Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and in vitro evaluation

M.J.B. Wissink\textsuperscript{a}, R. Beernink\textsuperscript{a}, J.S. Pieper\textsuperscript{b}, A.A. Poot\textsuperscript{a}, G.H.M. Engbers\textsuperscript{a}, T. Beugeling\textsuperscript{a}, W.G. van Aken\textsuperscript{a}, J. Feijen\textsuperscript{a,*}

\textsuperscript{a}Institute for Biomedical Technology, Polymer Chemistry and Biomaterials Group, Department of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands
\textsuperscript{b}Department of Biochemistry, Faculty of Medical Sciences, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

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Abstract

In the present study, heparin immobilization to a non-cytotoxic crosslinked collagen substrate for endothelial cell seeding was investigated. Crosslinking of collagen using \textit{N-\((3\text{-dimethylaminopropyl})-N'\text{-ethylcarbodiimide (EDC)}\) and \textit{N-hydroxysuccinimide (NHS)} resulted in a material containing 14 free primary amino groups per 1000 amino acid residues (E/N14C). At a fixed molar ratio NHS : EDC of 0.6, the amount of heparin covalently immobilized to E/N14C increased with increasing molar ratios of EDC to heparin carboxylic acid groups (Hep-COOH), to a maximum of approximately 5−5.5 wt% at a ratio of 2. Upon incubation in cell culture medium of endothelial cells, 4 to 7% of the immobilized heparin was released during 11 days.

Immobilization of increasing amounts of heparin to E/N14C progressively reduced activation of contact activation proteases. Optimal anticoagulant activity, as measured by thrombin inhibition, was obtained after heparin immobilization using a ratio of EDC to Hep-COOH of 0.2−0.4 (14−20 mg heparin immobilized per gram of collagen). Platelets deposited to (heparinized) E/N14C showed only minor spreading and aggregation, although heparin immobilization slightly increased the number of adherent platelets. The results of this study suggest that heparin immobilization to EDC/NHS-crosslinked collagen may improve the in vivo blood compatibility of this material.

Keywords: Vascular grafts; Collagen coating; Heparin immobilization; Contact activation; Thrombin inactivation; Platelet interaction

1. Introduction

Synthetic vascular grafts, made of Dacron or expanded Teflon, are widely used to replace occluded or diseased arteries in man. When used in large-diameter applications, synthetic vascular grafts show satisfactory patency rates. In small-diameter applications (inner diameter less than 5 mm), however, graft performance is disappointing due to stenosis and thrombus formation [1−4].

Endothelial cell seeding is an accepted approach to improve (small-diameter) graft performance. For successful endothelial cell seeding, a suitable substrate is required, which is not provided by Dacron or expanded Teflon grafts [5−7]. Since non-crosslinked collagen is a suitable matrix for the growth of endothelial cells in vitro [8−10], application of a collagen coating on synthetic vascular graft materials may result in a matrix suitable for in vivo endothelial cell seeding.

Collagen-coated vascular grafts have been developed to eliminate the procedure of pre-clotting of porous Dacron prior to implantation. In commercially available collagen-coated vascular grafts, collagen is crosslinked using glutaraldehyde or formaldehyde to reduce the in vivo resorption rate [11]. Both crosslink agents are incorporated into the collagen coating during crosslinking. Especially glutaraldehyde is known to induce cytotoxic reactions by release of (unreacted) glutaraldehyde or glutaraldehyde derivatives during in vitro or in vivo degradation [12−15], thus hampering endothelialization of the graft [13,16].

We have previously developed an alternative collagen coating for synthetic vascular grafts, crosslinked by
N-(3-dimethylaminopropyl)-N' -ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS). These crosslinking agents introduce ‘zero length’ amide-crosslinks between carboxylic acid groups from aspartic and glutamic acid residues, and e-amino groups from (hydroxy-)lysine residues [17]. EDC/NHS-crosslinked collagen is reported to be non-cytotoxic in vitro [15], and biocompatibility was observed in animal studies [18,19]. The supply of autologous endothelial cells for seeding is limited. Although vascular grafts with a confluent lining of endothelial cells can be obtained after expansion of cell numbers by in vitro culture, this approach introduces a long interval between the need of an endothelialized graft and its availability, and increases the risk of bacterial infection due to prolonged culture [20]. Therefore, per-operative cell seeding is the preferred method for endothelialization. This implies application of low cell seeding densities, leaving (large) parts of the collagen-coated graft surface exposed to blood in the period after cell seeding.

Collagen is a highly thrombogenic material, as is demonstrated from its use as a hemostatic powder or sponge [21]. Collagen induces platelet adhesion and aggregation as well as activation of intrinsic blood coagulation. To prevent graft failure resulting from thrombus formation when collagen is not yet completely covered by (proliferating) endothelial cells, development of a collagen matrix for endothelial cell seeding with improved blood compatibility is required.

A generally applied approach to improve the blood compatibility of biomaterials is covalent immobilization of heparin [22,23]. Heparin is an effective inhibitor of blood coagulation. Furthermore, immobilization of heparin is reported to reduce in vitro and in vivo platelet adhesion and aggregation [24-28], although increased platelet adhesion after heparin immobilization has been reported as well [29]. The effect of heparin immobilization on endothelial cell proliferation is not consistent: heparin immobilization is both reported to inhibit [30] as well as promote [31] proliferation of endothelial cells.

In the present study, heparin was immobilized to EDC/NHS-crosslinked collagen, also using EDC and NHS. Carboxylic acid groups of heparin were converted to reactive NHS-esters using EDC and NHS, and thereafter heparin was immobilized by reaction of NHS-activated carboxylic acid groups of heparin with residual primary amino groups in the EDC/NHS-crosslinked collagen matrix. The immobilization of heparin to EDC/NHS-crosslinked collagen was investigated, as well as the stability of the resulting heparinized matrices. To determine whether heparin immobilization improved the blood compatibility of the collagen matrix, contact activation, thrombin inhibition and deposition of blood platelets was studied in vitro.

2. Materials and methods

2.1. Materials

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany), and were of the highest purity available.

2.2. Collagen film preparation

All experiments were carried out using flat collagen films as model substrates. Type I insoluble collagen (1 g) derived from Bovine Achilles Tendon (Sigma, St.Louis, MO, C 99879, lot 23H7065) was swollen overnight in 0.52 M acetic acid solution (50 ml) at 4°C. The mixture was dispersed with 50 g of crushed ice for 4 min in a Philips Blender and thereafter homogenized for 30 min at 4°C using an Ultra-Turrax T25 (IKA labortechnik, Staufen, BRD). The resulting slurry was filtered through a series of filters (Cellector screen, Belco, Feltham, UK), with a pore size decreasing from 140 to 10 μm, mounted in 47 mm diameter Swinnex disc filter holders (Millipore, Ett-en-Leur, The Netherlands). After de-aeration at a pressure of 0.06 mbar, the resulting suspension was casted as a film with a thickness of 3 mm on a flat poly (ethylene terephthalate) (PET) surface, using a casting knife. After drying at room temperature, a collagen film with a thickness of approximately 50 μm was obtained.

2.3. Collagen crosslinking

Collagen films were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). In order to minimize hydrolysis of EDC, crosslinking was carried out in 0.05 M buffer of 2-morpholinoethane sulfonic acid (MES buffer, pH 5.40) [32]. Before crosslinking, dried collagen films were washed with MES buffer. Subsequently, the films were immersed in a solution of EDC and NHS in MES buffer, and gently shaken. For the crosslinking reaction, 1.731 g EDC and 0.415 g NHS in 215 ml MES buffer were used per gram of collagen (molar ratio EDC:NHS:collagen-carboxylic acid groups = 7.0:2.8:1.0). After 4 h, when the reaction was completed, the collagen was washed with 0.1 M Na2HPO4 solution (2 h) and demineralized water (four times for 30 min) [32,33]. The residual number of free primary amino groups in collagen after crosslinking was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, from Fluka, Buchs, Switzerland), according to a slightly modified procedure described by Wang et al. [34-36]. The shrinkage temperature of (crosslinked) collagen, indicating the resistance against thermal denaturation, was determined using Differential Scanning Calorimetry (DSC) [37].
2.4. \(^3\)H-labeling of heparin

Heparin sodium salt (Bufa Chemie, Castricum, the Netherlands) was used. This heparin preparation from porcine mucosa has the following characteristics [38]: \(\text{MW} = 12,500 \text{ g/mol (molecular weight distribution 3000–30 000 g/mol), activity} = 195 \text{ IU/mg, 18.75 mol of carboxylic acid groups (Hep–COOH)}\) per mol of heparin.

Heparin was tritiated using the method described by Hatton et al. [39], with slight modifications. Briefly, heparin (3.00 g) was dissolved in distilled water (400 ml), and the pH was adjusted to 8.0 with NaOH solution (4 m). \(\text{NaB}^{3}\text{H}_4\) (100 mCi, 7.5 Ci/mmol, Amersham, Amersham, UK) was added, and the reaction was allowed to proceed for 3 h at room temperature. The resulting \(^3\)H-labeled heparin was purified by dialysis at 4 °C against subsequently 2 m NaCl in phosphate-buffered saline (PBS, from NPBI, Emmer Compascuum, the Netherlands, pH 7.40), PBS (2 times) and distilled water (3 times). \(^3\)H-heparin was isolated by lyophilization and stored in a desiccator at room temperature. The yield was 2.80 g, with a specific activity of 76.9 kBq/mg. Free label content was less than 0.5%, as determined by gel filtration using a PD10-Sepharose column (Pharmacia, Upsala, Sweden).

2.5. Heparin immobilization

Typically, heparin immobilization onto crosslinked collagen films was performed as described below. Crosslinked collagen films were equilibrated with MES-buffer (0.05 M, pH 5.60) for at least 30 min. Carboxylic acid groups of heparin (Hep–COOH) were activated by adding EDC and NHS to a 2% (w/v) solution of (\(^3\)H-labeled) heparin in 0.05 M MES-buffer (pH 5.60), at a molar ratio of EDC : NHS : Hep-COOH of 0.4 : 0.24 : 1.0. After pre-activation for 10 min, 1 g of crosslinked collagen (containing 14 free primary amino groups per 1000 amino acid residues, E/N14C) was added to 188.3 ml of EDC/NHS-activated heparin solution, giving a molar excess of heparin to free collagen primary amino groups (Coll-NH\(_2\), 151 µmol/g collagen, calculated from 10.76 mol amino acids per gram of collagen [40]) of 2.0. After 2 h of reaction, the heparinized E/N14C, designated as E/N14C-H, was washed with 0.1 M Na\(_2\)HPO\(_4\) (2 h), 4 m NaCl (4 times for 24 h) and distilled water (3 times for 24 h).

Immobilization of heparin was investigated as a function of heparin pre-activation time (up to 60 min), immobilization time (up to 4 h), pH (4.5–6.5), the amount of heparin (concentrations up to 4% (w/v), molar ratio of heparin to Coll-NH\(_2\) up to 10), and amounts of EDC (molar ratio of EDC to Hep-COOH up to 5) and NHS (molar ratio of NHS to EDC of 0.2–1.0) used.

2.6. Determination of immobilized heparin

\(^3\)H-heparinized collagen films were dissolved in Lumasolve (2 ml, Lumac, Olen, Belgium) for 24 h at 60 °C. After addition of scintillation cocktail (20 ml, Optiphase HiSafe 3, Wallac, Milton Keynes, UK) the radioactivity of the samples was measured using a 1414 Winspectral liquid scintillation counter (Wallac, Turku, Finland). The amount of immobilized heparin was calculated from the specific activity of \(^3\)H-heparin.

The amount of immobilized non-radiolabeled heparin was determined using toluidine blue [41]. Circular samples with a diameter of 8 mm were incubated with 5 ml aqueous solution of toluidine blue (0.1 M HCl, 2 mg/ml NaCl, 0.4 mg/ml toluidine blue O zinc chloride double salt) for 4 h at room temperature, resulting in complexation of toluidine blue with heparin. Thereafter, samples were washed with distilled water (twice for 5 min). Subsequently, toluidine blue complexed to heparin was solubilized in 5 ml of a 1 : 4 (v/v) mixture of 0.1 M NaOH and ethanol. The extinction of the resulting solution was determined at 530 nm using an Uvikon 930 spectrophotometer (Kontron Instruments, Switzerland). The amount of immobilized heparin was calculated from a calibration curve obtained from EDC/NHS-crosslinked collagen containing various amounts of immobilized \(^3\)H-labeled heparin.

2.7. Localization of immobilized heparin

Alcian Blue staining was used for localization of immobilized heparin in crosslinked collagen. Samples were processed for paraffin sectioning using standard histological techniques. Briefly, after dehydration in a graded series of ethanol (50, 70, 96 and 100%) and xylene, the specimen were embedded in paraffin. The paraffin embedded samples were sectioned at 6 μm, and mounted on gelatin coated glass slides. Sections were deparaffinized in xylol (three times for 5 min), and rehydrated in a graded series of ethanol and demineralized water. After incubation in acetic acid solution (3% v/v, pH 2.5) for 3 min, sections were stained during 30 min using a solution of Alcian Blue 8GX (2% w/v) in 3% acetic acid solution [42]. After washing with de-mineralized water (15 min), the specimen were examined using a Zeiss Axiosclop (Zeiss, Jena, Germany).

2.8. Stability of heparinized collagen

\(^3\)H-heparinized E/N14C discs with a diameter of 10 mm (thickness 54 ± 6 μm, weight 2.5 ± 0.2 mg) were washed with PBS (twice for 10 min). Thereafter, the samples were transferred to endothelial cell culture medium (CM) containing 5% human serum, as used for endothelial cell culture, which for this experiment was supplemented with Na\(_3\) (50 μg/ml). CM consisted of...
a mixture of equal volumes of RPMI 1640 and M199 (both standard synthetic cell culture media), containing penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and glutamax (2 mm) (all from Gibco, Paisley, UK). Release of 3H-heparin was measured upon incubation in CM/5% serum (5 ml) at 37°C for 11 days on an orbital shaker. Supernatant medium was replaced every 24 h by 5 ml of fresh medium, after which 3H-heparin in the culture medium was determined. Scintillation cocktail (9 ml, Optiphase HiSafe 3) was added to culture medium (1 ml), and subsequently the radioactivity of the sample was measured using liquid scintillation counting.

2.9. Contact activation assay

Activation of intrinsic coagulation by (heparinized) E/N14C was measured using a chromogenic substrate for activated factor XII and plasma kallikrein (S2302, Chromogenix, Mölndal, Sweden) [43,44]. (Heparinized) E/N14C discs with a diameter of 15 mm (weight 5.6 ± 0.5 mg) were fixed in a 24 wells microtiter plate (Costar, Cambridge, MA) using silicon-rubber rings (Eriks, Alkmaar, the Netherlands). Glass was used as a reference substrate. After incubation with PBS for 24 h at 37°C, samples were washed with PBS (37°C) twice for 1 min. Fresh frozen, pooled citrated plasma from 12 healthy donors (Bloodbank Twente en Achterhoek, Enschede, the Netherlands) was thawed at 37°C, and diluted with PBS (37°C) to 25% (v/v). Test samples were incubated with 200 μl of diluted plasma at 37°C on an orbital shaker. After 15 min, contact activation proteases in the diluted plasma were assayed. A sample of 50 μl was incubated with 50 μl Tris-buffer (50 mM tris(hydroxymethyl)aminomethane, 0.15 M NaCl, pH 7.40) and 50 μl substrate solution (2.5 mg/ml S2302 in distilled water). The optical density of the solution at 405 nm was recorded as a function of time for 20 min using an ELISA reader (340 ATTC). The activity of the contact-activation proteases in the diluted plasma were assayed. A sample of 50 μl was incubated with 50 μl Tris-buffer (50 mM tris(hydroxymethyl)aminomethane, 0.15 M NaCl, pH 7.40) and 50 μl substrate solution (2.5 mg/ml S2302 in distilled water). The optical density of the solution at 405 nm was recorded as a function of time for 20 min using an ELISA reader (340 ATTC) and the absorbance at 405 nm was measured using an ELISA reader (340 ATTC). The activity of the heparinized collagen films was calculated using a calibration curve obtained from heparin solutions in PBS with concentrations ranging from 0 to 250 mU/ml.

2.10. Thrombin inactivation assay

The anticoagulant activity of immobilized heparin was determined using a thrombin inhibition assay, based on a method described by Chandler [45]. Briefly, the ability of immobilized heparin to mediate the inactivation of thrombin by ATIII was measured using a chromogenic substrate for thrombin (S2238). Heparinized collagen disks with a diameter of 10 mm (weight 2.5 ± 0.2 mg), were incubated with PBS for 24 h. After blotting on filter paper, the films were transferred to wells of a 48 well tissue culture cluster (Costar, Cambridge, MA) containing 50 μl PBS and 250 μl Tris-buffer. The Tris buffer used for this assay consisted of 50 mM Tris (pH 8.40) containing 1 g/l PEO 6000, 1 g/l BSA (Sigma) and 150 mM NaCl. The substrate solution consisted of 0.2 mg/ml S2238 (Chromogenix, Mölndal, Sweden) and 70 mU/ml purified human AT III (gift from CLB, Amsterdam, the Netherlands) in Tris-buffer. After addition of 150 μl substrate solution, the assay was started by adding 50 μl of a 1.2 U/ml solution of bovine thrombin (Sigma, T 4265) in Tris-buffer. After incubation for 10 min at 37°C on an orbital shaker, the reaction was stopped by adding acetic acid solution (50 μl, 40% v/v). Subsequently, 250 μl of the supernatant was transferred to a 96 well tissue culture cluster (Costar), and the absorbance at 405 nm was measured using an ELISA reader (340 ATTC). The activity of the heparinized collagen films was calculated using a calibration curve obtained from heparin solutions in PBS with concentrations ranging from 0 to 250 mU/ml.

2.11. Preparation of platelet suspensions

Fresh 'buffy coats' derived from whole blood units collected from healthy volunteers (Bloodbank Twente en Achterhoek) were diluted 2.5 times with Krebs–Ringer buffer (107 mM NaCl, 20 mM NaHCO3, 2 mM Na2SO4, pH 7.3) containing 19 mM trisodiumcitrate and 27 mM α-D-glucose, after which platelet-rich plasma (PRP) was obtained by centrifugation at 1500 g during 4 min. Subsequently, PRP was diluted with an equal volume of Krebs-Ringer buffer (pH 5.0) containing trisodiumcitrate (19 mM) and α-tyro-glucose (27 mM), giving a final pH of 6.1. Thereafter, platelets were washed according to the method described by Cazenave and labeled with 111In-DTPA (Amersham, Amersham, UK) as previously described [46]. Platelets were finally suspended in ABO-compatible platelet poor plasma (Bloodbank Twente en Achterhoek) at a concentration of 100 000/μl. The radioactivity of the platelets was 457 ± 15 cpm per million platelets.

2.12. Deposition of 111In-labeled platelets

Samples were fixed with a silicon rubber ring (Eriks) in custom-made Teflon sample holders, giving an exposed
(flat and circular) surface area of 4.10 cm² (calculated weight 13 ± 1 mg). Surfaces were equilibrated overnight at 37°C with PBS. After two additional washes with PBS (37°C), the surfaces were incubated with 485 µl ¹¹¹In-labeled platelets suspended in plasma, under static conditions at 37°C. After 1 h the platelet suspensions were removed, and the substrates were washed carefully with PBS (three times, 37°C). The number of deposited platelets was determined by measuring the radioactivity on the washed substrates, using a Compugamma 1282 γ-counter (LKB, Stockholm, Sweden).

An EDC/NHS-crosslinked albumin gel (bovine serum albumin, Sigma), prepared as described by Bos et al. [47], was used as a control surface. Briefly, to a solution of albumin in MES-buffer (200 mg/900 µl, pH 5.3) a solution of EDC (16.6 mg) and NHS (2.0 mg) in MES-buffer (100 µl, pH 5.3) was added. The mixture was pipetted in a petridish. After 4 h of crosslinking, the resulting albumin-gel (E/N-albumin) was extensively washed with PBS.

2.13. Scanning electron microscopy

Samples for scanning electron microscopy (SEM) analysis were fixed in a glutaraldehyde solution in PBS (2% w/v, 4°C) for at least 24 h. Thereafter, samples were dehydrated in a graded series of ethanol and air-dried. After sputter-coating with gold/palladium (10 nm), the samples were examined using an S-800 field emission SEM (Hitachi, Tokyo, Japan) at an acceleration voltage of 7 kV.

2.14. Statistical analysis

Results were expressed as mean ± standard deviation. For statistical analysis, a Student’s t-test (using GraphPad InStat, GraphPad Software, San Diego, CA) was used. Results were considered statistically different with \( p < 0.05 \).

3. Results

3.1. Collagen crosslinking

Upon crosslinking with EDC and NHS, the number of free primary amino groups per 1000 amino acid residues, which in native collagen amounts to 27 \[48\], decreased while the shrinkage temperature increased. At the conditions used, collagen crosslinking resulted in a material containing 14 free primary amino groups per 1000 amino acid residues (E/N14C). The shrinkage temperature increased from 55.4°C for non-crosslinked collagen (N-Coll) to 76.1°C for E/N14C.

3.2. Heparin immobilization

 Immobilization of ³H-heparin to E/N14C was maximal at pH 5.60 (as determined using a pH range from 4.5 to 6.5) in the presence of a heparin concentration of 2% (w/v) or higher (determined for heparin concentrations up to 4%). The immobilization reaction was complete within 2 h (data not shown).

Using these conditions, pre-activation of heparin with EDC and NHS for 5 to 30 min resulted in maximal heparin immobilization (Fig. 1A). Using a pre-activation time of 10 min and a molar ratio EDC : NHS : Hep-COOH of 0.4 : 0.24 : 1.0, the amount of immobilized heparin increased when the molar ratio of heparin to free primary amino groups of collagen (Coll-NH₂) was increased, leveling off above a ratio of 2 (Fig. 1B). Heparin immobilization using a molar ratio of heparin : Coll-NH₂ of 2.0 (10 min pre-activation, EDC : Hep-COOH = 0.4) was maximal at a molar ratio of NHS to EDC of between 0.4 to 0.6 (Fig. 1C). At a fixed molar ratio of NHS : EDC of 0.6 (10 min pre-activation, EDC : Hep-COOH = 0.4), the amount of immobilized heparin increased with increasing molar ratio of EDC to heparin-carboxylic acid groups (Hep-COOH), to a

![Fig. 1. Immobilization of ³H-heparin to E/N14 collagen as a function of pre-activation time of heparin (A), molar ratio of heparin to free primary amino groups in collagen (B), molar ratio of NHS to EDC (C), and molar ratio of EDC to heparin carboxylic acid groups (D) (n = 4, mean ± SD). Heparin concentration 2% (w/v), pH = 5.60, immobilization time 2 h. Heparin : Coll-NH₂ = 2, EDC : NHS : Hep-COOH = 0.4 : 0.24 : 1.0 (A); 10 min pre-activation, EDC : NHS : Hep-COOH = 0.4 : 0.24 : 1.0 (B); heparin : Coll-NH₂ = 2; 10 min pre-activation, EDC : Hep-COOH = 0.4, (C); Heparin : Coll-NH₂ = 2, 10 min pre-activation, NHS : EDC = 0.6 (D).](image)
maximum of approximately 5.5% heparin (w/w) at a ratio of 2 (molar ratio EDC : NHS : Hep-COOH = 2.0 : 1.2 : 1.0) (Fig. 1D). Based on these results a standard procedure for heparin immobilization to E/N14C was adopted, using 2% (w/v) heparin solution (pH 5.60), a fixed molar ratio of NHS to EDC of 0.6, a variable molar ratio of EDC : Hep-COOH of 0 to 2.0, heparin activation for 10 min, a molar ratio of heparin to free collagen primary amino groups of 2.0, and 2 h of immobilization reaction. In further experiments, the amount of heparin immobilized to E/N14C was predetermined by the molar ratio of EDC to Hep-COOH used for immobilization.

3.3. Localization of immobilized heparin

Alcian Blue is a cationic dye which can be used for quantitative determination of glycosaminoglycans in solution [42] and for selective staining of glycosaminoglycans in tissue sections [49]. Light-microscopic images of Alcian Blue stained sections of collagen–heparin films demonstrated homogeneous staining through the entire thickness of the specimen, in contrast to the non-heparinized crosslinked collagen which showed no staining using the same procedure (Fig. 2). This indicates that heparin is immobilized homogeneously throughout the entire thickness of the film.

3.4. Stability of heparinized collagen

Upon incubation of heparinized E/N14C matrices (E/N14C-H) with endothelial cell culture medium supplemented with 5% human serum partial release of immobilized heparin occurred (Fig. 3). When the molar ratio of EDC to Hep-COOH for heparin immobilization was increased, the release of \(^3\)H-heparin from E/N14C-H also increased. E/N14C heparinized using a molar ratio of EDC : Hep-COOH of 0.1 (E/N14C-H(0.1)) showed 7% heparin release after 11 days (0.54 ± 0.02 mg heparin per gram of E/N14C-H(0.1)), whereas E/N14C-H(0.4) and E/N14C-H(1.0) showed a release of approximately 5% of the immobilized heparin (1.01 ± 0.03 and 2.1 ± 0.1 mg of heparin released per gram of heparinized E/N14C, respectively). Heparin release leveled off after longer incubation times, but a plateau value was not observed during 11 days. Release of free \(^3\)H label, as determined using a PD10-column, was negligible.

3.5. Contact activation

Contact activation generated when diluted plasma was incubated with E/N14C was significantly lower compared to glass (Fig. 4A). Immobilization of heparin to E/N14C using molar ratios of EDC : Hep-COOH increasing from 0 (control, without EDC and NHS being used during the immobilization reaction) to 1.0 reduced contact activation. Significantly less contact activation was observed after immobilization of heparin at a ratio of EDC : Hep-COOH of 0.4 or higher.

The enzymatic activity of contact activation proteases adsorbed to glass after incubation with diluted plasma was lower compared to E/N14C or E/N14C-H (p < 0.05) (Fig. 4B). Collagen onto which heparin was immobilized did not contain significantly more protease activity than when no heparin was immobilized, except for E/N14C-H(0.6) and E/N14C-H(1.0). Results are expressed as mOD, measured after 45 min of reaction of surface adsorbed contact activation proteases and chromogenic substrate solution.

3.6. Thrombin inhibition

Inactivation of thrombin by heparinized E/N14C was determined as function of the molar ratio of EDC : Hep-COOH used for heparin immobilization (Fig. 5). Maximal thrombin inhibition was observed when heparin was immobilized using a molar ratio of EDC : Hep-COOH of 0.2–0.4 (14–20 mg heparin/g collagen), which resulted in
3.7. Platelet deposition

Deposition of $^{111}$In-labeled platelets was measured under static conditions, using platelets resuspended in human plasma. When compared to N-Coll, platelet deposition to E/N14C was significantly lower (Fig. 6). Platelet adhesion to E/N14C without immobilized heparin was comparable to platelet deposition onto heparinized E/N14C, except for E/N14C-H(0.4), onto which platelet deposition was significantly higher. The number of platelets deposited from plasma to E/N14C-H(0.4) and N-Coll were comparable. Platelet deposition onto E/N-Alb, which was used as a reference matrix, was very low compared to these substrates.

The morphology of platelets deposited onto (heparinized) E/N14C was studied using scanning electron microscopy. Platelets deposited on E/N14C and heparinized E/N14C demonstrated similar morphology (Fig. 7a–d). Most adherent platelets had developed pseudopodia. Only few completely spread platelets were seen, on both E/N14C and E/N14C-H. Platelet activation (pseudopod formation) had occurred on all surfaces and deposited platelets were mainly observed in clusters of activated platelets. On E/N-Alb, adherent platelets demonstrated limited pseudopodia formation. No spreading or platelet aggregates were observed (not shown).

4. Discussion

To obtain a non-cytotoxic collagen substrate suitable for endothelial cell seeding, collagen was crosslinked using EDC and NHS, which results in formation of amide crosslinks between carboxylic acid groups and free primary amino groups from amino acid residues in collagen [17]. Crosslinking of collagen was demonstrated by a decrease in free amino groups after crosslinking, and a corresponding increase in the shrinkage temperature. Denaturation of collagen is characterized by transition of the triple helix of the collagen molecule to a random coil, accompanied by a macroscopic shrinkage. Crosslinking results in stabilization of the triple helix structure, thus increasing the shrinkage temperature [50].
Thrombin for 10 min at 37°C was used. Substrates were incubated with platelets (100 000/μl) resuspended in human ABO-compatible platelet-poor plasma, for 1 h at 37°C. Heparin was immobilized onto E/N14C using molar ratios of EDC : Hep-COOH of 0, 0.05, 0.1, 0.2, 0.4, 0.6 and 1.0 (E/N14C-Hn), n represents the molar ratio of EDC : Hep-COOH, resulting in 2.6 ± 0.5; 4.4 ± 1.3; 7.6 ± 1.8; 14.2 ± 2.3; 20.3 ± 3.7; 30.6 ± 3.3 and 38.6 ± 2.2 mg immobilized heparin per gram of E/N14C, respectively. Thrombin inactivation by various surfaces during incubation with a solution of purified human ATIII and bovine thrombin for 10 min at 37°C.

Pre-activation times between 5 and 30 min resulted in decreased amounts of immobilized heparin, possibly due to hydrolysis of EDC- or NHS-activated carboxylic acid groups [32]. The increase in immobilized heparin, observed when the ratio of heparin to Coll-NH2 was increased (Fig. 1B), can be explained by simple reaction kinetics. The depletion of heparin from the pre-activated heparin solution was very low (<0.1%), at all ratios of heparin to Coll-NH2 studied. The heterogeneous system, immobilization of heparin in solution onto a solid surface, may account for inefficiency of the immobilization reaction. Electrostatic repulsion between (negatively charged) immobilized heparin and heparin in solution may determine maximal heparin immobilization at given reaction conditions, resulting in a plateau value of heparin immobilization at ratios of heparin to Coll-NH2 above 2. Increased heparin immobilization using increasing ratios of NHS : EDC (Fig. 1C) probably resulted from conversion of EDC-activated carboxylic acid groups to reactive NHS-esters, thus preventing side reactions like hydrolysis of EDC activated groups or an O-N-acyl shift. When increasing the ratio of EDC to Hep-COOH (Fig. 1D), more reactive NHS-esters or EDC-activated carboxylic acid groups are introduced per molecule of heparin, resulting in increased heparin immobilization. This is probably accompanied by increased numbers of covalent bonds introduced per molecule of immobilized heparin. Because of the gradual increase in the amount of immobilized heparin with increasing ratios of EDC to Hep-COOH, this ratio was used to control the amount of immobilized heparin. Materials with different amounts of immobilized heparin were used in blood compatibility studies described below.

In the present study, heparin immobilization to EDC/NHS-crosslinked collagen was performed using EDC and NHS as well, in order to prevent cytotoxicity due to heparin immobilization reagents. Collagen crosslinking and heparin immobilization were carried out in two successive procedures, to allow monitoring of both the crosslink density of the EDC/NHS-crosslinked collagen matrix as well as the heparin immobilization reaction. This control is needed, because we observed in earlier studies that the crosslink-density of the collagen matrix influences the proliferation of endothelial cells when seeded on EDC/NHS-crosslinked collagen [51].

After carboxylic acid groups of heparin (Hep-COOH) were activated using EDC and NHS, heparin was immobilized to E/N14C by formation of peptide bonds between activated carboxylic acid groups of heparin and free primary amino groups of crosslinked collagen. The heparin pre-activation time was not very critical with respect to the amount of heparin immobilized (Fig. 1A).

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Maximal heparin immobilization to E/N14C was approximately 5–5.5 wt%, which is higher than reported when using EDC [52] or 1-cyclohexyl-3-(2-(N-methylmorpholinomethyl)-carbodiimide, an alternative water soluble carbodiimide [53,54], for immobilization of heparin to non-crosslinked collagen (0.4–1.5 wt%). This indicates that the use of EDC in combination with NHS is a very efficient method for immobilization of heparin to collagen.
Alcian blue staining (Fig. 2) demonstrated heparin immobilization through the entire thickness of E/N14C-H(0.4). The heparin content of E/N14C-H(0.4) was approximately 2.2 wt% (Fig. 1C–D), which corresponds to 70 μg of heparin immobilized per cm² of crosslinked collagen (calculated from a weight of 2.5 mg/10 mm disc, see materials and methods section). Bokros et al. have determined that for a densely packed monolayer of immobilized heparin orientated ‘side on’ on a solid surface, a surface concentration of 0.1 to 0.2 μg heparin/cm² is needed [55]. For a solid surface with a monolayer of ‘end on’ orientated heparin, a heparin surface concentration of 2 μg heparin/cm² was calculated. Consequently, immobilization of 70 μg of heparin per cm² of E/N14C can not be only limited to the outer surface of the material, but has to occur throughout the bulk of the crosslinked collagen matrix, which confirms the results of the alcian blue staining assay.

After heparinization, the films were washed with 4 M NaCl solution until elution of heparin from the matrices.

Fig. 7. Representative SEM images of platelets deposited from human plasma onto various substrates during 1 h incubation at 37°C. Substrates: E/N14C (a), E/N14C-H(0.2) (b), E/N14C-H(0.4) (c) and E/N14C-H(1.0) (d). Original magnification 3000×.
was negligible (i.e. after 3–4 days, determined using \(^{3}H\)-heparin). During subsequent incubation of E/N14C-H for 11 days in endothelial cell culture medium (CM), however, 5–7% of the heparin was released (Fig. 3). This is most likely due to release of a residual fraction of non-covalently bound heparin not removed by washing with 4 M NaCl. Possibly, the high salt concentration used decreased the swelling of the heparinized collagen to such an extent, that part of the unbound heparin was entrapped within the collagen matrix during the washing procedure.

Increased heparin immobilization resulted in significantly increased swelling of heparinized E/N14C in PBS (data not shown). Increased swelling may facilitate a faster diffusion of non-covalently bound heparin out of the heparinized E/N14C matrix, explaining the faster (absolute) heparin release with increasing amounts of heparin immobilized.

The relatively slow and decreasing release rates indicate that a substantial amount of immobilized heparin will be present on or in the E/N14C-H matrix for prolonged periods of time. A continuous release of heparin from a matrix results in a micro-environment of heparinized blood near the blood–biomaterial interface, thus inhibiting thrombus formation. A minimal heparin release of \(40 \text{ ng/cm}^2\min\) is reported to create thrombo-resistant catheter surfaces or thromboresistant tubes, even at relatively high flow rates \([56–58]\). It is proposed that actual release can be much lower, because the remaining immobilized heparin also contributes to the thromboresistance of the surface \([58,59]\). The average rate of heparin release from E/N14C-H during the first hours of incubation with CM was calculated to be in the range of 0.1–0.4 ng/cm\(^2\)min, and release decreased with time. It is not likely that such low heparin release contributes to thromboresistance.

The effect of heparin immobilization to E/N14C on blood coagulation depends on both the extent of contact activation induced by heparinized EDC/NHS-crosslinked collagen and the anticoagulant properties of the heparinized material. Glass is a strong activator of intrinsic coagulation \([60]\). Compared to glass surfaces, contact activation by E/N14C was considerable (Fig. 4). Immobilization of heparin decreased the activation of the contact system significantly, although contact activation by E/N14C-H was still high when compared for example to heparinized polystyrene surfaces \([61]\) or surface-modified polyethylene \([62]\). The exact mechanism underlying reduced contact activation after heparin immobilization is not clear. Immobilized heparin is reported to inactivate factor XIIa in the presence of ATIII \([63,64]\). Alternatively, decreased non-specific adsorption of coagulation factors (FXII, HMWK) due to a modified surface chemistry after immobilization of heparin may result in reduced factor XII activation \([65]\).

The enzymatic activity of surface adsorbed contact activation proteases did not differ much between E/N14C and the heparinized E/N14C matrices (Fig. 4B), although a slight but significant increase was observed for E/N14C-H(0.6) and E/N14C-H(1.0). Results were comparable to the enzymatic activity of surface adsorbed contact activation proteases observed in other studies \([61,62,47]\).

Immobilization of heparin to E/N14C resulted in matrices with improved anticoagulant activity (Fig. 5). However, thrombin inactivation by heparinized E/N14C did not progressively increase with increasing amounts of immobilized heparin. E/N14C with 10.3–23.2 mg heparin immobilized per gram of collagen (obtained using a molar ratio EDC: Hep-COOH of 0.2–0.4) demonstrated maximal thrombin inhibitory activity. When using higher ratios of EDC: Hep-COOH, the anticoagulant activity of the obtained matrix decreased despite more immobilized heparin. Decreased thrombin inhibition using ratios of EDC: Hep-COOH > 0.4 may be due decreased accessibility of the ATIII binding site and/or decreased thrombin binding to the immobilized heparin, caused by an increased number of covalent bonds introduced between heparin and collagen.

The anti-thrombin activity of E/N14C-H(0.2) and E/N14C-H(0.4) was higher than the anti-thrombin activity of surfaces with an immobilized albumin–heparin conjugate \([61,47]\) or solid catheter surfaces to which heparin was covalently immobilized \([45]\). On the other hand, the thrombin inhibitory activity of E/N14C-H(0.2) and E/N14C-H(0.4), was lower than observed for surfaces that were heparinized via flexible spacers and surfaces containing ‘end point’ immobilized heparin (10–30 mU/cm\(^2\)) \([45,61]\). This indicates, in the present study direct immobilization of heparin onto collagen rendered a surface with relatively high anti-thrombin activity, although heparinized collagen with a higher anti-thrombin activity may be obtained using alternative immobilization techniques.

In vitro platelet aggregation by collagen can be reduced by chemical modification of collagen \([66,67]\), by distortion of the quaternary fibril structure of collagen \([68]\), or by immobilization of heparin \([26]\). Compared to N-Coll, platelet deposition was decreased for EDC/NHS-crosslinked collagen (Fig. 6). Crosslinking of collagen decreased platelet deposition probably due to a decrease in the number of free carboxylic acid groups of aspartic and glutamic acid residues during crosslinking \([69]\). Heparin immobilization to various surfaces was demonstrated to result in both increased \([29]\) as well as decreased in vitro platelet deposition \([25,26]\). In the present study, heparin immobilization to E/N14C did not influence the morphology of adherent platelets (Fig. 7). However, significantly increased numbers of deposited platelets were observed on E/N14C–H(0.4). Striking is the positive correlation between platelet deposition and thrombin inhibitory activity of heparinized E/N14C (Fig. 5 versus Fig. 6), which is in good agreement
with results of Bos et al. [47]. In addition, Lindon et al. reported increased platelet deposition onto heparinized polyethylene surfaces with higher ATIII-binding capacity [29]. Platelets contain heparin binding sites [70], which may mediate binding of platelets to immobilized heparin. Therefore, maximal platelet deposition onto E/N14C-H(0.4) might result from the same steric considerations as discussed above for the observed antithrombin activity. Alternatively, steric considerations may determine the ability of plasma proteins to bind to immobilized heparin. Maximal binding of plasma proteins like von Willebrand factor, fibronectin and thrombospondin [71], therefore may be responsible for the observed maximal platelet deposition onto E/N14C-H(0.4).

The effect of heparin release from E/N14C-H (Fig. 3) on the in vitro studies described above was not determined experimentally. Heparin release is, however, not likely to interfere with contact activation measurements (Fig. 4). It is reported that high heparin concentrations in plasma exposed to a heparinized surface did not influence activation of FXII by heparinized polyethylene [63]. Heparin release, however, may in part contribute to the observed in vitro thrombin inactivation (Fig. 5). The activity of heparin released from E/N14C-H(0.1), E/N14C-H(0.4) and E/N14C-H(1.0) during the assay was calculated to be approximately 0.1, 0.3 and 0.5 mU/cm², respectively. Although the actual activity of the immobilized heparin may be somewhat lower than depicted in Fig. 5 because of heparin release, the position of the observed maximum is not changed. Heparin in solution is reported to show variable results with regard to platelet aggregation and release. However, heparin did not influence platelet aggregation stimulated by a fibrillar collagen suspension [73]. Furthermore, heparin did not significantly inhibit adhesion of washed human platelets to collagen-coated glass at a concentration of 20 U/ml [74]. Therefore, heparin release from E/N14C-H is not expected to influence platelet deposition (Fig. 6) during the present study.

Several studies have shown that immobilization of heparin on collagen-coated vascular grafts reduces thrombogenicity in vivo. In a rabbit model, epoxy ether crosslinked heterografts show improved patency after heparin immobilization [75]. In dogs, comparable results have been found for gelatin-coated Corethane [76], and collagen or gelatin-coated ePTFE [75–77]. Although one has to be careful in extrapolating results of in vivo blood compatibility studies to actual in vivo thromboresistance, the results from the in vitro study presented here suggest that, compared to E/N14C, especially for E/N14C-H(0.4) improved in vivo blood compatibility might be expected. However, the results of the present study are not conclusive. Resuming, E/N14C-H(0.4) combined highest thrombin inhibition with lowest contact activation for all heparinized E/N14C surfaces. Although on E/N14C as well as E/N14C-H only limited platelet spreading or aggregation was observed, platelet deposition on E/N14C-H(0.4) was somewhat higher than observed for E/N14C, E/N14C-H(0.2) and E/N14C-H(1.0).

5. Conclusions

Heparin was immobilized to E/N14C using EDC and NHS. Using increasing molar ratios of EDC to heparin-carboxylic acid groups, increasing amounts of heparin were immobilized, up to a maximum of 5–5.5 wt%. Increased heparin immobilization onto E/N14C resulted in a progressive decrease in contact activation. Thrombin inhibition was maximal after heparin immobilization using a molar ratio of EDC to heparin-carboxylic acid groups of 0.2–0.4. E/N14C-H(0.4) demonstrated lowest contact activation in combination with the highest thrombin inhibition. Heparin immobilization resulted in a slightly increased platelet adhesion, platelet deposition was maximal on E/N14C-H(0.4). Immobilization of heparin did not affect the morphology of platelets deposited, and platelets adherent onto (heparinized) E/N14C demonstrated only limited spreading or aggregation. It is concluded that compared to E/N14C, especially for E/N14C-H(0.4) improved in vivo blood compatibility may be expected.

References


