Protamine Immobilization and Heparin Adsorption on the Protamine-Bound Cellulose Fiber Membrane

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Immobilization of protamine to the inner lumen of cellulose hollow fibers has been shown useful in preventing both heparin- and protamine-induced complications during an extracorporeal blood circulation procedure. The current study examined the effects of variables on the immobilization of protamine to cyanogen bromide (CNBr)-activated cellulose hollow fibers. The degree of protamine immobilization was controlled by three independent parameters: the amount of CNBr used during the activation process, the duration of the coupling process, and the protamine concentration in the coupling solution. By the adjustment of these parameters, cellulose fibers containing desired amounts of immobilized protamine (ranging from 1 to 20 mg of immobilized protamine per gram of dry fibers) were readily prepared.

Heparin adsorption to the protamine-bound cellulose fibers was also examined. The adsorption isotherm followed a Langmuir adsorption model. The amount of heparin adsorbed was dependent on both the heparin concentration in the substrate solution and the protamine loading on the fibers. The Langmuir adsorption constant K was estimated to be 0.37 ±0.06 mL/mg, whereas the saturation capacity Q_s of the protamine-bound fibers increased with increasing the protamine loading.

Key words: Heparin • protamine immobilization • cyanogen bromide activation • cellulose hollow fibers • Langmuir adsorption isotherm

INTRODUCTION

Heparin employed in current extracorporeal blood circulation procedures often leads to hemorrhagic complications.[5,11] Protamine sulfate employed clinically for heparin neutralization can cause adverse hemodynamic responses.[7,10] To prevent both types of complications, a new approach has been proposed.[22] The approach consists of placing a filter device containing immobilized protamine (i.e., the protamine filter) at the distal end of the extracorporeal circuit.[22] The protamine filter would remove heparin before heparin is returned to the patient, thereby eliminating heparin-induced bleeding. The protamine filter would also prevent protamine from entering the patient and thus minimize protamine-induced toxicities. Preliminary *in vivo* studies involving dogs demonstrated that the protamine filter removed heparin from the blood circuit and did not elicit any clinically significant hemodynamic responses in dogs.[24]

The objectives of this article are twofold. One objective is to address the immobilization of protamine to cyanogen bromide-activated cellulose fibers through a systematic study of variables that affect activation and immobilization. The effects of the amount of CNBr used during the activation process, the incubation interval during the coupling process, and the protamine concentration in the coupling solution on protamine immobilization were examined. The purpose of this study was to develop a protocol that could be used to optimize the immobilization process and achieve the desired protamine loading on the fibers.

The second objective is to characterize the adsorption pattern of heparin on the protamine-bound cellulose membrane. It has been reported that the binding between heparin and protamine is an electrostatic interaction.[18] Adsorption of charged molecules to an oppositely charged polymer surface has been successfully described by the Langmuir adsorption model.[6,13,26] To identify the adsorption pattern of heparin on the protamine-bound surface, we examined the adsorption kinetics with respect to the heparin concentration in the substrate solution and the protamine loading on the cellulose fibers. The results obtained from this study could be applied to assist in the design of different protamine filters for desired clinical uses and in the development of models for prediction of the behavior and function of the protamine filter.

MATERIALS

Heparin (sodium salt from porcine intestinal mucosa, 150 USP units/mg) used throughout the entire study was from the same lot purchased from Hepar Industries (Franklin, OH). Bio-Rad dye reagent was from Bio-Rad Laboratories (Richmond, CA). CNBr and protamine sulfate (grade III, Clupeine from herring) were purchased from Sigma (St. Louis, MO). All other chemicals were reagent grade and water was distilled and deionized.

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Cellulose hollow fibers were obtained by disassembling a Travenol (Deerfield, IL) Model 1500 CF hemodialyzer. Each hollow fiber has an internal diameter of 200 μm, a wall thickness of 10 μm, and is made of Cuprophane membrane with a nominal molecular weight cutoff of 10,000 daltons.[3] Unless otherwise stated, the fibers were cut to a uniform length of 15 cm. One gram of dry weight would normally consist of 295 pieces of the cut hollow fibers.

METHODS

Assays

Protamine concentration was determined by the protein assay developed by Bradford,[4] using the Bio-Rad dye reagent. This method has been found very sensitive for measuring protamine concentrations in the range of 1–5 μg/mL. Heparin concentration was determined by the Azure A dye metachromasia assay.[9] The assay is based on the shift of the absorption maximum of Azure A from 620 nm to 530 nm caused by the presence of heparin. The degree of shift is linearly related to the heparin concentration in the range of 1–10 μg/mL. Thus, by measuring the absorbance at 620 nm, heparin concentration can be accurately determined from a standard curve. The degree of activation was determined by measuring the cyanate ester content on the activated fibers, according to the method of Kohn and Wilchek.[12] The absorbance of the intense purple color generated by the reaction between the Konig's reagent and cyanate esters was measured at 588 nm, and the cyanate ester content was estimated using a molar absorptivity of $137 \times 10^6 M^{-1}$ cm^{-1}.[12]

Preparation of Fine Fiber Particles

To prepare fine fiber particles, intact hollow fibers were chopped with a razor blade to small fragments of about 100–200 μm in size, immersed in liquid nitrogen until frozen, and then ground in a crucible. The freezing and grinding were repeated several times until particles that were uniform in size (approximately 0.5–1.0 μm in diameter by the measurement of a light microscope) were obtained. Previous studies involving Sephadex and Sepharose beads indicated that repeated freezing and grinding of the beads resulted in a high degree of pore collapse.[1,2,16]

Investigation of Protamine Immobilization

Ground, fine-fiber particles were used for the investigation of protamine immobilization. Protamine was immobilized onto these ground fiber particles according to the method of March et al.[15] Variables affecting protamine immobilization such as the amount of CNBr used, the duration of incubation, and the protamine concentration in the coupling solution were examined. A general description of the immobilization procedures is listed later. One gram of dry weight of the ground fibers was mixed with 20 mL of CNBr solution at concentrations ranging from 0.05 to 0.4 g/mL. The CNBr solutions at different concentrations were prepared by combining adequate volumes of 1 g/mL of CNBr/acetonitrile solution with 2M Na_2CO_3 solution. The suspension mixture was vigorously agitated in a fume hood for 5 min, and the solution was filtered using a sintered glass funnel. The activated fiber particles were then washed with 70 mL each of distilled water, 1 mM HCl, and 0.1M $NaHCO_3$ (pH 8.3) containing 0.5M NaCl. Samples (0.1 g) of the suction-dried fiber particles were withdrawn and assayed for the cyanate ester concentrations. The remaining suction-dried fiber particles were incubated at room temperature with 5 mL of the protamine solution prepared in the coupling solution containing 0.1M $NaHCO_3$, 0.5M NaCl at pH 8.3. After the incubation, the fibers were washed with 70 mL of 0.25M phosphate buffer (pH 7.0) containing 0.5M NaCl to remove the nonspecifically adsorbed protamine. The amount of protamine immobilized on the fiber was calculated from the protamine concentrations in the coupling solution before and after the immobilization procedure, as well as that in the washing solution. The incubation time and protamine concentration were varied during the coupling process to examine their effects on protamine immobilization.

Fabrication of the Hollow Fiber Bundle

The Travenol Model 1500 CF hemodialyzer was disassembled, and the fibers were cut down to a length of 15 cm. Approximately 800–1000 of the cut fibers (about 3 g in weight) were collected and tied together at each end with a teflon film to form a bundle. The bundle was then housed in a tygon tubing that was fitted with a molded connector at each end. The junctions were sealed with epoxy, and the epoxy was cured at room temperature until it hardened.

Immobilization of Protamine onto the Hollow Fiber Bundle

The fabricated bundle prepared above was circulated with 200 mL of 1M sodium carbonate solution for 5 min. After the circulation, the bundle was placed in a fume hood and circulated for 5 min with 60 mL of 0.1 g/mL CNBr solution. The CNBr-activated bundle was then washed with 200 mL each of distilled water, 1 mM HCl solution, and 0.1M $NaHCO_3$ buffer (pH 8.3) containing 0.5M NaCl. Immediately following the washing steps, 20 mL of the protamine solution were circulated through the bundle at a flow rate of 50 mL/min over a period of 2 h at room temperature. The bundle was then washed with 200 mL of 0.25M phosphate buffer

(pH 7.0) containing 0.5*M* NaCl to remove the nonspecifically adsorbed protamine. The hollow fiber bundle thus prepared that contained immobilized protamine was defined as the "protamine filter."

Adsorption-Time Curve of Heparin on the Protamine Filter

Heparin solutions at concentrations ranging from 0.01 to 15 mg/mL were prepared by dissolving appropriate amounts of heparin in normal saline (i.e., 0.9% in NaCl). Fifty milliliters of the solution were placed in a reservoir and recirculated with a peristaltic pump through the protamine filter at a flow rate of 50 mL/min for 1 h. Samples were withdrawn from the reservoir at various time intervals and assayed for the residual heparin concentrations. Plain hollow fiber bundles (i.e., not treated with protamine) were used as the control to determine the amount of heparin that was adsorbed on the bundle through the nonspecific, physical adsorption.

Equilibrium Adsorption Isotherm

Ground fiber particles containing immobilized protamine, prepared by freezing and grinding the intact hollow fibers obtained from a protamine filter, were used for the adsorption isotherm studies. A sequence of test tubes containing 5 mL of the heparin solution at various heparin concentrations ranging from 0.01 to 15 mg/mL was prepared. To each tube, 0.5 g of the protamine-bound fiber particles were added. The tubes were placed on an orbital rotator and rotated at 200 rpm for 1 h at room temperature. Previous studies conducted in our laboratory indicated that the adsorption equilibrium was attained in 1 h, if the tube was rotated at speed greater than 50 rpm. The fiber particles were then removed by centrifugation and the equilibrium heparin concentration in the supernatant was measured by the Azure A assay. Plain ground fiber particles that were not treated with protamine were used as the control to assess the nonspecific heparin adsorption on the fibers. The amount of heparin specifically adsorbed to each gram of the protamine-bound fibers (Q) was estimated by substracting the nonspecifically bound heparin (i.e. the control, Q_C) from the total adsorbed heparin (Q_T) using the following equation:

$$Q = Q_T - Q_C = [V(C_0 - C)/W_t]_T - [V(C_0 - C)/W_t]_C \quad (1)$$

where C_0 and C are the initial and equilibrium (final) heparin concentrations, respectively, V is the volume of the heparin solution, and W_t is the dry weight of the ground fibers.

RESULTS

The protamine immobilization process consists of two steps: (1) activation of the hydroxyl groups on the cellulose membrane with CNBr to form reactive cyanate ester derivatives, and (2) coupling of protamine onto the activated cyanate ester groups through isourea linkages. Figure 1(a) shows the effect of CNBr concentration on fiber activation. At low CNBr concentrations (<0.1 g/mL), the cyanate ester concentration on the fibers increased in proportion to the increase in CNBr concentration. Doubling the CNBr concentration from 0.05 to 0.1 g/mL increased the cyanate ester content per gram of the fibers from 2.1 to 4.9 μmol. This cyanate ester/CNBr concentration ratio (i.e., the slope of the plot), however, dropped significantly at higher CNBr concentrations. As the CNBr concentration was raised from 0.2 to 0.4 g/mL, the cyanate ester concentration on the fibers increased by only 1.2-fold (from 6.6 to 8.1 μmol/g).

Coincidently, the change of protamine loading on the fibers at different CNBr concentrations followed a nearly identical pattern to that of the cyanate ester-CNBr curve [Fig. 1(a)]. When protamine loading was plotted vs. the cyanate ester content, a straight line with a correlation coefficient of 0.995 was observed [Fig. 1(b)]. Because a ratio of 50 mg (equivalent to 12.5 μmol) of protamine to 1 g of the activated fibers was generally used for immobilization, protamine was presumably in excess when compared to the cyanate ester content. The linear relationship observed in Fig-

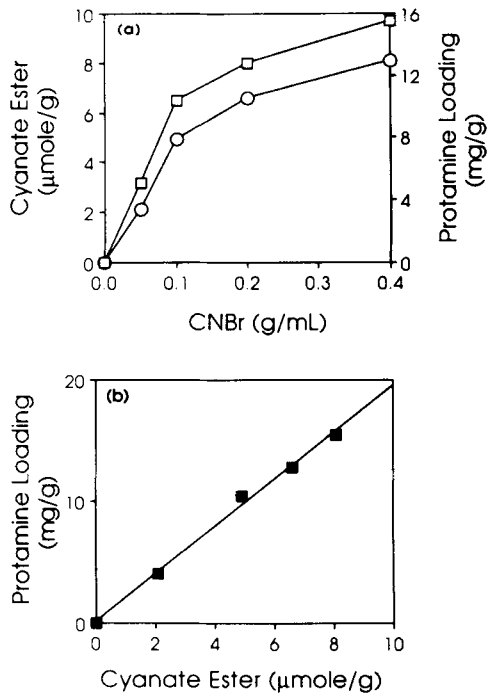

Figure 1. (a) Effect of cyanogen bromide concentration on cyanate ester content (○—○); and protamine loading on the cellulose fibers (□—□). (b) Relationship between the cyanate ester content and protamine loading on the cellulose fiber. Ground fiber particles were used for these studies. Experimental procedures were described in detail in the Methods. For each gram of ground fibers, 5 mL of the protamine solution (10 mg/mL) were used during the coupling process. The coupling time was 2 h.

ure 1(b) may therefore suggest the existence of certain stoichiometry between the reaction of protamine and the cyanate ester groups.

The effects of protamine concentration in the coupling solution and the duration of the coupling step on protamine loading are summarized in Table I. Up to a protamine concentration of 10 mg/mL, protamine loading on the fibers increased almost linearly with the change of protamine concentration in the coupling solution. Further increase of the protamine concentration from 10 to 20 mg/mL, however, only imparted a 50% increase in the protamine loading on the fibers. At a protamine concentration of 10 mg/mL and room temperature, protamine loading on the fibers also increased with the prolongation of incubation time, reaching a plateau at about 30 min of incubation. All immobilization studies were therefore conducted with an incubation time of longer than 30 min to ensure the completion of the coupling process. Control experiments using untreated fibers displayed a nonspecific protamine adsorption of about 0.8 mg/g of the untreated fibers.

When the heparin solution was placed in a reservoir and recirculated through the protamine filter at a flow rate of 50 mL/min, a drop in heparin concentration in the reservoir was observed. Figure 2 shows the adsorption vs. time curves for the protamine filters at heparin concentrations ranging from 0.1 to 10 mg/mL. All the curves appeared to follow a similar pattern: a rapid heparin adsorption that occurred within the first 10 min of recirculation, followed by an asymptotic approach of the heparin concentration to an equilibrium value. Table II demonstrated that heparin adsorption on the protamine filter was dependent on the protamine loading on the filter, increasing at higher protamine loadings. At a heparin concentration of 1 mg/mL, filters containing 5.0, 12.2, and 20.3 mg protamine per gram fiber (filters no. 5–7, respectively) removed 1.0, 1.8, and 3.3 mg heparin per gram fiber, respectively. In addition, heparin adsorption on filters with similar protamine loadings was strongly dependent on the initial heparin

Table I. Effect of protamine concentration and incubation time on protamine loading on the cellulose fibers.

Protamine concentration (mg/mL)	Incubation time (min)	Protamine loading (mg/g fiber)
2.5	120	5.6
5.0	120	10.2
10.0	120	20.8
20.0	120	29.8
10.0	3	5.6
10.0	10	9.1
10.0	20	9.8
10.0	30	10.0
10.0	60	10.5
10.0	120	10.7

Ground fiber particles were used for these studies. Experimental procedures were described in detail in the Methods.

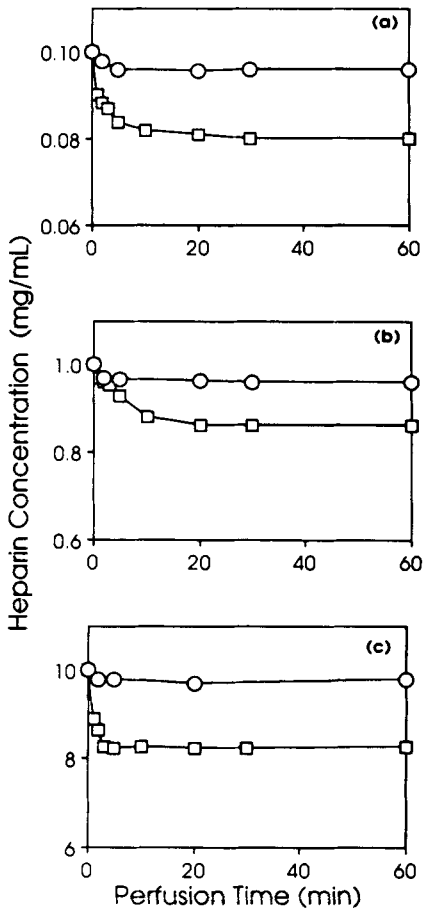

Figure 2. The change of heparin concentration in the reservoir. Intact hollow fiber bundles containing immobilized protamine (i.e., the protamine filters) were used for these studies. All the filters contained approximately 13 mg protamine per gram of the fibers. Experimental procedures were described in detail in the Methods. Hollow fiber bundles that were not treated with protamine were used as the control (○——○). The initial heparin concentration used were (a) 0.1 mg/mL, (b) 1 mg/mL, and (c) 10 mg/mL.

Table II. Effect of protamine loading and heparin concentration on heparin adsorption on the cellulose fibers.

Filter number	Protamine loading (mg/g filter)	Initial heparin concentration (mg/mL)	Amount of heparin adsorbed (mg/g fiber)
Plain filters (i.e., filters were not treated with CNBr)			
1	—	0.1	0.1
2	—	1.0	0.3
3	—	10.0	2.0
Protamine filters			
4	13.5	0.1	0.4
5	5.0	1.0	1.0
6	12.2	1.0	1.8
7	20.3	1.0	3.3
8	13.0	10.0	12.4

Intact hollow fiber bundles containing immobilized protamine (i.e., the protamine filters) were used for these studies. Heparin solution was recirculated through the protamine filter using a peristatic pump at flow rate of 50 mL/min for 1 h. Experimental procedures were described in detail in the Methods.

concentration in the solution (see filters no. 4, 6, and 8 in Table II). It is interesting to note that the amount of heparin adsorbed on the filter through nonspecific, physical adsorption was also dependent on the initial heparin concentration. Bundles that were not treated with protamine adsorbed 0.1, 0.3, and 2.0 mg heparin/g fiber at heparin concentrations of 0.1, 1.0, and 10.0 mg/mL, respectively.

Heparin adsorption on the protamine-bound fibers, as shown in Figure 3 by plotting the specific amount of heparin adsorbed (Q) vs. the equilibrium heparin concentration (C) in the solution, appeared to follow the Langmuir adsorption equation:[19]

$$Q = \frac{KQ_sC}{1 + KC} \tag{2}$$

where K and Q_s are the adsorption constant (mL/mg) and saturation capacity (mg/g fiber), respectively. At the low heparin concentration region (0.01–1.0 mg/mL), adsorption was first order, and the amount of heparin adsorbed was linearly proportional to the equilibrium heparin concentration. The adsorption in this case was determined by the equilibrium between heparin in the bulk solution and heparin adsorbed on the solid phase, which could be expressed by $Q = KQ_sC$ from the Langmuir equation. As the heparin concentration was increased from 1 to 10 mg/mL, the adsorption was mixed order in which the amount of heparin adsorbed was no longer approximately proportional to the substrate concentration. When the heparin concentration was further increased to above 10 mg/mL, the amount of heparin adsorbed reached a plateau that represented the saturation capacity ($Q = Q_s$) of the protamine-bound fibers. In this case, the adsorption was zero order and essentially independent of the substrate concentration. The K and Q_s values were estimated by using the double-reciprocal plots of the Langmuir adsorption isotherms observed in Figure 3. At three different protamine loadings, the adsorption constant K appeared to remain constant, whereas the saturation capacity Q_s varied with respect to the protamine loading. The average K value was estimated to be 0.37 ±0.06 mL/mg (i.e., $4.99 \times 10^6 M^{-1}$, assuming an average molecular weight of 13,500 daltons for heparin[23]). The saturation capacities were calculated to be 3.8, 6.2, and 9.2 mg/g fiber for the adsorbent containing 6.5, 12.7, 20.7 mg of immobilized protamine per gram of the fibers, respectively.

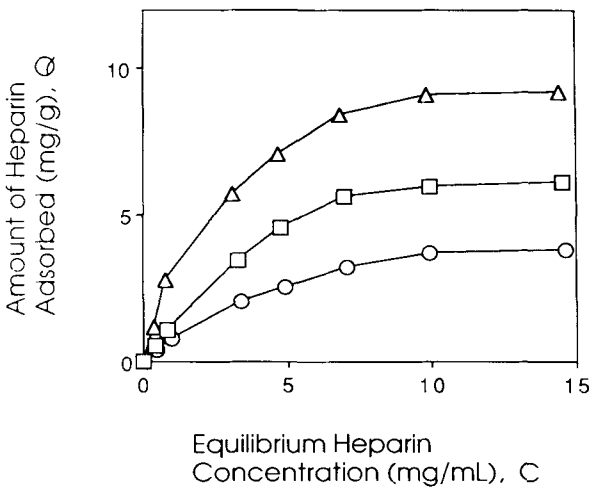

Figure 3. Equilibrium adsorption isotherm of heparin on the protamine-bound ground fiber particles. Fine fiber particles containing immobilized protamine were prepared according to the procedures described in the Methods. Experimental procedures were also described in detail in the Methods. Protamine loading on the fiber was: ○—○, 6.5 mg/g; □—□, 12.7 mg/g; and △—△, 20.7 mg/g.

DISCUSSION

To develop a protamine filter for extracorporeal blood heparin removal, protamine sulfate was immobilized onto regenerated cellulose hollow fibers.[24] A detailed investigation of the parameters affecting the immobilization process was conducted in order to establish the optimal procedures for protamine immobilization. Ground fiber particles were selected for the immobilization studies for two major reasons. One reason was that only ground fibers would offer the opportunity for a uniform cyanogen bromide activation of the fiber matrices that, in a sense, was crucial for accurate and consistent evaluation of the immobilization process. The other reason was that by using ground fibers and vigorous agitation, the intrinsic parameters would be measured in the absence of mass transfer resistance to macromolecular species such as protamine.

Protamine immobilization onto the CNBr-activated fibers followed a bimolecular reaction mechanism, and was dependent on both the cyanate ester concentration on the fibers and protamine concentration in the coupling solution. Increasing the concentration of either of these two reactants (e.g., increasing the cyanate ester concentration by using a larger quantity of CNBr for fiber activation) resulted in an increase of the protamine loading on the fibers. In addition, protamine loading on the fibers was also dependent on the duration of the coupling step, increasing with the prolongation of the incubation time. At room temperature and using sufficient reactant concentrations, the immobilization process was completed within an incubation period of about 30 min.

A linear relationship was observed when the protamine loading on the fibers was plotted against the cyanate ester concentration [Fig. 1(b)]. The slope of the plot yielded a ratio of 1.7 (mg/μmol), suggesting that one μmol of cyanate ester would bind 1.7 mg of protamine. If one assumes an average molecular weight of 4000 daltons for protamine,[7] a molal stoichiometric ratio of 0.43 (mole-protamine/mole-cyanate ester) was estimated for the reaction between protamine and the cyanate ester groups. Thus, in spite of the presence of abundant amino groups such as arginine and lysine resi-

dues on the protamine molecule, each protamine molecule was linked to the support material through only two or three isourea linkages. Because a certain number of cyanate ester groups may have already been leached off the support by hydrolysis[20] before protamine can be attached to them, the molal stoichiometric ratio of 0.43 is an apparent value and only represents the lower limit.

Previous studies involving the use of CNBr-activated materials for protein immobilization have addressed the potential instability of the cyanate ester derivatives caused by hydrolysis.[2,20] A recent investigation conducted in our laboratory indicated that after 1 h of incubation at room temperature of the CNBr-activated agarose beads in the coupling solution (0.1*M* $NaHCO_3$, 0.5*M* NaCl, pH 8.3), nearly 50% of the cyanate ester groups were leached off the beads because of hydrolysis.[25] The results presented in this article appeared to confirm these findings. As shown in Table I, the protamine loading on the fibers increased with increasing the protamine concentration in the coupling solution. If hydrolysis had not occurred, then the amount of available cyanate esters on the fibers would have remained unchanged. Under conditions where protamine was in large excess in comparison with the cyanate ester groups, and where the incubation period was carried out long enough to assure the completion of protamine immobilization (e.g., 2 h of incubation), the protamine loading on the fibers would have been independent, rather than dependent (as seen in Table I) on the protamine concentration in the coupling solution. These results suggested that the cyanate ester derivatives were not stable under the experimental conditions employed, and were continuously inactivated during the coupling process. Because of this continuous inactivation, protamine loading on the fibers was governed by the competition of two reaction kinetics: the kinetics of coupling of protamine to the cyanate ester groups, and the kinetics of hydrolysis of the cyanate ester groups. For fibers possessing the same degree of CNBr activation, increasing the protamine concentration in the coupling solution would increase the kinetics of protamine coupling and, as a consequence, would increase the protamine loading on the fibers.

In summary, protamine loading on the fibers appears to be controlled by three independent parameters: the CNBr concentration during the activation step, the duration of the coupling step, and the protamine concentration in the coupling solution. The CNBr concentration is normally fixed at 0.1 g/mL, because above this concentration structural deterioration may be induced, thus weakening the mechanical strength of the fibers. The incubation period is generally held to one hour or slightly longer so that completion of the immobilization process is assured. Therefore, it becomes evident that altering the protamine concentration in the coupling solution is the easiest and most convenient means to control the protamine loading on the fibers.

A recirculation system consisting of a substrate reservoir was adopted to characterize heparin adsorption by the protamine filter. This was for the purpose of simulating conditions close to those of blood circulation where the protamine filter would eventually be applied. A flow rate of 50 mL/min was used for the recirculation because it simulated the clinical flow rate employed in hemofiltration. Heparin adsorption vs. time curves appeared to follow a similar pattern: a rapid drop in heparin concentration in the reservoir with time, with an asymptotic approach to a steady-state value. This adsorption pattern has also been shown by other investigators in their studies of antigen removal using antibody-linked immunoadsorbent.[15] It appeared that the adsorption process was controlled by local equilibrium rather than diffusional limitation; otherwise, heparin concentrations in the reservoir would have changed exponentially.[17] In addition, if mass transfer inside the fiber lumen had been rate limiting, the mass transfer coefficient would have changed with the cube root of the fluid velocity inside the lumen.[17] Operating the protamine filter at various flow rates, however, did not show this type of behavior (data not shown).

The adsorption isotherm was characterized using the protamine-bound fiber particles obtained by grinding the intact protamine filter. As previously discussed, the Cuprophane membrane employed for the construction of the hollow fibers has dense structures with a nominal molecular weight cutoff of 10,000 daltons.[3] Diffusion of heparin, which has an average molecular weight of 13,000 daltons, is likely to be hindered because of the size exclusion effect of the membrane pores. Protamine molecules with an average molecular weight of 4,000 daltons, however, are relatively small and may diffuse through the membrane pores during the immobilization process. Should this occur, then only protamine molecules located at the surfaces of the fibers are subject to heparin binding. It has been reported that repeated freezing and grinding of Shephadex or Sepharose beads result in a high degree of pore collapse.[1,2,16] The use of ground, protamine-bound fiber particles for the adsorption isotherm studies is thus to assure the exposure of all the protamine molecules, both on the fiber surface and within the fiber matrix pores, to heparin binding. Assessment of the amount of heparin adsorbed on both the intact and ground protamine-bound fibers suggests, however, that protamine molecules with long rod shapes and high positive charges may not diffuse into the membrane pores at all during the immobilization process. Under a similar protamine loading of about 20 mg/g, the same initial heparin concentration (1 mg/mL), and an identical heparin solution/fiber (volume/weight) ratio, 1 g of intact fibers adsorbed 3.3 mg of heparin, whereas 1 g of ground fibers adsorbed nearly 3.1 mg.

Adsorption of biologic molecules to polymeric materials such as those of heparin to DEAE-cellulose membrane,[6] proteins to hydrophobic polymers,[13] and bovine

serum albumin to cross-linked chitosan membranes[26] have been satisfactorily analyzed by the use of the Langmuir adsorption model [Eq. (1)]. Results in Figure 3 showed that heparin adsorption on the protamine-bound membrane also followed the Langmuir adsorption model. Adsorption was first order at low substrate concentrations, and gradually became zero order at high substrate concentrations. The double-reciprocal plot ($1/Q$ vs. $1/C$) gave an adsorption constant (K) of 0.21 $\pm$0.05 mL/mg for the protamine-bound membrane, and a saturation capacity that was dependent on the protamine loading on the membrane. The K value was consistent with those observed in the adsorption of acids on a basic membrane,[26] implicitly confirming the results that heparin-protamine interaction was primarily an electrostatic interaction.[18]

Results obtained from preliminary animal studies appeared to agree with the proposed adsorption model. A similar adsorption versus time curve was observed in the in vivo heparin removal by the protamine filter.[6] In addition, at a testing dose of 2 units of heparin per milliliter of blood (equivalent to a heparin concentration of about 0.02 mg/mL), the in vivo heparin removal was nearly independent of the protamine loading on the filter.[24] As shown in Figure 3 and also reflected by the Langmuir adsorption model [Eq. (2)], at such a low bulk heparin concentration, the amount of heparin adsorbed (Q) was relatively insensitive to the change in protamine loading on the filter.

Blood heparin levels encountered during the clinical extracorporeal blood circulation procedure are often in the range of 4–8 units/mL (equivalent to a concentration range of 0.04–0.08 mg/mL).[8] As shown in Table II, 1 g of intact hollow fibers containing 13.5 mg of immobilized protamine could adsorb 0.4 mg of heparin at a heparin concentration of 0.1 mg/mL. If the total inner surface area provided by 1 g of intact fibers is given by the equation of Area = πDLn,[21] where D is the inside diameter of the fiber lumen, L is the length of the fiber, and n is the number of fibers per gram of dry weight, then 1 g of fibers that consists of 295 pieces of intact hollow fibers with a length of 15 cm and an inside diameter of 200 μm would provide a surface area of approximately 278 cm^2. This would give an adsorption capacity of 0.11 nmol of heparin per square centimeter of the protamine-bound surface. Thus, for a total heparin dose of 3000–10,000 units employed clinically, 75–250 g of the protamine-bound hollow fibers would be required to reverse fully the anticoagulant activity of heparin. Such fiber weights would be equivalent to one to three hemodialyzers currently in use clinically.

The present study provides the foundation for developing an in vivo protamine filter. Information gathered from the investigation on protamine immobilization will be used to optimize the design and preparation of different protamine filters for desired clinical uses. In addition, adsorption parameters determined in the current study will be used to assist the development of models for the prediction and evaluation of the behavior and heparin removal characteristics of the protamine filter. A mathematical model that utilizes a plug flow reactor connected in series to a stirred tank reactor to simulate the in vivo operation of the protamine filter (i.e., the plug flow reactor) in the cardiovascular circulatory system (i.e., the stirred tank reactor) is currently under evaluation.

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