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Immobilization of heparin on a silicone surface through a heterobifunctional PEG spacer

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Abstract

A novel method of immobilizing heparin on a silicone surface through a heterobifunctional PEG spacer was used yield well defined surfaces with highly active surface immobilized heparin and low non-specific protein adsorption. The heparin surface density achieved using this technique was 0.68 μg/cm^2. Sessile drop water contact angles showed increased hydrophilicity of the silicone surface after PEG modification and a further decrease in the contact angles following the grafting of heparin. High specificity for ATIII with little fibrinogen adsorption was noted in plasma adsorption studies. This ATIII adsorption was mediated by the heparin layer, since surfaces modified with PEG only did not adsorb significant quantities of AT. The thrombin resistance of the heparin modified surfaces was demonstrably greater as measured by a chromogenic thrombin generation assay. The results suggest that the heterbifunctional PEG linker results in a high density of active heparin on the surfaces.

Keywords: Silicone; PEG spacer; Heparin; Thrombogenic; Fibrinogen; Antithrombin III

1. Introduction

Silicones have a long history of use as biomaterials in applications ranging from intraocular lenses to catheters as a result of their good bulk properties, reasonable biocompatibility, processability and low cost. However, the relatively high surface hydrophobicity of these materials, which results in the adsorption of significant amounts of protein [1], constrains their use in biomedical applications. While potentially desirable in some applications [2,3], the non-specific adsorption of proteins is generally considered detrimental to performance, as it has been widely demonstrated that the initially adsorbed protein layer is responsible for mediating subsequent biological effects [4]. In blood contacting applications, the non-specific adsorption of plasma proteins to an artificial surface is known to mediate subsequent thrombotic and immunogenic effects with potentially catastrophic results. Therefore, it is necessary to alter the surface properties of PDMS in order to render the material more biocompatible.

Heparin, a heterogeneous extended polymer of repeating sugar units with molecular weight ranging from 3000 to 30,000 and an average molecular weight of 15,000, and related compounds are among the most frequently used therapeutic agents for thrombin regulation. The utility of heparin-modified surfaces including heparin-modified silicone surfaces, however, has been mixed [5,6]. Heparin contains a key pentasaccharide sequence that binds to the inhibitor antithrombin; this complex has a significantly increased reaction with serine proteases including thrombin compared with free heparin [7]. The maintenance of an intact three-dimensional structure, particularly one in which the accessibility of the key active pentasaccharide is maintained, is necessary for activity of the heparin molecule.

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One method to stabilize biomolecules at interfaces is to tether them through a poly(ethylene glycol) (PEG) spacer [8], which presumably has the additional advantage of reducing the non-specific adsorption of proteins [9]. There have been a number of studies where heparin has been immobilized to a variety of surfaces using a PEG spacer [10]. Heparin immobilization via a PEG spacer has been demonstrated to enhance its bioactivity relative to direct immobilization [11–16].

Most PEG derivatives used for linking of biomolecules are limited to homobifunctional polymers and polymers with one reactive terminus and one unreactive terminus [17]. However, tethering of biological molecules via a homobifunctional PEG can result in a lower surface density due to the potential for multiple reactions between the PEG termini and the surface. The ability to tether biological molecules via a heterobifunctional polymer is therefore expected to result in more homogeneous, higher density surfaces.

We have previously reported on a method for generating surfaces with a high PEG density that show significant reductions in the non-specific adsorption of plasma proteins [18]. Herein we report on the surface first heparinization of PDMS surfaces via a heterobifunctional PEG spacer and the subsequent biological properties of the heparinized material.

2. Materials and methods

2.1. Reagents

PEG monoallylether (average MW 500) was obtained as a gift from JuTian Chemical Co. (Nanjing, China). It was dried by azeotropic distillation with toluene before use. *N*,*N*′-Disuccinimidyl carbonate, *o*-xylene (97%, anhydrous), triethylamine (99%), acetonitrile (99%, anhydrous), Karstedt's Pt catalyst (2–3 wt% in xylene, $[(Pt)_2(H_2C{=}CH{-}SiMe_2OSiMe_2CH{=}CH_2)_3]$), 2-mercaptoethanol, CF_3SO_3H were purchased from Aldrich Chemical Co. The synthesis of α-allyl-ω-*N*-succinimidyl carbonate-poly(ethylene glycol) has been previously described [19]. Sylgard 184 and DC1107 ($(MeHSiO)_n$) were purchased from Dow Corning (Midland, MI). Sephadex G-25 columns were obtained from Sigma. Heparin, toluidine blue and *N*-*p*-tosyl-gly-pro-arg *p*-nitroanilide were purchased from Sigma Aldrich. Platelin® was obtained from Organon Teknila Corp., (Durham, NC, USA). Fibrinogen and antithrombin III (ATIII) were obtained from Enyzme Research Laboratories. Plasma poor ACD plasma was prepared from freshly drawn human blood (~25 donors), aliquoted and stored at −70 °C before use. Toluene was dried by refluxing over Na prior to distillation and MeOH was dried by refluxing over Mg and was distilled before use.

2.2. Elastomer preparation and modification

Sylgard silicone elastomers were prepared according to the procedure provided by Dow Corning. After curing, the silicone elastomer films were punched into disks, approximately 5 mm in diameter and 0.5 mm thick. The disks were washed with hexane and dried under vacuum prior to further modification.

Silicone elastomers were functionalized with Si–H groups under acidic conditions in the presence of $(MeHSiO)_n$ (DC1107) and methanol as described previously [18]. The Si–H modified silicone surfaces were incubated in a solution of 2-methoxyethyl ether as solvent and α-allyl-ω-*N*-succinimidyl carbonate-poly(ethylene glycol) (80:20 wt%:wt%, 3 mL). Pt-catalyst (platinum-divinyltetramethyldisiloxane complex, 1 drop) was added and the mixture was stirred for 15 h at room temperature. Following modification, the PEG modified surfaces were washed thoroughly with dry acetone and dried under vacuum.

The *N*-succinimidyl carbonate PEG (NSC-PEG) grafted surfaces were immersed in phosphate buffered saline (PBS, pH 8.0) containing the heparin (10 mg/mL) for 6 h. Control surfaces were prepared by modification with allyl-PEG-methoxy. Prior to further testing, surfaces were rinsed three times with PBS for 10 min each (30 min total) and dried under vacuum.

2.3. Surface characterization

2.3.1. Heparin density

Heparin density on the modified surfaces was determined as previously described [20,21]. Briefly, a series of heparin standard solutions (0–20 μg/mL) were prepared. Toluidine blue (0.0005%, 1.0 mL) solution and either 0.1 mL of the heparin standard or the heparin modified surface were placed in a centrifuge tube and mixed for 30 s. To this, 1 mL of *n*-hexane was added and the mixture separated into two layers. After the organic layer was removed, the absorbance of the aqueous layer at 631 nm was measured by UV spectrophotometry. Heparin density on the polymer surface was determined by comparison with standard results and was expressed by mass per unit surface area (μg/cm^2).

2.3.2. Water contact angle

Advancing and receding sessile drop water contact angles were measured using a Ramé Hart NRL C.A goniometer (Mountain Lakes, NJ) using Milli-Q water (18 MΩ/cm) with a drop volume of approximately 0.02 mL. Contact angles were also measured using the captive bubble method. Results are presented as the average of at least six measurements on three different surfaces.

2.3.3. X-ray photoelectron spectroscopy (XPS)

XPS was performed at Surface Interface Ontario, University of Toronto, using a Leybold Max 200 X-ray photoelectron spectrometer with a MgK-α non-monochromatic X-ray source. The spot size used in all cases was 2 × 4 mm. Survey scans were performed from 0–10,000 eV. Both low resolution and C1s high-resolution analyses, with a scan width of 20 eV were performed. The raw data were analyzed and quantified using the Specslab software (specs Gmbh, Berlin).

2.4. Protein adsorption from plasma

Fibrinogen was labeled with ^{125}I (ICN Pharmaceuticals, Irvine CA) using the ICl method [22], and passed through an