antithrombin (AT) [12]. These limitations have led to the investiga-

tion of other anticoagulants, including direct thrombin inhibitors such as hirudin, hirulog and PPACK [13–15], as well as a range of heparinoids.

Our laboratory has developed a novel covalent complex of antithrombin and heparin (ATH) with increased pentasaccharide content in the heparin moiety and high anticoagulant activity compared to standard heparin [16,17]. ATH has many advantages over heparin, including both catalytic and direct AT activity, reduced non-specific binding of plasma proteins and the ability to inhibit surface-bound coagulation factors [18]. As a surface modifying agent, ATH has an advantage over heparin in that attachment can take place through the AT moiety, leaving the heparin effectively “free”. In addition, the heparin is expected to be directed away from the surface and to be able to interact more efficiently with blood components such as thrombin.

In previous studies ATH was shown to have potential as a biomaterial surface modifier on polyurethane grafts and catheters [19–22]. The present work attempts to elucidate in more detail the interactions of surface-immobilized ATH using gold as a better defined substrate more amenable to precise surface characterization. Once optimized, the various chemistries studied on gold for attachment of ATH can be applied to more practical substrates for blood contacting materials, such as synthetic polymers. In a previous communication on gold–ATH [23], we described the surface preparation to create well-defined surfaces through direct immobilization, using DSP, a short linker molecule, and with PEO

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as a linker/spacer. Detailed surface characterization and protein interaction studies were carried out and demonstrated that, in comparison to analogous heparin-modified surfaces, ATH could bind greater amounts of AT from plasma. In the present work, the biological activity of these surfaces has been further investigated. AT binding both biospecifically (through the pentasaccharide sequence of heparin) and non-specifically, inhibition of factor Xa, platelet adhesion from flowing whole blood and plasma coagulation are reported.

2. Materials and methods

2.1. Materials

Human AT was purchased from Affinity Biologicals Inc. (Ancaster, ON, Canada). Unfractionated heparin (UFH) was Grade I-A sodium salt from porcine intestinal mucosa (Sigma–Aldrich, Oakville, ON, Canada). ATH synthesis and purification was performed as described previously [16]. Briefly, AT and UFH were heated to 40 °C for 14 days followed by addition of NaBH₃CN and further heating at 37 °C for 5 h. ATH was purified by butyl agarose hydrophobic chromatography to remove excess UFH followed by DEAE Sepharose anion exchange chromatography to remove unreacted AT. Dithiobis(succinimidyl propionate) (DSP) was from Sigma–Aldrich (Oakville, ON, Canada) and N-hydroxysuccinimide (NHS)-terminated PEO ester disulfide, MW 1100, was from Polypure (Oslo, Norway). NaBH₄ was from Caledon Laboratories (Georgetown, ON, Canada) and NaIO₄ was from Aldrich (St Louis, MO, USA).

2.2. Surface preparation

Gold surfaces were prepared as described previously [23]. Silicon wafers, 0.5 mm thick and polished on both sides, were coated with a titanium adhesion layer and 1000 Å of gold (Silicon Valley Microelectronics, Santa Clara, CA, USA). Four inch diameter gold coated wafers were diced into 0.5 × 0.5 cm squares for protein adsorption and activity studies or into four equal pieces for platelet studies. Gold was cleaned for 5 min in a boiling aqueous solution containing 1 part hydrogen peroxide, 1 part ammonium hydroxide and 5 parts Milli-Q water, followed by extensive rinsing in Milli-Q water. Surface modification methods were as described elsewhere [23]. Briefly, gold surfaces were modified directly or functionalized by incubation with either DSP (20 mM in DMSO) or PEO NHS ester disulfide (1 mM in ethanol) for 2 h at room temperature, rinsed with solvent and dried in a stream of nitrogen. NHS functional groups on the distal ends of DSP and PEO were then able to react with amino groups of ATH or heparin.

Surfaces were transferred to a solution of ATH in phosphate-buffered saline (PBS) (0.1 mg ml⁻¹) or, for parallel modification with heparin, a solution of heparin in PBS (1.0 mg ml⁻¹) and incubated overnight at room temperature. Surface modification schemes using these approaches are shown in Ref. [23].

2.3. Grazing incidence reflection Fourier transform infrared (GIR-FTIR) spectroscopy

GIR-FTIR spectra of the surfaces were obtained with a Nicolet 6700 FTIR spectrometer (Thermo Scientific) with Smart SAGA

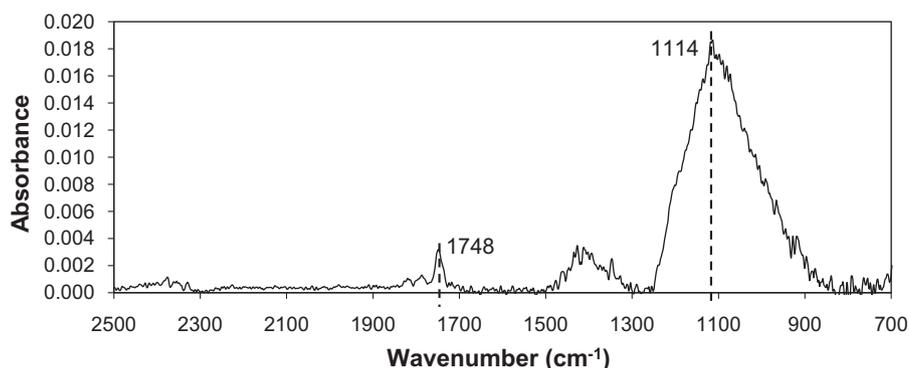


Fig. 1. GIR-FTIR spectrum of Au following modification with PEO-NHS showing characteristic stretching frequencies of NHS groups at 1748 cm⁻¹ (C=O) and 1114 cm⁻¹ (C–O).

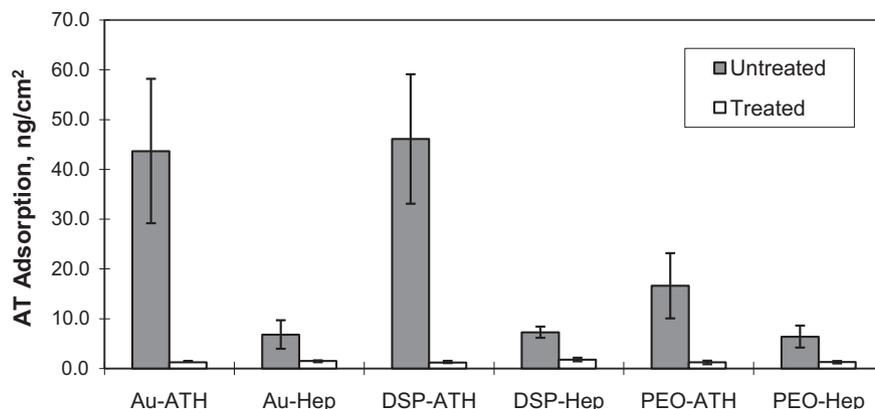


Fig. 2. AT adsorption from plasma to modified surfaces treated and not treated with NaIO₄ and NaBH₄, demonstrating the effect of destroying the active pentasaccharide sequence of the surface immobilized heparin. Data are mean ± SD, n ≥ 6.

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