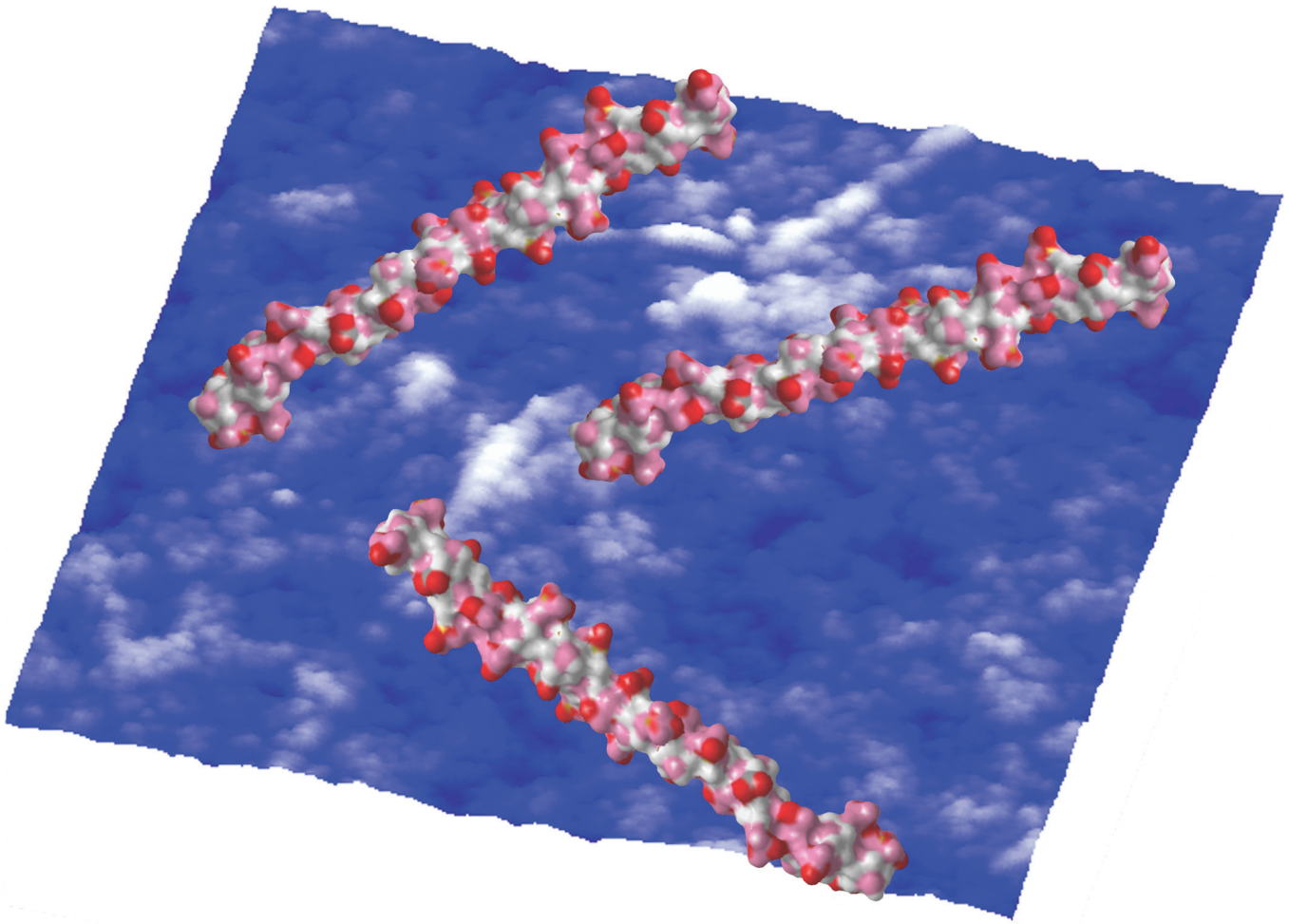

Highlighting research results from Linnaeus University (Kalmar, Sweden), Uppsala University, (Uppsala, Sweden), the University of Nottingham (Nottingham, UK), the University of Portsmouth (Portsmouth, UK), and the University of Stuttgart (Stuttgart, Germany).

Heparin molecularly imprinted surfaces for the attenuation of complement activation in blood

Polymer films molecularly imprinted with surface-immobilized heparin have been developed and investigated using goniometry, AFM, and XPS. The capacity of the polymer films to suppress activation of the complement cascade was established through a series of studies using whole (human) blood.

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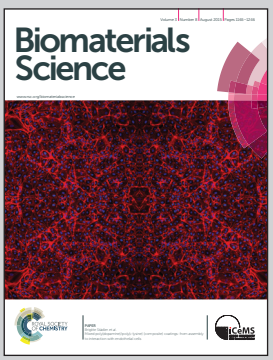

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Heparin molecularly imprinted surfaces for the attenuation of complement activation in blood

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Heparin-imprinted synthetic polymer surfaces with the ability to attenuate activation of both the complement and the coagulation system in whole blood were successfully produced. Imprinting was achieved using a template coated with heparin, a highly sulfated glycosaminoglycan known for its anticoagulant properties. The *N*,*N*′-diacryloylpiperazine—methacrylic acid copolymers were characterized using goniometry, AFM and XPS. The influence of the molecular imprinting process on morphology and template rebinding was demonstrated by radioligand binding assays. Surface hemocompatibility was evaluated using human whole blood without anticoagulants followed by measurement of complement activation markers C3a and sC5b-9 and platelet consumption as a surrogate coagulation activation marker. The observed low thrombogenicity of this copolymer combined with the attenuation of complement activation induced by the molecular imprint offer potential for the development of self-regulating surfaces with important potential clinical applications. We propose a mechanism for the observed phenomena based upon the recruitment of endogenous sulfated glycosaminoglycans with heparin-like activities.

1. Introduction

Control of blood activation upon interaction with foreign materials is the central issue in the attenuation of patient response to implantation or to a process, *e.g.* dialysis or a cardio-pulmonary bypass, which involves direct contact between the defense systems of the blood and structures placed in an internal human biological environment.[1]

The initial event that takes place when blood comes into contact with a foreign object involves rapid adsorption of proteins to the material surface.[2–4] This, in turn, triggers platelet adhesion and activation of the coagulation and complement systems.[5,6] The ability to design a material that does not provoke an adverse host response, and thus possesses good biocompatibility, is important on account of the need for vast number of devices for interaction with blood in clinical medicine.

The primary function of the complement system is to defend an organism against pathogens. Complement is activated when host recognition molecules encounter non-self structures, *e.g.* microbial carbohydrates or artificial (bio) materials. This leads to activation of the central component, C3, by proteolytic enzyme complexes, convertases, into the anaphylatoxin C3a that, together with C5a, activates and recruits phagocytes, and C3b that binds to target surfaces and promotes phagocytosis.[7] At host surfaces this system is under delicate regulation at multiple steps in order to protect autologous cells from complement-mediated damage. Surfaces of non-self origin lack these mechanisms resulting in inflammatory reactions that in the case of medical device with large surface areas could be systemic without administration of soluble inhibitors, *e.g.* heparin.

The main event in the coagulation cascade is the enzymatic activation of prothrombin to thrombin by factor Xa in complex with factor Va. Thrombin is a potent activator of platelets and

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cleaves fibrinogen to fibrin. The main coagulation inhibitor is the serine protease inhibitor antithrombin (AT), which inactivates many of the enzymes in the cascade, the major targets being thrombin and factor Xa. In contrast to endogenous surfaces, synthetic surfaces do not possess coagulation inhibitors resulting in an increased risk for thrombosis. Heparin coated surfaces overcome this mainly through adsorption of the coagulation inhibitor AT, thus enhancing its activity.[8] However, the complexity of the coagulation cascade, and the diversity of roles that heparin itself plays in the body, mean that heparin coated surfaces might have unexpected *in vivo* effects.[9,10] Consequently, surfaces with 'heparin-like' functionality, or surfaces that can recruit heparin or other sulfated carbohydrates are of considerable interest. We have been developing an alternative strategy for controlling the activating properties of a surface by creating materials designed to exhibit specific binding but overall moderate affinities for a regulatory target molecule such as heparin or other sulfated carbohydrates. In principle, surfaces capable of selectively recruiting a patient's endogenous glycosaminoglycans (GAGs) or administered heparin would provide a self-regulating biomaterial.

One strategy for preparing such materials is molecular imprinting,[11,12] a means to produce synthetic polymers with antibody-like recognition behavior.[13] The technique utilizes a molecular template to direct the three dimensional arrangement of polymerizable structural building blocks (monomers) through complex formation.[14] Stabilization of the complexes through polymerization and subsequent removal of the template reveals a surface capable of selectively rebinding the template molecular structures or analogues thereof. Generally, biomacromolecular templates present challenges to the technique due to the difficulties associated with the release of large template structures from a three dimensional polymer matrix and the necessity for working in aqueous media.[15] It was envisaged that the use of a water compatible polymerization system,[16] in conjunction with a surface based imprinting format could address these challenges.[17–19] In particular, we reasoned that utilizing heparin as a template would produce materials with self-regulating characteristics through recognition of endogenous heparin-like substances. Recent reports of efforts to develop heparin-selective electrochemical sensors using molecular imprinting provided further support for the potential of this strategy.[20–22]

2. Materials and methods

2.1 Preparation of template surfaces

Glass microscope slides (26 × 76 mm) were immersed overnight in solutions of H_2SO_4 (aq, 0.1 M) and then rinsed sequentially with water and acetone before treatment (45 min, 37 °C) with a solution of (3-aminopropyl)trimethoxysilane (APTMS, Fluka, >97%) in acetone (5%, v/v). The slides were rinsed with acetone and then water before treatment (1 h, 22 °C) with an aqueous solution (pH 5.2 adjusted with H_2SO_4) of heparin (Bioiberica, Barcelona, Spain, mean M_w 12 kDa, 110 IU mg^{-1}, 7.5%, w/v) containing glutardialdehyde (GA, Merck, 3%, v/v). After incubation, the slides were rinsed with water, dried and stored at 8 °C until further use.

2.2 Quantification of heparin bound to template surface

The surface concentration of heparin was determined by Corline Systems AB, Uppsala, Sweden using toluidine blue staining. The AT binding capacity of the immobilized heparin, with a Corline™ Heparin Surface (CHS) as reference, was determined by a colorimetric assay based on the fact that AT in complex with heparin inhibits coagulation factor FXa, performed as described.[23] Briefly, the surfaces were incubated with AT, then washed and incubated with FXa (both from Enzyme Research Laboratory Inc., South Bend, IN, USA). The remaining FXa in solution was determined using a chromogenic substrate specific for FXa (S-2222, Chromogenix, Milano, Italy), thus giving an indirect estimate of the amount of bound AT.

2.3 Stability of immobilized heparin surfaces

Template surfaces and CHS references (coated on glass) were incubated with citrate plasma in the chamber model described below. After incubation, detection was carried out by incubating aliquots of plasma with AT. Thereafter, FXa was added and the amount of active FXa (*i.e.* that had not been inhibited by the complexes formed between AT and the heparin that had detached from the surfaces) was determined as described above.

2.4 Synthesis of heparin-imprinted polymer surfaces

Microscope slides were cleaned (5 min, 80 °C) in a series of washing solutions containing 4% NH_3 (aq), 5% H_2O_2 (aq), 4.5% HCl (aq) and 3.8% H_2O_2 (aq), then carefully rinsed with water, acetone, dry tetrahydrofuran and dry toluene and were immediately transferred into a solution comprised of (3-methacryloxypropyl)trimethoxysilane (3 mL, Sigma), triethylamine (0.3 mL) and in dry toluene (150 mL). After 24 h at 22 °C the surfaces were washed with dry toluene, dry tetrahydrofuran and dry acetone, and dried.

For a batch of five polymer films, diacryloylpiperazine (288 mg, Fluka, >99%) was dissolved in water (400 μL) by sonication, and 4.6 μL methacrylic acid (MAA, Merck) was added. The solution was sparged with nitrogen, 40 μL 33% (w/v) ammonium persulfate (Sigma) and TEMED (4 μL, 30%, v/v) was added and 120 μL of the solution was spread between the two surfaces. The monomerized and template surfaces were separated using spacers (140 μm) cut from teflon membrane filters (Pall Corp., MI, USA). After polymerization (16 h), the surfaces were carefully separated by treatment with an aqueous ammonia solution (5%, w/v), followed by sequential washes with this solution and then with aqueous 4 M Guanidine HCl. The resultant polymers were inspected for completeness of surface coverage, absence of scratches and air bubbles using a light microscope. Reference polymers were synthesized as described above, but instead of a heparinized glass

template, a clean glass surface was used. The polymer films were stored in 20% ethanol at 8 °C for up to 6 months.

2.5 Heparin adsorption/desorption studies

Polymer surfaces were equilibrated in 20 mM phosphate buffer, pH 7.5 (30 min) before PMMA masks (26 × 76 × 10 mm containing 8 wells with i.d. 6 mm) were mounted over the polymer slides. The wells were incubated with ^{3}H-heparin (American Radiolabeled Chemicals Inc., St Louis, MO, USA; 0.4 mCi mg^{-1}, M_w 6–20 kDa) solutions (100 μL, 1 h, 22 °C) in the concentration range 0.75 to 75 μg mL^{-1} diluted in phosphate buffer. Aliquots (50 μL) were removed and mixed with scintillation cocktail (Beckman Ready Safe™, Sweden, 2 mL) and counted in a liquid scintillation counter (Beckman LS 6000, Sweden). The values were normalized to avoid day-to-day variability and the difference between reference and imprinted polymers was determined. Seven individual experiments were performed (n = 3 to 12 wells). Studies were also performed with the competing ligand chondroitin sulfate A from bovine trachea (Sigma-Aldrich, St Louis, MO, USA), 75 μg mL^{-1} or heparin (Bioiberica) in phosphate buffer, were incubated together with 7.5 μg mL^{-1} of ^{3}H-heparin (*i.e.* the concentration where selective binding was seen for the MIP compared to the reference, see section 3.4).

In separate experiments, polymer surfaces (imprinted and reference) were incubated for 45 min at 22 °C with sodium heparin (Bioiberica, 8 mg mL^{-1} in 0.9% NaCl) and washed for 15 min with 0.9% NaCl (aq). The degree of desorption of heparin from these surfaces after incubation in plasma was studied as described above for immobilized heparin surfaces.

2.6 Heparin treatment of equipment and preparation of blood

All equipment used in contact with blood (the slide chamber, tubes, pipette tips, *etc.*) was furnished with a CHS according to the manufacturer's recommendations. The surface concentration of heparin was 0.5 μg cm^{-2}, with an AT binding capacity of 2–4 pmol cm^{-2}.[24] Whole blood (25 mL) from healthy volunteers who had received no medication for 10 days was collected in 50 mL Falcon® tubes (Becton Dickinson, San José, CA, USA) without the addition of soluble heparin, or other anticoagulants, and was used within 5 min in the slide chamber model described below. Blood was drawn from healthy blood donors who had received no medication for at least 10 days. The study was performed with the consent of the Ethical Committee of the University of Linköping, Sweden (#03-520).

2.7 Test surface (slide) chambers

The effect on coagulation and complement activation (below) as well as the leakage of heparin from different surfaces (above) was determined using a slide chamber model that consists of a polymethylmethacrylate (PMMA) microscope slide-sized two-well tray (well-volume 1.65 mL), each of which were filled with 1.3 mL of blood (activation studies) or citrate plasma (heparin release).[25] Thereafter, the test surfaces (template, reference and imprinted polymers, CHS or glass) were attached as a lid with clips, creating two circular chambers. The device was rotated for 60 min vertically at 22 rpm in a 37 °C water bath. Reference glass surfaces were cleaned in 5% (w/v) ammonium persulfate at 60 °C for 60 min and rinsed in MilliQ water prior to use.

2.8 Incubation of surfaces with blood

Imprint, reference and glass surfaces, incubated with heparin as above, or with 0.9% NaCl only, and heparin surfaces (template and CHS coated onto glass) were exposed to whole blood (without added anticoagulants) in the chamber model as described above. After incubation, EDTA was added to a final concentration of 10 mM, to terminate further activation of complement and coagulation. The experiment was performed seven times (four different donors) with duplicate analyses with similar results.

2.9 Platelet consumption

The platelets remaining in the blood samples were counted using a Coulter A^{c}T diff™ hematology analyzer (Coulter Corporation, Miami, FL, USA). The blood was centrifuged (10 min, 2200*g*, 4 °C) within 30 min. and the plasma stored at −70 °C.

2.10 Complement activation

Complement activation was monitored as the generation of C3a and sC5b-9 complexes in plasma by enzyme immunoassays (EIAs) as described previously.[26] In the C3a assay the monoclonal antibody 4SD17.3 was used for capture and biotinylated polyclonal rabbit anti-C3a and HRP-conjugated streptavidin (Amersham, Little Chalfont, UK) for detection.[26] In the sC5b-9 assay, anti neoC9 monoclonal antibody aE11 (Diatec AS, Oslo, Norway) was used for capture and polyclonal rabbit anti-C5 antibodies and HRP-conjugated anti-rabbit immunoglobulin (Dako A/S, Glostrup, Denmark) for detection.[27] Zymosan-activated serum served as standard and values are given as ng mL^{-1} (C3a) or arbitrary units per ml (AU mL^{-1}; sC5b-9).

2.11 Physical characterization of polymer surfaces

Surface free energies of the polymer surfaces were determined by contact angle goniometry performed with a Krüss G10 goniometer (Krüss Optronic, Hamburg, Germany) fitted with an enclosed thermostated cell and interfaced to image-capture software (Drop Shape Analysis 1.00.14.0, Krüss Optronic, Hamburg, Germany). For advancing (θ_a) contact angle experiments, measurements of air bubbles (2–8 μL) using the captive bubble technique were recorded at 20 °C using either doubly distilled water, diiodomethane (Fluka, >98%) or ethylene glycol (Sigma, >99%). Surfaces were equilibrated (2 × 50 min) in the solvent before contact angle experiment was performed and measurements were carried out with 4–9 bubbles on a minimum of two independently prepared surfaces to obtain an average contact value.

Images of reference and imprinted polymers were acquired using an atomic force microscope (AFM, TopoMetrix TMX2000

Discoverer Scanning Probe Microscope, ThermoMicroscopes, UK) with a 70 × 70 × 12 μm^3 tripod piezoelectric scanner. Topography measurements were conducted using "V" shaped silicon nitride cantilevers each bearing an integrated standard profile tip (length 200 μm, nominal spring constant (K) 0.032 N m^{-1}; Part. no. 1520-00, ThermoMicroscopes, Santa Clara, CA, USA). Topographic imaging was performed in air and aqueous buffer using a closed wet cell, modified to allow variable temperature adjustment. Contact mode imaging utilized an applied load and scan rate limited to *ca.* 1 nN and 3 Hz, respectively, to minimize compression and lateral damage to polymer grafts and underlying surfaces.

2.12 Statistical treatments

Statistical significance was calculated with Student's *t*-test using GraphPad Prism® 3.02 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean values ± SEM. For blood-based studies one-way ANOVA was employed followed by Bonferroni's multiple comparison test.

3. Results

3.1 Preparation of template surfaces & quantification of heparin bound to template surface

The surface concentration of heparin on the template surface was shown by toluidine staining to be 0.14 ± 0.01 μg cm^{-2} (12 ± 1 pmol cm^{-2}) (n = 7). The AT binding capacity of the template surface was determined to be 4.4 ± 0.2 pmol cm^{-2} compared to the CHS 2.8 ± 0.3 pmol cm^{-2}.

3.2 Stability of heparin bound to template surface

The stability of the immobilized heparin was demonstrated by determining the presence of active heparin-AT complex, after incubation of the template surface with citrated plasma. The amount of heparin released into the plasma from a surface area was determined to be 8.8 ± 1.5 pmol cm^{-2} (n = 7).

3.3 Synthesis of heparin-imprinted polymer surfaces

Heparin-imprinted and reference polymers were synthesized according to the strategy summarized in Fig. 1. Although readily obtained, the yield of surfaces of suitable quality (size) for use in studies varied between the heparin-imprinted (40%) and glass reference systems (75%). The polymers were brittle in the dry state, which necessitated their storage in aqueous solution. X-ray photoelectron spectroscopy studies (XPS, Table 1) confirmed the successful elution of template from the surface, as reflected in an S-content of <10 pmol cm^{-2}.

3.4 Polymer-heparin binding

Polymer binding characteristics were studied using a radioligand binding assay with ^{3}H-heparin (M_w 6–20 kDa) in the concentration range from 0.75 to 75 μg mL^{-1}. The imprinted polymer showed higher affinity for heparin relative to the reference polymer over a narrow concentration interval. The difference between reference and imprinted polymers, the selective binding 23 ± 19 pmol cm^{-2}, was statistically significant at a heparin concentration of 7.5 μg mL^{-1} (t-test with unequal variances, p = 0.009), where the imprinted and reference surfaces bound 340 and 317 pmol cm^{-2}, respectively. The presence of chondroitin sulfate (CS) (75 μg mL^{-1}) resulted in a 33 ± 6% reduction in heparin binding to the imprinted polymer surface, while the same concentration of heparin resulted in a reduction of 39 ± 13% (n = 3). More detailed studies using higher concentrations were not possible due to saturation of the surface, and the use of lower concentrations was limited by the activity of the radioligand.

3.5 Release of heparin from heparin incubated imprinted and reference polymer surfaces

The polymer surfaces were tested for their ability to release heparin into plasma after saturation with heparin. Following incubation with 8 mg mL^{-1} heparin, ≈1000-fold more than used for the radioligand binding studies, followed by rinsing, the surfaces were then incubated with plasma. The plasma was analyzed for heparin content as above. This experiment showed that the amount of heparin released from the imprinted polymer, 0.036 ± 0.008 IU mL^{-1} (18 ± 4 pmol cm^{-2}), was lower than for the reference polymer, 0.060 ± 0.020 IU mL^{-1} (31 ± 10 pmol cm^{-2}) (n = 7).

3.6 Surface contact induced platelet consumption

Imprinted and control polymer surfaces as well as unmodified glass surfaces, after saturation with soluble heparin followed by incubation with 0.9% NaCl or incubated with NaCl only, were tested in the blood chamber model. The platelet loss was significantly lower for all polymer surfaces compared to the unmodified glass (Fig. 2A). There were no significant differences between heparin-imprinted or control polymer surfaces in the platelet loss results. Preincubation of the heparin-imprinted surface with soluble heparin did not affect the decline in platelet counts (44 ± 11%). All platelets were consumed after contact with glass, regardless of whether or not the glass had been preincubated with soluble heparin.

3.7 Complement activation upon contact between imprinted surfaces and whole blood

The activation of complement by glass is significant, and provides a meaningful reference for comparison with treated glass surfaces.[28] The diacryloylpiperazine-methacrylic acid copolymer used in this study was found to be as potent as unmodified glass with respect to complement activation. This high complement activating capacity was efficiently overcome by the heparin imprinting process (Fig. 2B and C). Neither the levels of generated C3a nor of sC5b-9 complexes were affected by preincubation with soluble heparin. In absolute numbers, 635 ± 75 ng mL^{-1} of C3a was generated in blood after contact with imprinted surfaces without heparin contact, and 740 ± 155 ng mL^{-1} with heparin imprints that had been preincubated with heparin (Fig. 2B). The corresponding values for reference polymers are 1355 ± 195 ng mL^{-1}, and 1370 ± 90 ng mL^{-1}, respectively. Levels of 1155 ± 205 ng mL^{-1} were detected in samples

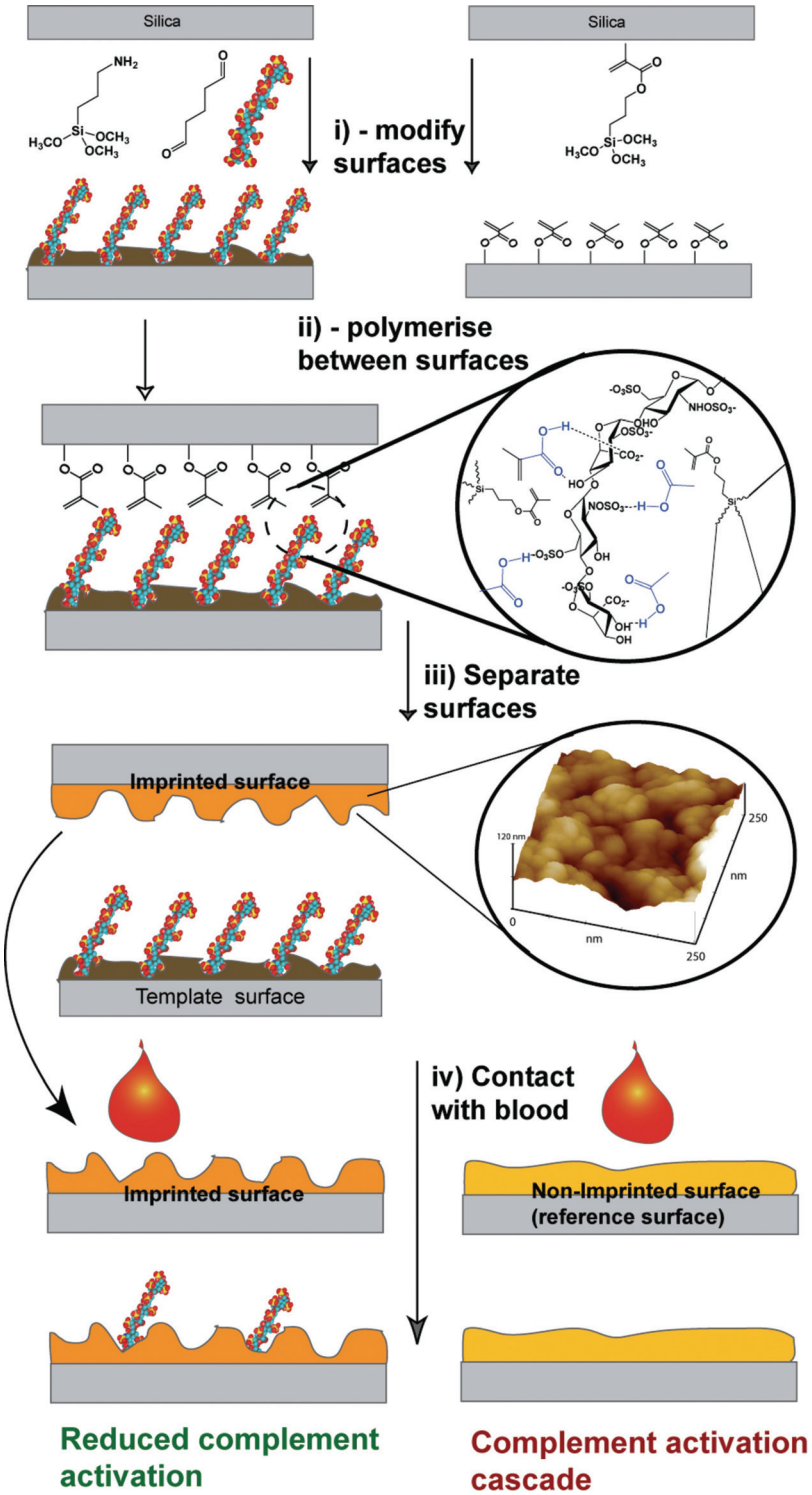

Fig. 1 Schematic representation of the surface imprinting process. Silica glass slides are reacted with 3-aminopropyltrimethoxysilane (APTMS) and glutardialdehyde in the presence of heparin to generate the template surface. A polymerizable layer is formed on a second slide by treatment with 3-methacryloxypropyl)trimethoxysilane (i). Polymerisation of a solution containing methacrylic acid (MAA) and diacryloylpiperidine (DAP) is carried out between the glass slides held at a defined distance (140 μm) by the use of spacers to generate an imprinted surface (ii). Non-imprinted (reference) surfaces are prepared using MAA and DAP but with a non-templated surface. Separation of the upper and lower slides and extensive washing exposes the imprinted surface (iii, AFM shown in inset) with a 'memory' for heparin. Exposure of imprinted surfaces to blood leads to lower complement activation than occurs for the reference surfaces (iv).

Table 1 XPS analysis – elemental composition of polymers[a]

	Carbon	Nitrogen	Oxygen	Sulfur	Total (%)
Found (non-imprinted)	69.7 ± 0.3	9.0 ± 0.1	20.5 ± 0.1	0.10 ± 0.1	99.2
Found (imprinted)	68.4 ± 0.2	8.7 ± 0.1	22.1 ± 0.1	0.18 ± 0.2	99.2

[a] Performed at Fraunhofer Institute for Interfacial Engineering and Biotechnology.

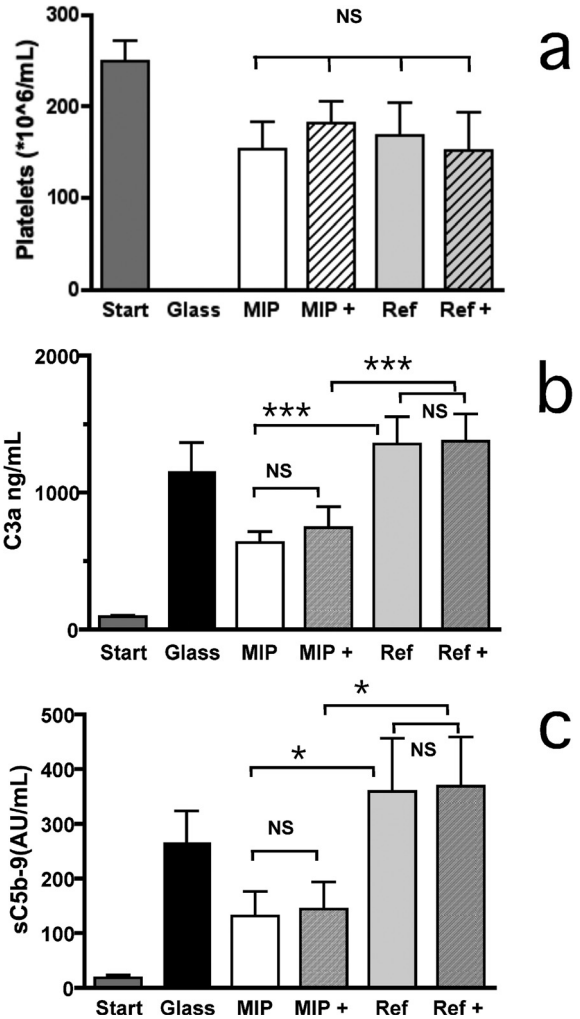

Fig. 2 Platelet counts and detection of complement activation products C3a and sC5b-9 complexes after contact between whole blood and imprint and control surfaces. The tested surfaces consisted of glass, heparin-imprinted surfaces before (MIP) or after (MIP+) saturation with soluble heparin, and reference polymers before (Ref) or after (Ref+) saturation with heparin. Panel a: platelet count after incubation of blood without anticoagulant additives for 1 h in the blood chamber model. Complement activation was monitored as the generation of C3a (Panel b) and sC5b-9 complexes (Panel c). Data are presented as means ± SEM Panel A five experiments, and Panels B and C seven experiments, each performed in duplicate, using blood from four different donors (*p < 0.05, ***p < 0.001, NS = not significant).

that had been in contact with glass, and no effect was seen upon preincubation with heparin (data not shown). Similar observations but with even greater differences between heparin-imprinted and reference polymer surfaces were seen when the end product of the complement cascade, sC5b-9, was measured (Fig. 2C). The heparin-imprinted surfaces induced the formation of 130 ± 45 AU of sC5b-9 per mL with heparin saturation and 140 ± 50 AU mL^{-1} without. Corresponding values for the polymer reference were 360 ± 95 AU mL^{-1} and 365 ± 90 AU mL^{-1}, respectively. The values detected after contact with glass were 265 ± 60 AU mL^{-1} and did not change upon preincubation with soluble heparin (data not shown).

3.8 Physical characterization of the polymer

Atomic Force Microscopy (AFM) imaging of the polymer films was conducted in water using contact mode to establish eventual differences in topography and/or roughness, Fig. 3.

Imprinted and reference polymer surface energies were determined by contact angle goniometry using the captive bubble technique.[29] The contact angles (water, diiodomethane and ethylene glycol) and surface free energy components were determined, Table 2. Advancing water contact angles, θ_w, for the surfaces were determined to be 35.7 ± 0.6° and 34.4 ± 0.9°, respectively.

4. Discussion

Full blood compatibility requires that the surface in contact activates neither coagulation nor complement cascades. While heparinization, which is always optimized for minimizing coagulation rather than complement activation,[30] has been used for decades to produce surfaces with minimal activating character, alternatives not relying on the use of substances of biological origin are most desirable. We envisaged that a surface capable of recruiting exogenous heparin (or endogenous analogues such as chondroitin sulfate (CS) or serglycin) from a patients' own blood during an extra-corporal procedure such as during coronary-pulmonary bypass surgery should have the possibility to be self-regulating thus diminishing the risk of thrombosis.

Molecular imprinting[11,12] was perceived as a means to produce surfaces with such self-regulatory function. However, to achieve imprinted polymer surfaces capable of recognizing heparin in its biologically active conformation necessitates a polymerization system amenable to use in aqueous media and capable of supporting monomer–template interactions while not compromising the three dimensional structure of the macromolecular template. Polymers comprised of diacryloylpiperazine

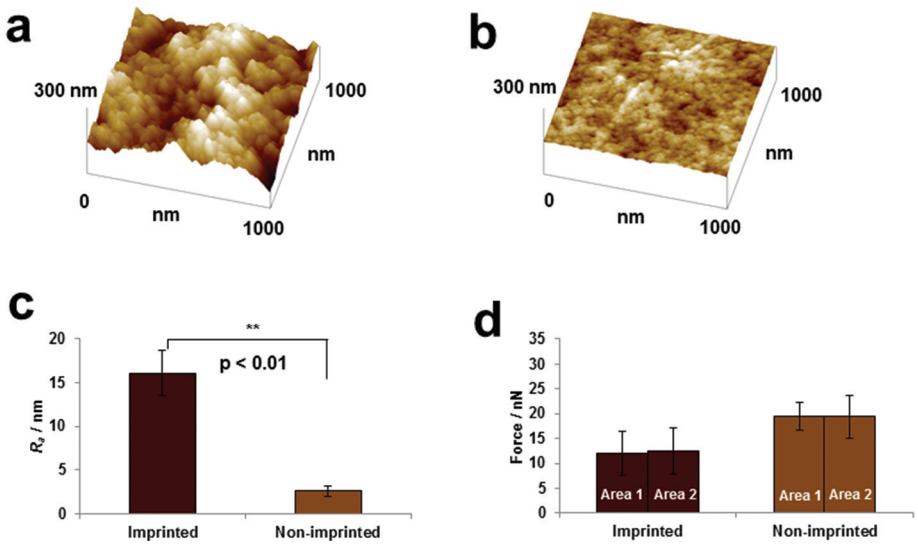

Fig. 3 Atomic force microscopy of imprinted (a) and non-imprinted (b) surfaces. Topographic imaging was performed in air and aqueous buffer using a closed wet cell. Contact mode imaging utilised an applied load and scan rate limited to *ca.* 1 nN and 3 Hz, respectively, to minimize compression and lateral damage to polymer grafts and underlying surfaces. Surface roughness data (c) shows significantly higher roughness (*t*-test, unequal variances) for the imprinted surface than for the control. Adhesion forces (d) of Si_3N_4 tip-to-surface for both imprinted and non-imprinted surfaces were significantly lower ($p < 0.001$) than for glass and mica (data not shown), but there was no significant adhesion force difference between the imprinted and non-imprinted surfaces ($p > 0.05$).

Table 2 Advancing contact angles of water, diiodomethane (DIM) and ethylene glycol (EG) on polymer surfaces and surface free energies of these systems

	Contact angle, θ_a [a] (°)			Surface energy[b] (mJ m^{-2})			
Polymer	H_2O	DIM	EG	γ_S^{LW}	γ_S^+	γ_S^-	γ_S^{tot}
Ref	36 ± 3	52 ± 2	35 ± 2	33 ± 1	0.1 ± 0	54 ± 3	39 ± 1
MIP	34 ± 5	61 ± 2	42 ± 2	28 ± 1	0.1 ± 0	63 ± 4	34 ± 1

[a] Each contact angle value is the mean of at least four bubbles on a minimum of two independently prepared polymer samples. [b] Differences between γ_S^{LW}, γ_S^- and γ_S^{tot} were all statistically significant ($p < 0.05$) as determined by *t*-test (two-sample assuming unequal variances).

and methacrylic acid have previously been demonstrated to be suitable for imprinting in aqueous media.[16] However, the physical size of a macromolecular structure such as heparin prohibits its use in conjunction with established imprinting strategies (bulk polymerization) on account of the difficulties associated with the removal of the template from the polymer. To avoid this problem, and in order to have a material to which blood proteins have access a surface imprinting strategy was adopted, Fig. 1. Whitcombe and colleagues have described the preparation of quasi-2-dimensional surface imprints (a textured 2-D polymer surface) of inorganic materials,[17] and a related approach has been adopted by the groups of Ratner[19] and Shea[18] for proteins and peptides. Heparin-derivatized template surfaces were prepared with a surface concentration of 12 ± 1 pmol cm^{-2}. The theoretical surface loading (close packing) of heparin (mean M_w 12 kDa) was estimated to 7 pmol cm^{-2} based on the heparin structure determined from NMR studies by Mulloy *et al.*[31] and the assumptions that all heparin molecules lie flat on the surface and that charge repulsion does not influence packing density. A higher packing density indicates that portions of the heparin structure are free in solution, *i.e.* that on average only a portion of each heparin molecule is physically bound to the derivatized surface, a process driven by repulsive coulombic interactions.

The conformation of immobilized heparin is critical, *i.e.* that the active pentasaccharide is exposed and can bind AT. The quality of the template surface was therefore determined by the efficiency of which AT bound to the surface bound heparin (4.4 ± 0.2 pmol cm^{-2}), and was found to be comparable to that of commercial heparin surfaces (2.8 ± 0.3 pmol cm^{-2}). The stability of the immobilized heparin upon contact with plasma was reflected in the very low level of soluble heparin-AT complex, 8.8 ± 1.5 pmol cm^{-2}. This value, close to the detection limit of the assay, indicates that the template surface is as stable as the reference CHS, *i.e.* with no leakage of heparin into the blood.[32]

Imprinted and reference polymer surfaces were synthesized following the strategy depicted in Fig. 1. Importantly, imprinted surfaces were shown by XPS to have no significant concentration of residual heparin. Radioligand binding studies revealed that the imprinted polymer surfaces had greater affinity for heparin, a function that could be attenuated by the presence of the endogenous GAG CS.

These studies using whole blood demonstrate that the influence of the heparin template on the polymer resulted in a surface capable of attenuating complement activation, as determined through studying two separate points in this

cascade system. Furthermore, as pre-incubation of surfaces with soluble heparin did not influence complement activation, it must be concluded that the specific characteristics of the heparin molecularly imprinted polymer surface are directly responsible for the biological activity of the surface. In studies using surfaces pre-incubated with heparin, the amount of heparin released from the imprinted polymer, 0.036 ± 0.008 IU mL^{-1}, was lower than for the reference polymer, 0.060 ± 0.020 IU mL^{-1}, indicating that heparin is bound more strongly to the imprinted surface than to the reference. This was supported by the results of XPS studies. This concentration can be compared to the patient blood concentration normally employed during bypass surgery (up to 3 IU mL^{-1}).[33] Moreover, since the amount of heparin released from the surfaces is very low, the effect seen on C3a and sC5b-9 measurements cannot be a result of heparin leaking since no effect on complement activation is seen at concentrations of heparin below 0.5 IU mL^{-1}.[34] Importantly, the diacryloylpiperazine-methacrylic acid co-polymer has an inherently low thrombogenicity, which when coupled with the relatively low complement activating character induced by the molecular imprinting process renders a material with enhanced hemocompatibility.

Unraveling the mechanisms underlying the unique behavior of the imprinted polymer films when in contact with blood is challenging. As heparin is not an endogenous component of blood an important question arises, namely, to what component(s) present in blood can we attribute the observed differences in the hemocompatibility of the two surfaces? The main proteoglycan present in all blood cells consists of the protein serglycine, decorated with multiple CS chains.[35] The concentration of CS in plasma has been demonstrated to increase by 4 μg mL^{-1} upon platelet activation.[28] We hypothesize that the interaction of the polymers with this complex endogenous proteo-GAG provides the basis for the differences in degree of complement activation of the two surfaces. This is supported by the capacity of CS to attenuate the binding of radiolabeled heparin to the imprinted polymer surface. Indeed, incubation of imprinted surfaces with radiolabeled heparin in the presence or absence of CS demonstrated CS recognition by the imprint surface. Moreover, we propose a role for the glycosaminoglycan serglycin in the observed compatibility of the molecularly imprinted surfaces with the complement cascade. Yoshimi *et al.*[20] have reported the synthesis of bulk imprinted (2-(methacryloxy)-ethyl)trimethylammonium chloride acrylamide–acrylamide–*N*,*N*′-methylenebisacrylamide copolymer films for electrochemical detection of heparin over the concentration range 0.05–0.5 μg mL^{-1}. In the present study we have been able to probe concentration ranges down to the pM domain through the bioassays deployed here. The reported presence of higher sulfur-levels, *i.e.* residual heparin, on the polymer coated electrodes makes direct comparisons with the surface template materials reported in the present study difficult. This was reported to arise from the covalent immobilization or network-entrapment of the template, factors motivating the use of surface templating strategies. However, the selectivity for heparin, or lack of selectivity for CS, they reported is in contrast to the suppression of heparin binding by CS seen when working with the polymer systems used here. We attribute this to the significantly greater number of charged sulphated residues in heparin relative to CS that results in a significant difference in the numbers of strong ion-pairing interactions with the quaternary ammonium ion between the ligands and polymer-coated electrode, though even morphological features could contribute. A similar heparin detection range was reported by Li *et al.*[21] using a methacrylic acid–ethyleneglycol dimethacrylate copolymer, again prepared by bulk polymerization rather than the surface templating approach used here.

In view of the known activation of the coagulation cascade *via* contact of blood with synthetic surfaces,[36] surfaces were investigated using AFM in order to establish if there were any marked differences in topography and/or roughness of imprinted surfaces compared to controls, Fig. 3. Importantly, there were no significant differences in the overall topographies at the micron scale and both polymer films displayed features corresponding to interconnected agglomerates (~50–75 nm in diameter), spaced evenly across the surface. These agglomerates were indicative of the macroporous polymer structure formed by cross-linking in a porogenic solvent. Separation of the polymer aggregates was <10 nm. Further analysis indicated a marginally increased overall roughness in the case of the heparin-imprinted polymer surface, than in the case of the reference polymer, with a difference in height over the areas studied of ±1 nm for the imprinted surfaces than for the control polymer. Generally speaking, complement activation is proportional to blood accessible surface area. In this case, the imprinted polymer surface was less complement activating than the reference, as reflected by the observed levels of sC5b-9 and C3a, despite a greater apparent surface area. Together with the platelet consumption and complement activation studies, this data provides further support for the role of the heparin template induced influence on polymer structure on the behavior of the imprinted polymer when in contact with blood.

In an attempt to determine the influence of the template surface on the corresponding polymer at a higher level of chemical detail, the surface free energies of reference and imprinted materials were determined by contact angle goniometry using the captive bubble technique.[29] The contact angles for water, diiodomethane and ethylene glycol as well as the surface free energy components were determined, Table 2. The advancing water contact angle, θ_w, for the reference and imprinted surfaces indicated that both surfaces were similar with respect to hydrophilic character. The hydrophilic nature of the material itself may explain the strong complement activation seen when presented to blood in the absence of a heparin surface imprint.[37]

Examination of the Lewis base and Lifschitz van der Waals components revealed noticeable differences between reference and imprinted polymers, suggesting a higher electron pair density at the imprinted surface. This may be explained by an

enrichment of methacrylic acid residues (reported as carboxylates during contact angle measurements at pH 7) at the imprinted surface through prior binding to polar and hydrogen bonding sites on the heparin chain during the templated polymerization. The methacrylic acid residues were likely to have been non-ionized during the initial imprinting, but would have been converted to carboxylates during contact angle studies in distilled water: the Lewis base content thus reports the *relative* surface content of these residues. Similarly, the localized higher polarity of the imprinted surface was confirmed through the reduced van der Waals' component compared to the non-imprinted material. The low magnitude of the Lewis acid components for both surfaces suggested that residual non-ionized carboxylic acid functionality contributed little to the measured total surface energy during contact angle analysis. The total surface free energies of the polymer films were determined to be 39 mJ m^{-2} for the reference and 34 mJ m^{-2} for the imprinted surface, which are similar to those obtained for other polyacrylamides.[38] The indication of functionalities complementary to the template, and especially their surface enrichment, provides further support for the presence of sites with selectivity for an active form of heparin or similar sulfated polysaccharides, such as serglycine, on the surface of the imprinted polymers.

Collectively, these data provide strong support for the presence of heparin selective sites in the molecularly imprinted polymer film. These results suggest the use of molecularly imprinted polymer films as potential autoregulatory surface coatings for materials to be placed in contact with blood.

5. Conclusions

The capacity to attenuate complement cascade activation with heparin molecularly imprinted surfaces provides the first example of the design and fabrication of synthetic self-regulating surfaces for the attenuation of biological responses in blood. The results presented here offer significant potential for clinical use due to the demonstrated suppression of thrombo-inflammatory processes. The use of surface molecular imprinting protocols in conjunction with polymers compatible with aqueous systems offers further possibilities for the regulation of biological processes with biomimetic materials. The stability of these materials, *e.g.* to autoclave sterilization,[39] offers particular advantages with respect to their use in medical device development. Furthermore, the use of whole blood systems for the evaluation of biomaterials provides a holistic perspective of significance for eventual clinical applications.

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