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Design of a new type of coating for the controlled release of heparin

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Abstract

Thrombus formation at the surface of blood contacting devices can be prevented by local release of heparin. Preferably, the release rate should be constant for prolonged periods of time. The minimum heparin release rate to achieve thromboresistance will be different for various applications and should therefore be adjustable. In this study a new type of heparin release system is presented which may be applied as a coating for blood contacting devices. The system is based on the covalent immobilization of heparin onto porous structures via hydrolysable bonds. This approach was evaluated by the immobilization of heparin onto a porous cellulosic substrate via ester bonds. Cuprophan was used as a model substrate and *N,N'*-carbonyldiimidazole as a coupling agent. Heparinized Cuprophan incubated in phosphate buffered saline showed a release of heparin due to the hydrolysis of the ester bonds between heparin and Cuprophan. The release rate could be easily adjusted by varying the amount of coupling agent used during immobilization. Cuprophan with a rather stable heparin coating (release rate: 6.1 $mU/cm^2 \cdot h$) and Cuprophan which shows a substantial release of heparin (release rate up to 23.0 $mU/cm^2 \cdot h$) could be prepared. Except when the release was relatively high, release rates were constant for at least 1 week. Storage of the release system at ambient conditions up to 6 months or sterilization by means of steam, ethylene oxide exposure, or gamma irradiation did not affect the release properties. It was concluded that this concept for a heparin release system is highly promising to prepare thromboresistant surfaces for various blood contacting devices.

Keywords: Antithrombogenicity; Controlled release; Heparin; Hydrolysis of covalent bonds; Porous structures

1. Introduction

The application of blood contacting devices can be complicated by the formation of thrombi at their surface. Heparin is a very powerful anticoagulant. Therefore, an often applied strategy to prevent thrombus formation is to covalently immobilize heparin at the surface [1,2]. Immobilized heparin may interact with factors of the coagulation cascade thereby preventing thrombus formation. A disadvantage is that due to immobilization, the mobility of heparin is limited, even when spacers are used. As a result the activity of immobilized heparin can be strongly reduced as compared to unbound heparin [3–5]. Another strategy to prevent thrombus formation is to create a microenvironment of heparin at the surface of blood contacting devices [6–11]. Despite much research, the minimum heparin concentration which is required to achieve thromboresistance in such a microenvironment is still unknown. In litera-

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ture, concentrations of 0.2–7.0 μg/ml have been mentioned [12,13]. A microenvironment of heparin can be achieved by the immobilization of heparin at the surface and a subsequent release of heparin from the surface into blood. Since heparin is consumed in blood and transported along with the blood flow, a continuous release of heparin from the device is necessary to maintain the heparin concentration near the surface at the same level. Also with regard to the minimum release rate necessary to guarantee thromboresistance, there is some debate. Obviously, the minimum release rate will depend on the blood flow rate at the site of application and the geometry of the blood contacting device. Besides the consumption of heparin, these factors determine the depletion of heparin from the microenvironment [12]. But also for one particular application there is some confusion. Tanzawa et al. [11] stated that a catheter inserted into the inferior vena cava in dogs should release 4×10^{-2} μg/cm^2·min to ensure thromboresistance. In other reports, however, a ten times lower minimum release rate has been claimed for the same application [10,14]. Others proposed that the release rate can be much lower because heparin remaining immobilized at the surface will also display antithrombogenic activity [12,15–17].

Many types of heparin release systems have been developed. In most of these systems, heparin is immobilized by dispersing heparin within the biomaterial [7,18,19] or by ionic binding of heparin onto the biomaterial [6,8–11,14,20–22]. In most cases, after incubation in aqueous salt solutions, plasma, or blood, the heparin release rate is initially very high but decreases very rapidly in time [6,7,9,20,21,23–25]. Usually no release of heparin can be detected anymore already after 1–24 h while for many applications a release during a period of days to weeks is required. Only incidentally a prolonged release of heparin has been claimed [8,10,11,14,22].

From the discussion above, it is clear that there is a need for a heparin release system with which the release rate remains constant for a prolonged period of time and of which the release rate can be easily adjusted.

In this study, a new concept is presented for a heparin release system which meets these requirements. This concept is based on a combination of two different approaches. Firstly, heparin is covalently immobilized onto a substrate via hydrolysable bonds. By changing the heparinization conditions, the number of covalent bonds between heparin and the substrate can be adjusted and therefore also the release rate. Secondly, the substrate has a porous structure. By using a porous structure, a very high surface area is available for heparin immobilization. A very high amount of immobilized heparin guarantees the continuous supply of heparin for prolonged periods of time to be released. Furthermore, because of the very high amounts of immobilized heparin, a constant release rate for prolonged periods of time may be expected. A release system according to this concept applied as coating for blood contacting devices may assure thromboresistance for long-term applications. In this study, a porous cellulosic membrane (Cuprophan) is used as a model substrate and *N,N′*-carbonyldiimidazole (CDI) is used as a coupling agent to immobilize heparin via hydrolysable bonds.

2. Materials and methods

2.1. Materials

Cuprophan 150 M was a gift from AKZO (Wuppertal, Germany). Formamide (synthesis grade, Merck, Darmstadt, Germany) was purified and dehydrated according to a method as described by Verhoek et al. [26] and stored beneath a blanket of dry nitrogen. Heparin (sodium salt) from porcine intestinal mucosa (activity: 177 U/mg) was purchased from Diosynth (Oss, The Netherlands). Heparin also from porcine intestinal mucosa of which 55–60% of the sodium ions was substituted by benzyltrimethylammonium ions (heparin-triton B) was purchased from HBG (Enschede, The Netherlands). Chromogenic substrate S_{2222} was purchased from Chromogenix (Mölndal, Sweden). Polyethylene glycol (6000 g/mol, PEG); human antithrombin III (AT III, Sigma, St Louis, USA) was purified using a heparin-Sepharose column [3,27] (Sigma). Bovine albumin (Alb) and human activated factor X (factor Xa) were purchased from Sigma. Partial thromboplastin time (PTT) reagent was obtained from Boehringer (Mannhein, Germany). CPDA-1 plasma (plasma supplemented with citrate, phosphate, dextrose, and adenine) was purchased from Rode Kruis Bloedbank Twente-Achterhoek (Enschede, The Nether-

lands). Phosphate buffered saline (PBS, pH 7.4) was purchased from NPBI (Emmercompascuum, The Netherlands). Acetone, benzoic acid, benzyl alcohol, CDI, cyclohexanol, D_2O, glacial acetic acid, 1 N HCl, NaCl, 1 N NaOH, NaN_3, 4-phenyl-1-butanol, P_2O_5, toluidine blue O zinc chloride double salt, tris(hydroxymethyl)-ammonium methane (Tris) (all analytical grade) were purchased from Merck. All chemicals were used without further purification except when mentioned.

2.2. Heparinization of Cuprophan

Cuprophan was heparinized by means of a procedure as described by Engbers et al. [28]. Heparin-triton B was dissolved in dry formamide beneath a blanket of dry nitrogen. CDI was added to the solution under vigorous stirring. Thereafter the pressure was reduced to about 10^{-2} mBar. After 60 min, the pressure was elevated to 1 atm with dry nitrogen after which the reaction mixture was transferred to Cuprophan membranes which had been thoroughly rinsed with formamide to remove glycerol and water. The immobilization reaction was also carried out beneath a blanket of dry nitrogen. After immobilization, the reaction was quenched by replacing the reaction mixture by demineralized water. Subsequently the membranes were thoroughly rinsed with demineralized water then incubated in an aqueous 4 M NaCl solution. Thereafter, the membranes were again thoroughly rinsed with demineralized water, soaked in a 10 wt% solution of glycerol in demineralized water and then dried by air exposure.

Immobilization was performed at weight ratios heparin/CDI of 5, 10, and 30 yielding membranes with 60–85 μg immobilized heparin per cm^2 membrane surface area (defined as the outer surface of the membrane). The membranes were either not sterilized or sterilized by steam, ethylene oxide (EtO) exposure, or gamma irradiation using standard procedures [29].

2.3. Activated partial thromboplastin time (APTT) assay

The anticoagulant activity of solutions of heparin in PBS was determined by means of an APTT assay. Fresh frozen human CPDA-1 plasma was thawed at 37°C and stored on ice. To 50 μl heparin solution in PBS, 50 μl plasma and 50 μl PTT reagent was added and mixed for 3 min at 37°C. Thereafter, 50 μl of an aqueous 20 mM $CaCl_2$ solution was added. The suspension was mixed again and the coagulation time was determined at 37°C using a coagulatometer (LC-6, Lode, Groningen, The Netherlands). A calibration curve was obtained by measuring the coagulation time of solutions of heparin in PBS of known activities (0–0.7 U/ml). The calibration curve was used to calculate the anticoagulant activity of the heparin solutions.

2.4. Factor Xa inactivation assay

The anticoagulant activity of solutions of heparin in PBS was also determined by means of a factor Xa inactivation assay. Therefore, solutions of substrate S_{2222} (2.0 mg/ml)/AT III (70 mU/ml) and solutions of factor Xa (0.4 U/ml) were prepared using a buffer solution consisting of an aqueous solution of 50 mmol/l Tris, 1.0 g/l PEG, 1.0 g/l Alb, and 150 mmol NaCl which was adjusted to pH 8.4. To 150 μl of substrate S_{2222}/AT III solution, 40 μl of the heparin solution was added. Subsequently, 50 μl of the factor Xa solution was added. The resulting mixture was shaken for 10 min at room temperature. Thereafter, the reaction was quenched by adding 70 μl of 40 vol% acetic acid. Subsequently, 200 μl of the solution was taken from which the extinction was measured at 405 nm. A calibration curve was obtained using solutions of heparin in PBS of known activities (0–20 mU/ml). The calibration curve was used to calculate the activity of heparin in the solutions.

2.5. Toluidine blue solution assay

A toluidine blue assay was developed to study the effects of various treatments of heparin (see below) on the capacity of heparin to complex with toluidine blue or to determine the heparin concentration in solution. To 2 ml of a heparin solution in aqueous 0.01 N HCl/0.2 wt% NaCl or PBS, 2 ml of a freshly prepared solution of 0.04 wt% toluidine blue in aqueous 0.01 N HCl/0.2 wt% NaCl, was added. The mixture was gently shaken for 4 h during which the formed heparin/toluidine blue complex precipitated. Subsequently, the mixture was centrifugated at

1000×g for 10 min. The amount of toluidine blue in the heparin/toluidine blue complex (i.e. the amount of toluidine blue in the precipitate) was determined as follows. The precipitate was rinsed with aqueous 0.01 N HCl/0.2 wt% NaCl and then dissolved in 5 ml of a 4:1 (v/v) mixture of ethanol and aqueous 0.1 N NaOH. The extinction of the resultant solution was measured at 530 nm. To obtain a calibration curve, the same procedure was carried out with solutions of untreated heparin of known concentrations. The calibration curve was used to relate the capacity of treated heparin to complex with toluidine blue with untreated heparin or to determine the concentration of heparin in solution.

2.6. Toluidine blue membrane assay

The amount of immobilized heparin was determined by a procedure as described by Smith et al. [30] and modified for our purposes. The membranes were cut in discs with a diameter of 7 mm. The immobilized heparin was stained by incubating the membrane in 5 ml of a freshly prepared solution of 0.04 wt% toluidine blue in aqueous 0.01 N HCl/0.2 wt% NaCl. After 4 h of gently shaking, the membrane was rinsed twice with demineralized water and then incubated into 5 ml of a 4/1 (v/v) mixture of ethanol and aqueous 0.1 N NaOH. In this medium the heparin/toluidine blue complex is broken and toluidine blue is released into the fluid phase. After complete decolourization of the membrane, the extinction of the fluid phase was measured at 530 nm. The extinction was related to the amount of immobilized heparin using the calibration curve as described under 'toluidine blue solution assay'. The results of experiments using radiolabeled heparin confirmed that the heparin/toluidine blue ratio in the complex was the same for immobilized heparin and heparin in solution (data not shown).

2.7. Activation of heparin with CDI

Theoretically, CDI can activate carboxylic acid as well as hydroxyl groups [31] of heparin. The reaction of carboxylic acid groups with CDI results in the evolution of CO_2 whereas during the reaction of hydroxyl groups with CDI no CO_2 is formed (see Fig. 1a). The activation of heparin was therefore studied by measuring the evolution of CO_2 using an experimental set-up as shown in Fig. 2. To a solution

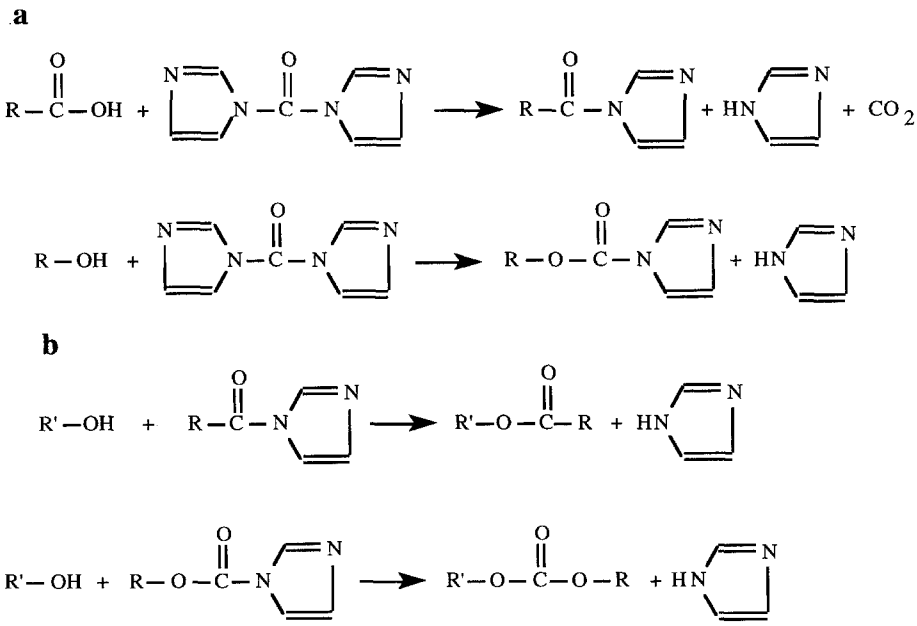

Fig. 1. (a) Activation reactions of carboxylic acid and hydroxyl groups with CDI. (b) Coupling reactions of CDI-activated carboxylic acid groups and CDI-activated hydroxyl groups with hydroxyl groups.

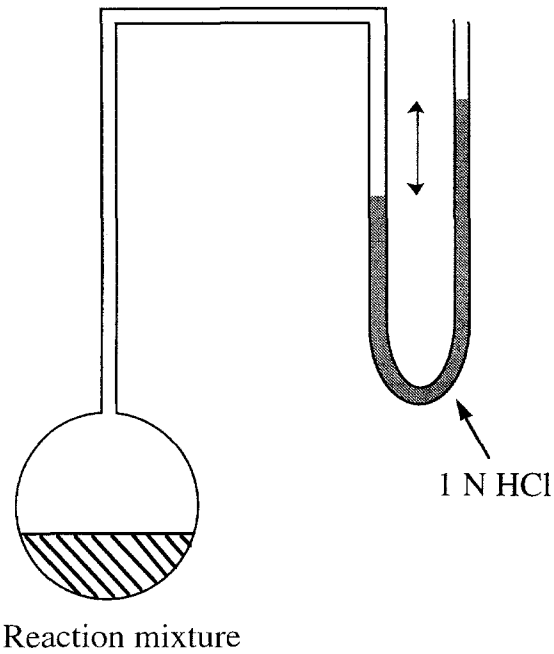

Fig. 2. Schematic drawing of the experimental set-up for the measurement of CO_2 release.

of 666 mg heparin-triton B in 5 ml formamide, 1 mmol CDI was added. After 1 h the amount of CO_2 released from the reaction mixture was measured. Subsequently, a large excess of glacial acetic acid was added to allow any remaining CDI to react. The amount of CO_2 released from the reaction mixture after addition of glacial acetic acid was measured again. A calibration curve was obtained by measuring the amount of CO_2 released after addition of an excess of glacial acetic acid to solutions of various amounts of CDI in formamide. The calibration curve was used to relate the amount of released CO_2 to the amount of activated carboxylic acid groups of heparin and the amount of unreacted CDI after 1 h of activation. These values were used to calculate the amount of reacted hydroxyl groups of heparin.

2.8. Immobilization of model compounds onto Cuprophan

Benzoic acid and benzyl alcohol were used as model compounds for carboxylic acid and hydroxyl groups of heparin, respectively. The reactions of CDI-activated carboxylic acid and CDI-activated hydroxyl groups with the hydroxyl groups of Cuprophan yield ester and carbonate bonds, respectively [31] (see Fig. 1b). To immobilize the model compounds onto Cuprophan, the same procedure was applied as used for the immobilization of heparin onto Cuprophan. The immobilization times and the concentrations of the model compounds were varied. The amounts of immobilized benzoic acid or benzyl alcohol were determined as follows. The membranes were incubated in 0.1 N NaOH for 24 h at ambient temperature. FTIR-spectroscopy revealed that after incubation all covalent bonds between the model compounds and Cuprophan were broken (data not shown). The extinction of the NaOH/benzoic acid and NaOH/benzyl alcohol solutions was determined at 266 nm and 256 nm, respectively. Calibration curves were used to calculate the concentrations of the model compounds.

2.9. Coupling of heparin to model compounds

Cyclohexanol and 4-phenyl-1-butanol were used as model compounds for Cuprophan. Heparin was covalently linked to the model compounds by means of the same procedure as used for the immobilization of heparin onto Cuprophan except that after activation of heparin with CDI, the reaction mixture was added to solutions of the model compounds in formamide instead of to Cuprophan. After coupling, the reaction mixtures were precipitated in and thoroughly washed with acetone. Subsequently the precipitates were dried over P_2O_5 in vacuo and then dissolved in D_2O. The resulting solutions were analysed with proton decoupled ^{13}C-NMR spectroscopy.

2.10. Release of heparin from heparinized Cuprophan in PBS

Non-sterilized and sterilized heparinized Cuprophan was incubated in tubes containing PBS. PBS was supplemented with NaN_3 (final concentration: 0.02 wt%) to prevent bacterial growth. The tubes were placed in a water bath of 37°C and gently shaken. At different time intervals, samples of PBS were taken and/or the membranes were rinsed with water, soaked into a 10 wt% solution of glycerol in water, and then dried by air exposure. The amount of

heparin released into PBS was determined by means of the APTT assay and in some cases also by means of the toluidine blue solution assay and the factor Xa inactivation assay. The amount of heparin remaining immobilized onto Cuprophan was determined by means of the toluidine blue membrane assay.

2.11. Effect of storage

Non-sterilized Cuprophan heparinized at a weight ratio of heparin/CDI of 30 was exposed to air at ambient temperature for various time intervals up to 6 months. Thereafter, the membranes were incubated in PBS at 37°C for 1 week. Before and after incubation the amount of immobilized heparin was determined by means of the toluidine blue membrane assay.

2.12. Effects of various treatments of heparin on the complexation of heparin with toluidine blue and the anticoagulant activity of heparin

The sodium salt of heparin was dissolved in PBS/NaN_3 and kept at 37°C for 1 week. Subsequently, the solution was dialysed against demineralized water at 4°C for 2 days, during which the dialysate was refreshed twice a day, and then lyophilized. In other control experiments heparin-triton B was dissolved in formamide and allowed to react with various amounts of CDI for 2 days. Subsequently, heparin was precipitated by pouring the reaction solution into a large excess of acetone. The precipitate was thoroughly washed with acetone and then dried by air exposure. Subsequently, heparin was dissolved in aqueous 4 M NaCl to hydrolyse CDI-activated groups of heparin and to convert the triton B salt of heparin into its sodium salt (triton B/Na^+ =1/100 (mol/mol)). After three days, the solution was dialyzed against water at 4°C. After 1 day, an additional amount of NaCl was added (triton B/Na^+ 1:5, mol/mol). Dialysis was continued for 5 day during which the dialysate was refreshed twice a day. Thereafter the solution was lyophilized. In a third series of experiments, heparin-triton B was dissolved in aqueous 4 M NaCl and treated likewise. Photospectrometry confirmed that during the procedure heparin-triton B was completely converted into its sodium salt (extinctions of triton B dissolved in water are at 257, 262, and 268 nm, detection level was less than 1% of the original triton B concentration, data not shown).

The treated heparin samples were evaluated for their capacities to complex with toluidine blue (toluidine blue solution assay) and their anticoagulant activity (APTT assay).

3. Results

3.1. Activation of heparin with CDI

The activation of heparin with CDI was studied by measuring the evolution of CO_2. After 1 h of activation, the reaction was quenched by the addition of an excess of glacial acetic acid. It was found that after quenching no CO_2 released. Therefore it is concluded that after 1 h of activation, all CDI had been reacted. From the amount of CO_2 released during activation, it was calculated that 69.7±9.3 mol% of CDI had activated carboxylic acid groups (n=3). It can be assumed that CDI only reacts with carboxylic acid and hydroxyl groups of heparin [31]. Therefore, the remaining amount of CDI, 30.3±9.3 mol% had activated hydroxyl groups.

3.2. Immobilization of model compounds onto Cuprophan

Benzoic acid and benzyl alcohol were used as model compounds to compare the reactivity of CDI-activated carboxylic acid and CDI-activated hydroxyl groups towards the hydroxyl groups of Cuprophan. As shown in Fig. 3, CDI-activated carboxylic acid groups reacted much more rapidly with the hydroxyl groups of Cuprophan than CDI-activated hydroxyl groups (note the different time scales in Fig. 3a and b). The initial reaction rate shows a linear relationship with the concentration of the model compounds (see Fig. 4). Therefore, the immobilization of the model compounds onto Cuprophan was for both cases a first order process for the model compounds. Also from these graphs it is clear that the reaction rate for the immobilization of CDI-activated benzoic acid (reaction constant defined as the slope of the line in Fig. 4: $0.18 \cdot 10^{-3}$ cm/h) was much higher

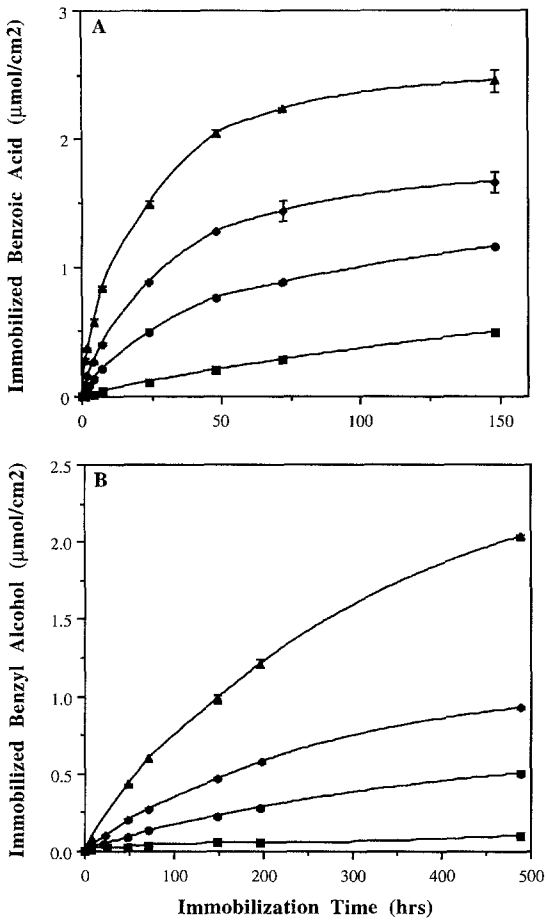

Fig. 3. Immobilization of CDI-activated model compounds onto Cuprophan as a function of time at different concentrations of the model compounds: 0.1 mmol/ml ■, 0.3 mmol/ml ·, 0.5 mmol/ml ♦, 1.0 mmol/ml ▲, $n=3\pm$S.D. (A) Benzoic acid. (B) Benzyl alcohol.

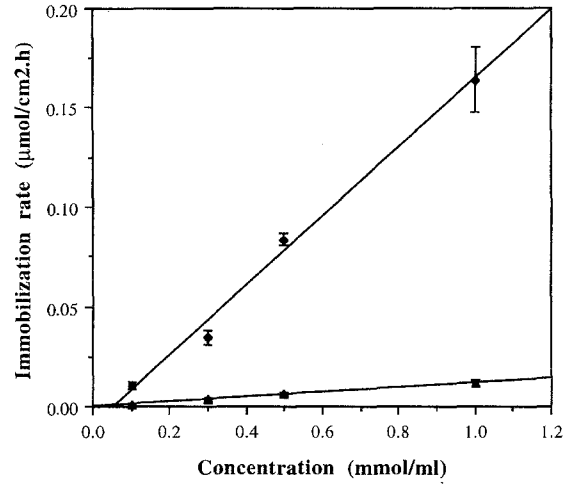

Fig. 4. Initial rate of immobilization of CDI-activated model compound onto Cuprophan as a function of the concentration of the model compounds. Benzoic acid ♦, benzyl alcohol ▲.

than that of CDI-activated benzyl alcohol (reaction constant: $0.012\cdot10^{-3}$ cm/h).

3.3. Coupling of heparin to model compounds

The immobilization mechanism of heparin onto Cuprophan was also studied using cyclohexanol and 4-phenyl-1-butanol as model compounds for secondary and primary hydroxyl groups of Cuprophan respectively. The products of the reactions between CDI-activated heparin and the model compounds were analyzed by means of ^{13}C-NMR spectroscopy. ^{13}C-NMR spectroscopy is a suitable technique to investigate whether ester or carbonate bonds were formed since these bonds yield distinct peaks at 167 ppm and 155 ppm, respectively. In the ^{13}C-NMR spectra of both reaction products, strong signals at 167 ppm were observed while no signals at 155 ppm could be detected. Apparently, the reactions of CDI-activated heparin with secondary as well as primary hydroxyl groups were dominated by the formation of ester bonds while carbonate bonds were not or hardly formed.

3.4. Release of heparin from heparinized Cuprophan in PBS

The release of heparin from heparinized Cuprophan in PBS at 37°C was investigated. The release of heparin during 1 week of incubation as determined by means of the APTT-assay as a function of time was linear for Cuprophan heparinized at weight ratios heparin/CDI of 5 and 10 while for Cuprophan heparinized at a weight ratio heparin/CDI of 30 this linearity was lost after three days of incubation (see Fig. 5). The amount of released heparin after 1 week of incubation was also determined by means of the factor Xa inactivation assay and by both toluidine blue assays. The data were analysed by means of ANOVA analysis of variance and considered as significantly different when $P<0.05$. The results of

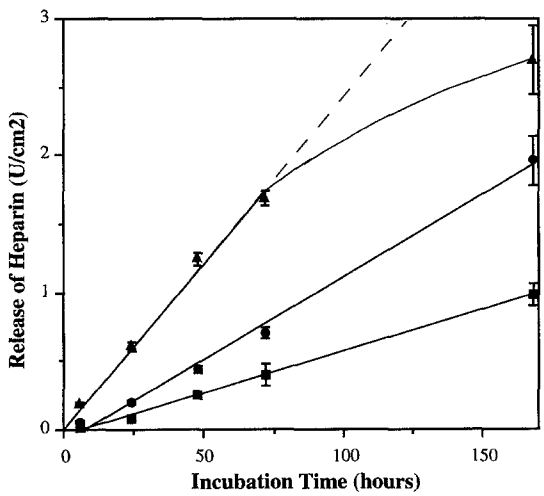

Fig. 5. Release of heparin from Cuprophan heparinized at various heparin/CDI ratios (weight ratio heparin/CDI of 5 ■, 10 ·, 30 ▲) after incubation in PBS at 37°C as a function of time as determined by means of the APTT assay (n=4±S.D.).

the APTT assay did not significantly differ from those of the factor Xa inactivation assay (see Table 1). Also the two different toluidine blue assays did not significantly differ. However the biological assays yielded much lower values for the amount of released heparin than the toluidine blue assays ($P<$ 0.001). After 8 weeks of incubation of Cuprophan heparinized at a weight ratio heparin/CDI of 30 in PBS at 37°C, the amount of released heparin as determined by means of the APTT was 6.46±0.81 U/cm^2. Assuming that the released heparin has the same activity as that of the starting material, the amount of released heparin equals to 36.5±4.6 μg/cm^2. The amount of released heparin as determined by measuring the amount of immobilized heparin before and after incubation by means of the toluidine blue membrane assay was 46.8±1.7 μg/cm^2. Apparently, the relative difference between the amount of released heparin as determined by means of the APTT and toluidine blue assay decreases with increasing incubation times.

Furthermore, the effects of various sterilization methods on the release of heparin were investigated. The data were analysed by means of ANOVA analysis of variance and considered as significantly different when $P<0.05$. It appeared that sterilization of heparinized Cuprophan by means of steam, EtO exposure, or gamma irradiation did not significantly affect the amount of released heparin during 1 week of incubation in PBS at 37°C as measured by means of the APTT assay ($P<0.001$; see Table 2).

3.5. Effect of storage

The effect of storage on the release properties of heparin from heparinized Cuprophan was investigated. Cuprophan heparinized at a weight ratio of heparin/CDI of 30 was exposed to air at ambient temperatures for various periods of time up to 6 months. Thereafter, the membranes were incubated

Table 1
Release of heparin from Cuprophan heparinized at different heparin/CDI ratios expressed in wt% (±S.D.) of the initial amount of immobilized heparin after 1 week of incubation in PBS at 37°C as determined by four different methods

Method of analysis	Heparin/CDI=5[a]	Heparin/CDI=10[a]	Heparin/CDI=30[a]
APTT[b,c]	7.3±0.6	13.3±0.9	23.2±1.2
Factor Xa inactivation[b,c]	7.2±0.3	13.3±3.1	25.4±3.2
Toluidine blue membrane[d]	14.8±2.3	28.2±5.2	46.3±2.6
Toluidine blue solution[d,e]	13.6±2.2	27.1±2.7	39.5±5.0

[a]Weight ratio applied during heparinization.
[b]n=4.
[c]The assays yield the release of heparin in U. The release of heparin expressed in μg was calculated assuming that the activity of heparin in the release medium was the same as that of the starting material (177 U/mg). The amount of immobilized heparin before incubation was determined by means of the toluidine blue membrane assay (n=3). The results of both assays were used to calculate the release of heparin expressed in wt% of the initially amount of immobilized heparin.
[d]n=3.
[e]The assay yields the release of heparin in μg. The amount of immobilized heparin before incubation was determined by means of the toluidine blue membrane assay (n=3). The results of both assays were used to calculate the release of heparin expressed in wt% of the initial amount of immobilized heparin.

Table 2
Release of heparin from non-sterilized and sterilized heparinized Cuprophan after 1 week of incubation in PBS at 37°C expressed in wt% (±S.D.) of the initial amount of immobilized heparin[a]

Sterilization method	Heparin/CDI=5[b]	Heparin/CDI=10[b]	Heparin/CDI=30[b]
Non-sterilized	7.3±0.6	13.3±0.9	23.2±1.2
Steam	6.5±0.2	14.7±1.1	26.5±2.1
EtO exposure	5.6±0.4	12.4±0.8	25.3±2.2
Gamma-irradiation	6.1±0.4	12.3±0.6	21.9±1.1

[a]The amount of heparin in the release medium was determined by means of the APTT assay (n=4). The assay yields the release of heparin in U. The release of heparin expressed in μg was calculated assuming that the activity of heparin in the release medium was the same as that of the starting material (177 U/mg). The amount of immobilized heparin before incubation was determined by means of the toluidine blue membrane assay (n=3). The results of both assays were used to calculate the release of heparin expressed in wt%.
[b]Weight ratio applied during heparinization.

in PBS at 37°C for 1 week. The release of heparin was determined by measuring the amount of immobilized heparin before and after incubation by means of the toluidine blue membrane assay. The results (see Table 3) show that exposure of heparinized Cuprophan to air at ambient temperatures up to six months did not significantly affect the amount of immobilized heparin (Student's t: $P>0.05$). Although the results of the release measurements were somewhat scattered, the release as a function of storage time showed no clear increase or decrease.

3.6. Effects of various treatments on the complexation of heparin with toluidine blue and the anticoagulant activity of heparin

It was investigated whether treatment of heparin with CDI affects the properties of heparin. In control experiments also the effects of the exchange of sodium ions by triton B and subsequent exchange of triton B by sodium ions and the dissolution of heparin in formamide and PBS were investigated. Therefore, the effect of these treatments on the anticoagulant activity (APTT-assay) and the capacity of heparin to complex with toluidine blue (toluidine blue solution assay) was determined. As shown in Table 4 these treatments had moderate effects on the anticoagulant activity and no significant effects (Student's t: $P>0.05$) on the capacity of heparin to complex with toluidine blue.

4. Discussion

The aim of this study was to evaluate a new design for a coating which releases heparin at a constant rate for prolonged periods of time and of which the release rate can be adjusted. It was hypothesized that a coating consisting of a porous structure onto which heparin is immobilized via hydrolysable bonds should meet these requirements. To evaluate this hypothesis, Cuprophan was used as

Table 3
Effect of storage time on the amount of immobilized heparin and the release properties of Cuprophan heparinized at a weight ratio heparin/CDI of 30 (n=3, ±S.D.)

Storage time(months)	Amount of immobilized heparin (μg/cm^2)[a]		Release of heparin(%)
	Before incubation	After incubation[b]	
0	63.2±3.1	—	—
1	65.2±0.1	37.5±1.0	42.5±1.3
2.5	63.6±1.0	40.3±1.7	36.6±2.1
4	63.3±2.5	34.0±0.3	46.2±2.3
5	65.9±2.5	39.9±0.3	39.4±3.7
6	65.2±2.9	41.3±2.2	36.6±6.3

[a]Determined by means of the toluidine blue membrane assay.
[b]Membranes were incubated in PBS/NaN_3 at 37°C for 1 week.

Table 4
Effects of various treatments on the complexation of heparin with toluidine blue and the anticoagulant activity of heparin

Treatment	Toluidine blue solution (n=3, ±S.D.)[a]	APTT (n=4, ±S.D.)[b]
CDI(30/1)[c]	96.4±2.2	83.4±3.3[g]
CDI(5/1)[c]	97.2±1.1	92.2±4.5
Triton B-Hep/form[d]	96.3±0.9	88.7±3.0[g]
Triton B-Hep/NaCl[e]	100.5±2.1	95.2±5.4
Na-Hep/PBS[f]	103.1±6.5	102.8±4.8

[a]Degree of complexation of toluidine blue with treated heparin relative to untreated heparin in %.
[b]Anticoagulant activity determined by means of the APTT assay of treated heparin relative to untreated heparin in %.
[c]Triton B-heparin dissolved in formamide was treated with CDI (weight ratios heparin/CDI of 5 and 30). Subsequently treated heparin was successively precipitated in and thoroughly washed with acetone, dissolved in aqueous 4 M NaCl (3 days), dialysed against water (6 days), and lyophilized.
[d]Triton B-heparin was successively dissolved in formamide, precipitated in and thoroughly washed with acetone, dissolved in aqueous 4 M NaCl (3 days), dialysed against water (6 days), and lyophilized.
[e]Triton B-heparin was successively dissolved in aqueous 4 M NaCl (3 days), dialysed against water (6 days), and lyophilized.
[f]Na-heparin was dissolved in PBS/NaN_3 and stored for 1 week at 37°C. The solution was subsequently dialysed against water (2 days) and then lyophilized.
[g]Significantly different from Na-Hep/PBS (Student's t: $P<0.05$).

a model substrate and CDI was used as a coupling agent to immobilize heparin.

The results of the present study confirm the hypothesis. Cuprophan heparinized by means of the CDI-activation procedure forms an ideal system for the controlled release of heparin. The release profiles can be adjusted very easily by changing the heparin/CDI ratio applied during heparinization. Furthermore, the heparin release rate of Cuprophan heparinized at weight ratios heparin/CDI of 5 and 10 remained constant for at least 1 week of incubation in PBS at 37°C. When the rate of heparin release is relatively high as found in the case of Cuprophan heparinized at a weight ratio heparin/CDI of 30, a constant heparin release rate was limited to 3 days of incubation after which the release rate gradually decreased.

The effect of the heparin/CDI ratio on the release properties clearly indicates that the release of heparin is due to the cleavage of covalent bonds. The more CDI is used, the more covalent bonds between heparin and Cuprophan will be formed. Therefore, the release rate will decrease when the heparin/CDI ratio is decreased. FTIR-spectroscopy also confirmed that the release of heparin is due to the cleavage of covalent bonds. The carbonyl groups of the covalent bonds between heparin and Cuprophan give rise to an absorption peak around 1740 cm^{-1}. After 1 week of incubation, the surface area of this peak was much smaller (data not shown).

CO_2-release experiments and experiments with model compounds showed that activation of heparin with CDI yields more than twice as many activated carboxylic acid groups than activated hydroxyl groups and that activated carboxylic acid groups react much faster with hydroxyl groups of Cuprophan than activated hydroxyl groups. Furthermore, in ^{13}C-NMR spectra of products of the reaction between CDI-activated heparin with model compounds only ester bonds and no carbonate bonds could be identified. The results of the CO_2 release experiments and the model reactions strongly indicate that heparinization of Cuprophan by means of the CDI activation procedure yielded (nearly) only ester bonds and no (or hardly any) carbonate bonds. These findings are in agreement with the results of Staab [31] who studied the coupling of small organic compounds using CDI. He also found that ester bonds are more easily formed than carbonate bonds. It is well-known that ester bonds are susceptible to hydrolysis. Therefore, it is concluded that the release of heparin is due to the hydrolysis of ester bonds between heparin and Cuprophan. The rate of hydrolysis of these bonds was relatively high. The

model compound, benzoic acid, was also immobilized onto Cuprophan via ester bonds. Incubation of these membranes in PBS resulted in a very slow hydrolysis of the esters bonds. The difference may be caused by the fact that heparin is negatively charged in PBS. These negative charges may have a catalytic effect on the hydrolysis of ester bonds.

Exposure of heparinized Cuprophan to air at ambient temperature up to six months did neither significantly affect the amount of immobilized heparin nor the release properties. Apparently, the covalent bonds between heparin and Cuprophan are stable towards moisture in the air at ambient temperature for a long period of time. It can be expected that due to the hydrophilicity of heparinized Cuprophan water will absorb from the air. Absorption of water may lead to hydrolysis of the ester bonds between heparin and Cuprophan. As found, exposure to air at ambient temperature did not result in significant hydrolysis of covalent linkages between heparin and Cuprophan. Possibly not enough water had absorbed but also temperature effects may have played an important role. The membranes were exposed to air at ambient temperature whereas the release experiments were carried out at 37°C. It is known that the hydrolysis of ester bonds can strongly depend on temperature. Preliminary experiments indeed showed that the release of heparin from heparinized Cuprophan incubated in PBS dramatically decreases when the temperature is decreased (data not shown). Sterilization of heparinized Cuprophan by means of steam, EtO exposure, or gamma irradiation did not affect the release properties. Apparently, also these treatments had no effect on the covalent linkages between heparin and Cuprophan. Furthermore neither significant cleavage of heparin chains nor modification of immobilized heparin had occurred during sterilization.

The release of heparin from heparinized Cuprophan after 1 week of incubation in PBS at 37°C was investigated by means of the APTT, factor Xa inactivation, toluidine blue membrane, and toluidine blue solution assays. The APTT and factor Xa inactivation assays are based on the anticoagulant activity of heparin whereas both toluidine blue assays are based on the complexation of toluidine blue with the negative charges of heparin. To compare the results of the four different assays, the release of heparin in wt% of the initial amount of immobilized heparin was calculated with the assumptions that heparin released from the membrane had the same anticoagulant activity and complexed with toluidine blue in the same ratio as the starting material (see Table 1). Because these calculations yielded much lower values for the release determined by both biological assays than those determined by both chemical assays, it is clear that the assumptions mentioned above are not valid. It was therefore investigated whether the properties of heparin were affected during the immobilization procedure and/or incubation in PBS at 37°C. These control experiments, however, revealed that these treatments had no or moderate effects on the anticoagulant activity of heparin or the capacity of heparin to complex with toluidine blue (see Table 4). The results of the APTT and the toluidine blue membrane assays were in better agreement when heparinized Cuprophan was incubated in PBS at 37°C for 8 weeks instead of 1 week. These results indicate that the differences between the results of biological and chemical assays can be ascribed by the following mechanism. Heparin is a polydisperse polysaccharide containing fractions of various anticoagulant activities and molecular weights. Because the anticoagulant activity of heparin increases with increasing molecular weight [32–34], the number of covalent bonds between heparin and Cuprophan will increase with increasing anticoagulant activity. The less covalent bonds between a heparin molecule and Cuprophan exists, the easier the heparin molecule will be released. Therefore, the anticoagulant activity of heparin released from the membrane will increase with the incubation time until all heparin is released. At that time, the average activity of all heparin released will be the same as that of the starting material. The gross chemical structures of heparin fractions of different anticoagulant activities are very similar [33,34]. Therefore, the complexation characteristics of heparin with toluidine blue will be independent of the anticoagulant activity. Consequently, the capacity of heparin released from the membrane to complex with toluidine blue will not change with the incubation time and will be the same as that of the starting material. Therefore the differ-

ences between the calculated values for the release of heparin in wt% using the results of the biological and chemical assays will be large at short incubation times but will decrease with increasing incubation times.

Heparinization of Cuprophan by means of the CDI-activation procedure yielded membranes with 60–85 μg of immobilized heparin per cm^2 membrane surface. These amounts of immobilized heparin are much higher than when only a monolayer of heparin at the surface of the membrane would have formed. In that case a density of 0.1–2.0 μg of immobilized heparin per cm^2 is expected [35]. Cuprophan has a porous structure with pores of 25 Å in diameter when swollen in water [36]. Colton et al. [37] and Klein et al. [38] showed that Cuprophan is permeable for heparin. Therefore, it can be assumed that heparin is also immobilized in the pores of the membrane. Given the amount of heparin released from the membrane, it is clear that heparin immobilized in the pores also contributes to the release. The effects of the diffusion characteristics of heparin in the porous matrix on the release profiles, however, remain unclear. A high amount of immobilized heparin is necessary to allow for a release of substantial amounts of heparin during prolonged periods of time. Those amounts of immobilized heparin can never be reached when the immobilization of heparin is restricted to the surface of the membrane. The results of our study show that application of porous structures can overcome this problem because the pores provide a very large surface area for the immobilization of high amounts of heparin.

As mentioned above, the relatively high heparin release rate, as observed with Cuprophan heparinized at a weight ratio heparin/CDI of 30, remained constant for 3 days after which it decreased. Most likely, this decrease was due to the substantial depletion of the amount of immobilized heparin. Although the initial release rate of Cuprophan heparinized at a weight ratio heparin/CDI of 30 is relatively high, it may not be sufficient to assure thromboresistance. Furthermore, a constant release rate of 3 days may be too short. It can be expected that if more heparin is immobilized, the release rate will be higher and will be constant for a prolonged period of time. An increased amount of immobilized heparin can for example be achieved by the application of membranes with increased thickness and/or porosity. Furthermore, the release rate can be increased by increasing the heparin/CDI ratio applied during heparinization.

Application of the concept for a heparin release system as described in this study will not be restricted to cellulosic membranes as substrates. Also other porous substrates might be applicable providing that these materials possess hydroxyl groups which are necessary to immobilize heparin via hydrolysable bonds by means of the CDI-activation procedure. Because not only the heparin/CDI ratio but also the substrate characteristics can be varied, controlled release systems with a very broad range of heparin release rates which remain constant for prolonged periods of time can be established. These controlled release systems used as coatings may successfully prevent thrombus formation at the surface of blood contacting devices for various applications.

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