Covalently-Bound Heparin Makes Collagen Thromboresistant
Jeffrey F.W. Keuren, Simone J.H. Wielders, Anita Driessen, Michel Verhoeven, Marc Hendriks, Theo Lindhout

Objective—Blood compatibility of artificial surfaces depends on their immunogenic and thrombogenic properties. Collagen’s weak antigenicity makes it an attractive candidate for stent coatings or fabrication of vascular grafts. However, the thrombogenic nature of collagen limits its application. We examined whether heparinization can make collagen more thromboresistant.

Methods and Results—Collagen was heparinized by crosslinking collagen with extensively periodate oxidized heparin and/or by covalently bonding of mildly periodate oxidized heparin. Both ways of heparinization have no effect on platelet adhesion and could not abolish induction of platelet procoagulant activity. However, thrombin generation was completely prevented under static and flow conditions. The functionality of immobilized heparin was confirmed by specific uptake of antithrombin, 13.5±4.7 pmol/cm² and 1.95±0.21 pmol/cm² for mildly and heavily periodated heparin, respectively.

Conclusions—These results indicate that immobilization of heparin on collagen, even as a crosslinker, is a very effective way to prevent surface thrombus formation. These data encourage the application of heparinized collagen as stent-graft material in animal and eventually human studies. (Arterioscler Thromb Vasc Biol. 2004;24:613-617.)

Key Words: collagen, heparin, thrombogenicity, thrombosis, blood flow

Cardiovascular disease, including vascular stenosis, is still the leading cause of death in Western society. Obstructive atherosclerotic disease, causing angina pectoris or even myocardial infarction, is currently treated by the implantation of a stent or through bypass surgery. Unfortunately, attempts to implant vascular grafts with a small diameter are not successful because of thrombogenic and inflammatory reactions.1-6 Thus, to enhance blood compatibility, a surface has to be designed that is both anti-immunogenic and thromboresistant. Collagen has been widely used in medical applications, including skin replacement, bone substitutes, and artificial valves.7,8 Recently, a number of investigators attempted to use modified collagen as a vascular graft material.9-12 Potential advantages of the natural biological polymer collagen are its weak antigenicity and high tensile strength, which can resist high arterial blood pressures. Furthermore, collagen is a suitable substrate for endothelial cell growth in vitro,11 which makes its application as an artificial vessel even more attractive. Nevertheless, the prothrombotic properties of collagen13 are a major drawback in its applicability in blood contacting devices.

To make collagen more thromboresistant, Wissink et al14 coupled the sulfated polysaccharide, heparin, to collagen. An in vitro assay showed that the immobilized heparin was functionally active as it accelerated the thrombin-antithrombin (AT) reaction. In addition, this group demonstrated that crosslinking of collagen had an adverse effect on the antigenicity and degradation rate of collagen, but stimulated endothelial cell adhesion and proliferation.11,12 The suitability of collagen as a vascular graft material was also emphasized in a rabbit study, which established that collagen-grafts were completely endothelialized after one month of implantation.10

This study was undertaken to get a better understanding of the precise antithrombotic functions of immobilized heparin. Three types of heparinized collagen (heparin-crosslinked collagen, heparinized heparin-crosslinked collagen, and heparinized EDC/NHS-crosslinked collagen) were evaluated for their thrombogenicity by measuring platelet adhesion, platelet activation, and thrombin generation.

Methods
Preparation of Collagen and Heparinized Collagen Sheets
Four different types of type I collagen (Syntacoll) sheets were prepared; namely, noncrosslinked collagen (NC), heparin-crosslinked collagen (HC), heparinized EDC/NHS-crosslinked collagen...
lager (HEC), and heparinized heparin crosslinked collagen (HHC). For a detailed description, see http://atvb.ahajournals.org.

**Heparin Assay**

In all (static) experiments collagen and heparinized collagen sheets, mounted in the wells of a 48-well microtiter plate (Corning), were repeatedly rinsed in Hepes buffer (5 mmol/L Hepes, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L MgCl₂, 1 mg/mL BSA, pH 7.45) to remove unbound heparin. The amount of heparin in the rinse-buffer was measured by adding a 30 μL aliquot into 112.5 μL AT (0.66 μmol/L). After an incubation of 3 minutes at 37°C, 7.5 μL bovine factor Xa (1 μmol/L) was added. At timed intervals, 25-μL samples were transferred into a cuvette containing 425 μL Hepes buffer containing 20 mmol/L EDTA and 50 μL chromogenic substrate S2765 (2 mmol/L, Chromogenix). The amount of heparin was calculated from the rate of disappearance of the factor Xa activity. A reference curve was constructed from heparin (190 U/mg; Diosynth) that was used to prepare heparinized collagen.

**Platelet Adhesion and Procoagulant Activity**

Venous blood was obtained from volunteers who denied taking any medication in the 2 weeks before sampling. Washed platelets were prepared as described.13 Wells were blocked with 20 mg/mL BSA in Hepes buffer for 15 minutes. This was followed by incubation with 1 μg/mL Oregon Green-labeled annexin V (NeXins) and 3 mmol/L CaCl₂ in Hepes buffer. Nonbound annexin V was removed with Hepes buffer containing 20 mmol/L EDTA. Fluorescence tracings as a result of thrombin generation were recorded with a temperature controlled microplate fluorometer (SPECTRAMax GEMINI XS, Molecular Devices) at 37°C with an excitation wavelength of 485 nm and emission wavelength of 535 nm. A reference curve was constructed with known amounts of Oregon Green-labeled annexin V.

**Determination of Specific Antithrombin Binding to Heparinized Collagen**

Specific binding of AT to heparinized collagen was determined as described.14 See http://atvb.ahajournals.org for further information.

**Thrombin Generation**

Collagen films with adherent platelets were exposed to 300 μL of citrated PFP or AT depleted plasma (Biopool) containing 250 μmol/L of the fluorogenic thrombin substrate Z-GGR-AMC (Bachem). Coagulation was started by the addition of 20 mmol/L of CaCl₂, in Hepes buffer containing 20 mmol/L EDTA and 200 μmol/L LA T to remove nonbound heparin. The amount of recovered annexin V was measured in a spectrophotometer (SLM) with an excitation wavelength of 485 nm and emission wavelength of 535 nm. A reference curve was constructed with known amounts of Oregon Green-labeled annexin V.

**Statistical Analysis**

The data were expressed as mean±SD. To determine the statistical significance of differences, probability values were obtained with a nonparametric test for two (Mann–Whitney Test) or more (Kruskal–Wallis Test) independent variables.

**Results**

**Effect of Heparinization on Platelet Adhesion**

Prior to experimentation, collagen films were extensively rinsed to remove any nonbound heparin. Heparin in the effluent was assayed as described. The collagen films were then incubated with washed platelets. Platelet adhesion was assessed from SEM micrographs (Figure 1A, middle). It is clearly seen that all adherent platelets formed pseudopods (Figure 1A, bottom). No significant differences in platelet adhesion to NC, HC, HEC, and HHC were observed, indicating that heparinization of collagen does not affect platelet adhesion (Figure 1B).

**Effect of Heparinization on Collagen-Induced Platelet Procoagulant Activity**

Collagen-adherent platelets were examined for their loss of plasma membrane phospholipid asymmetry, utilizing the property of annexin V to bind only to cell membranes...
containing anionic phospholipids such as phosphatidylserine. The collagen surfaces, except HHC, did not bind annexin V. No significant difference could be observed in the amount of annexin V bound to platelets on NC and HC (P=0.42, Mann–Whitney test), indicating that multi-point coupled heparin, when used as a crosslinker of collagen, does not prevent the collagen-induced activation of adherent platelets (Table 1). However, annexin V binding was significantly reduced for HEC and HHC surfaces with adherent platelets (P≤0.01, Kruskal–Wallis test). It is apparent that immobilization of heparin, using mildly periodate oxidized heparin, partially inhibits collagen-induced exposure of anionic phospholipids in the outer leaflet of the platelet plasma membrane.

### Annexin V Binding

The AT binding capacities of the different collagen surfaces are shown in Figure 2. The NC surface bound hardly any AT (0.09±0.08 pmol/cm²). When the collagen surfaces contained heparin, either as crosslinker (HC) or immobilized on crosslinked collagen (HHC or HEC), significant amounts of AT bound to these surfaces. HC bound 1.95±0.21 pmol/cm² AT, which is about 25-fold more than NC. Interestingly, heparinization of HC greatly increased the AT binding capacity (23.8±4.1 pmol/cm²). It is very likely that the mild periodate oxidation procedure used to immobilize heparin to the HC preserves the binding sites for AT to a larger extent than the modification of the crosslinker heparin. This notion is supported by the finding that HEC also binds relatively high amounts of AT (13.5±4.7 pmol/cm²).

### Antithrombin Binding

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### Thrombin Generation at Collagen Surfaces under Static Conditions

Collagen surfaces with adherent platelets were exposed to recalcified citrated platelet-free plasma, and thrombin generation was continuously monitored as described. It was first established that under the conditions of the assay, collagen does not generate thrombin in the absence of adherent platelets. Thus, under the conditions of the experiment, thrombin generation requires the presence of (activated) platelets (data not shown). When NC surfaces with adherent platelets were exposed to PFP, thrombin activity became detectable after a lag phase of about 12 minutes, and a peak level of thrombin was reached after about 20 minutes (Figure 3, left). In contrast, when the same experiment was performed with HC, HEC, or HHC, thrombin generation was not detected. To examine the contribution of heparin cofactor II, heparinized collagen surfaces with adherent platelets were exposed to AT-depleted plasma. In contrast with normal PFP, significant amounts of thrombin were generated on heparinized collagen films. Maximal levels of thrombin were reached after approximately 20 minutes for HC and 25 minutes for HEC and HHC surfaces (Figure 3, right). The sustained levels of thrombin activity in the case of AT-depleted plasma indicate that heparin cofactor II hardly contributes to the inactivation of thrombin in plasma. Consequently, heparinized-collagen surfaces do not inhibit thrombin generation in AT-depleted plasma.

### Thrombin Generation at Collagen Surfaces under Flow Conditions

Stents were wrapped in a collagen sheet, inserted in silicone tubes (inner diameter 1.5 mm), expanded, and perfused with recalcified whole blood. Figure 4 shows the thrombin concentration measured at the outlet of the flow system as a function of the perfusion time. In the control, an empty silicone sleeve with (Figure 4, △) and without (Figure 4, ▲) an inserted stent, traces of thrombin appeared 16 and 18 minutes after the start of the perfusion, respectively. Non-
of phosphatidylyserine in the outer leaflet of the plasma membrane. This result appears to contrast with findings of other investigators, who demonstrated that heparin coatings significantly decreased platelet adhesion.\textsuperscript{22,23} However, our observation that platelet adhesion on collagen is not inhibited by the presence of immobilized heparin is in line with a previously reported study that demonstrated that heparin immobilization on carbodiimide crosslinked collagen even slightly increased the number of adherent platelets.\textsuperscript{14} Contradictory findings in the effects of immobilized heparin on platelet adhesion might be related to the type of surface to which heparin is bound.

Collagen-induced platelet procoagulant activity is mediated by the interaction of collagen with its platelet receptor, GPVI.\textsuperscript{24} It is apparent that crosslinking collagen with heparin did not inhibit the loss in phospholipid membrane asymmetry, as monitored by annexin-V binding. In contrast, collagen-induced platelet procoagulant activity was significantly reduced when collagen films were treated with mildly periodate oxidized heparin, implying that under these circumstances, when the polysaccharide chain of heparin is almost fully intact, immobilized heparin interferes with platelet activation by collagen. Whether immobilized heparin affects the interaction of collagen with its primary platelet receptor GPVI or with a secondary one (P2Y12 and/or P2Y12) awaits further experimentation. Because these experiments were performed with adherent platelets that were extensively washed, heparin-accelerated inactivation of traces of thrombin is highly unlikely. Interference of heparin with platelet activation by collagen was previously demonstrated under both static and flow conditions.\textsuperscript{25–27} One study showed that under static conditions or at low shear rate, immobilized heparin did not affect platelet deposition to collagen, but strongly inhibited the platelet release reaction.\textsuperscript{26} Another study in rats revealed that under flow conditions (low and high shear rate), heparin released by stimulated mast cells did not block \(\alpha_\text{IIb}\beta_\text{IIIa}\)-dependent platelet adhesion, but attenuated subsequent platelet activation as well as fibrinogen binding to platelets.\textsuperscript{27} Together, these results support the notion that mildly periodate oxidized heparin may interfere with collagen-induced platelet activation (ie, release reaction and exposure of negatively charged phospholipids in outer leaflet of the plasma membrane).

Exposure of heparinized collagen films with adherent (activated) platelets to recalcified platelet-free plasma demonstrated that, independent of the method of heparinization, thrombin generation is abolished completely during the time of the experiment (60 minutes). We surmise that the amount of AT (±2 pmol/cm\(^2\)) that binds to collagen films crosslinked with highly periodated heparin is already sufficient to prevent thrombin generation at these surfaces. These results extend earlier findings from our group illustrating that AT bound to immobilized heparin is probably the most effective way to prevent thrombus formation at an artificial surface.\textsuperscript{19,28,29} To confirm that the thromboreistance of the heparinized collagen surfaces was indeed AT-dependent, thrombin generation was measured with AT-depleted plasma. While thrombin generation on heparinized collagen with adhering platelets was strongly inhibited when exposed to normal plasma,
significant thrombin generation was observed when the same experiment was performed with AT-depleted plasma, suggesting that the thromboresistance of heparinized collagen surfaces is fully dependent on the presence of AT. The physiological relevance of our findings was established in a perfusion setup, where stents coated with heparinized collagen films were exposed to flowing whole blood. While an explosive thrombin formation was observed in blood that passes the nonheparinized collagen-coated stents, stents coated with heparinized collagen appeared to be fully thromboresistant. We postulate that immobilization of heparin effectively catalyzes the thrombin–antithrombin reaction, whereby levels of surface-located thrombin remain below the threshold values that are needed to exert its positive feedback reactions, such as activation of the cofactors factors V and VIII and generation of procoagulant cell membranes, that regulate thrombin formation. The in vitro finding that heparinized collagen films are highly thromboresistant encourages further research on the application of collagen in medical devices in contact with blood.

References
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Methods section: supplementary information to be published online only

Preparation of Collagen and Heparinized Collagen Sheets

All experiments were performed with collagen sheets with a thickness of approximately 20 µm. Collagen type I (0.25 g) derived from bovine achilles tendon (Syntacoll) was suspended in 100 ml deionized water (pH adjusted to 2 with HCl). The collagen suspension (2.5 mg/ml) was stirred for at least 3 hours. 22.5 g suspension was then poured into a flat polystyrene container with a bottom surface area of 5x5 cm² and dried under ambient conditions to obtain a sheet with a collagen density of 2.25 mg/cm².

Collagen crosslinking with heparin. Collagen was crosslinked using periodate treated heparin. Heparin (50 mg/ml) was treated with NaIO₄ (3.3 mg/ml) in a 0.05 M phosphate buffer, pH 6.9. The solution was stirred overnight in the dark. Under stirring, 1.5 ml heparin solution was added to 80 g collagen suspension (2.5 mg/ml) and pH adjusted to 11 with NaOH. The suspension was then stirred for 10 minutes. Finally, 8 mg NaCNBH₃ was added and 23.5 g suspension poured into a polystyrene container with a bottom surface area of 5x5 cm² and dried under ambient conditions.

Collagen crosslinking with EDC/NHS. Collagen films of 5x5 cm were crosslinked by the addition of 20 ml 0.2M MES buffer pH=5.5, containing 2.5 mg EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) and 1.2 mg NHS (N-hydroxysuccinimide). After incubation on a shaker for 4 hours, films were thoroughly rinsed with deionized water and allowed to dry at room temperature.

Heparinization of heparin- and EDC-cross-linked collagen. Mildly periodate oxidized heparin was prepared by mixing a solution of 5 mg/ml heparin in 0.05 M phosphate buffer pH=6.88 with 0.165 mg/ml NaIO₄. After overnight incubation, the heparin solution was diluted (1:5) into 0.2 M acetate buffer pH=4.66 containing 0.2 mg/ml NaCNBH₃. The
collagen films were incubated in 20 ml of this solution overnight while gently shaken. After that, films were thoroughly rinsed with deionized water and dried at room temperature.

**Determination of Antithrombin Binding to Heparinized Collagen**

The AT binding assay was performed as previously described.\textsuperscript{17} Briefly, thoroughly rinsed heparinized collagen films were incubated with 5 \( \mu \)M bovine AT. After one hour, unbound AT was removed by rinsing extensively with Hepes buffer. AT bound to immobilized heparin was eluted with heparin (5 U/ml). AT was measured by mixing 15 \( \mu \)l of the heparin eluant with 85 \( \mu \)l Hepes buffer containing 12 nM thrombin and 1.2 U/ml heparin. The residual thrombin activity was measured after five minute incubation at 37 °C using the thrombin substrate S2238. The amount of AT was calculated from the amount of inactivated thrombin.