Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 306 (2005) 132–141
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

# Strategies for improving the functionality of an affinity bioreactor

Tanya Wang [b], Zhiqiang Yang [b], Emel Emregul [c], Allan David [b,d], Joseph P. Balthasar [e], Junfeng Liang [f], Victor C. Yang [a,b,*]

[a] *School of Chemical Engineering, Tianjin University, Tianjin 300072, China*
[b] *College of Pharmacy, University of Michigan, Ann Arbor, MI 48109-1065, USA*
[c] *Faculty of Science, Department of Chemistry, University of Ankara, Ankara 06110, Turkey*
[d] *Industrial Science and Technology Network (ISTN) Inc., York, PA, USA*
[e] *College of Pharmacy, University of Buffalo, Buffalo, NY 14260, USA*
[f] *Department of Chemistry and Chemical Biology, Stevens Institute of Technology, Hoboken, NJ 07030, USA*

Received 30 June 2005; received in revised form 14 September 2005; accepted 15 September 2005
Available online 24 October 2005

**Abstract**

Heparin employed in extracorporeal blood circulation (ECBC) procedures (e.g. open heart operations) often leads to a high incidence of bleeding complications. Protamine employed in heparin neutralization, on the other hand, can cause severe adverse reactions. We previously developed an approach that could prevent both heparin- and protamine-induced toxic side effects concomitantly. This approach consisted of placing a hollow fiber-based bioreactor device containing immobilized protamine (termed a "protamine bioreactor") at the distal end of the ECBC procedure. This protamine bioreactor would remove heparin after heparin served its anticoagulant purpose in the ECBC device, thereby eliminating heparin-induced bleeding risks. In addition, this protamine bioreactor would prevent protamine from entering the patients, thereby aborting any protamine-induced toxic effects. Both in vitro and in vivo studies have successfully demonstrated the feasibility of this approach.

Despite promises, early findings also revealed two shortcomings that must be overcome for the protamine bioreactor to be applied clinically. The first drawback was that the cyanate ester linkages, involved in conjugating protamine to the bioreactor device, were unstable and prone to hydrolysis, resulting in the leakage of a significant amount of protamine into circulation during application of the protamine bioreactor. The second deficiency was that the capacity of the protamine bioreactor in heparin removal was rather low, owing to the limited surface area of the hollow fibers for protamine immobilization and subsequently heparin adsorption.

In this paper, we present novel strategies to overcome these two limitations. A new conjugation method based on the use of 4-(oxyacetyl)phenoxyacetic acid (OAPA) as the activating reagent was employed to yield stable linkages, via the abundant arginine residues of protamine, onto the hollow fibers. Results showed that while the amount of protamine immobilized oneach gram of fibers was relatively comparable between the OAPA and the previous CNBr activation methods (7.45 mg/g versus 7.69 mg/g fibers), there was virtually no detectable leaching of immobilized protamine from the bioreactor by the OAPA method,

* Corresponding author at: Department of Pharmaceutical Sciences, College of Pharmacy, The University of Michigan, 428 Church Street, Ann Arbor, MI 48109-1065, USA. Tel.: +1 734 764 4273; fax: +1 734 763 9772.
*E-mail address:* vcyang@umich.edu (V.C. Yang).

0378-5173/$ – see front matter 
doi:10.1016/j.ijpharm.2005.09.011

comparing to 35% leaching of protamine by the previous CNBr method following 72 h of storage of the bioreactor in PBS buffer at 37 °C.
To improve the capacity and functionality of the protamine bioreactor, two novel approaches were adopted. Long chain and high molecular weight poly-lysine was linked to the hollow fibers, prior to protamine coupling, to create multiple layers of immobilized protamine for subsequent heparin adsorption. In addition, a poly(ethylene glycol) (PEG) chain was inserted between protamine and the hollow fibers to yield a three-dimensional, free dynamic motion for immobilized protamine. Preliminary observations indicated that a four- to five-fold enhancement in heparin adsorption was attained by utilizing each of these new approaches. Aside from their current use, these new strategies can also be employed generically to improve the functionality of any affinity-type bioreactor. Indeed, efforts have been made recently in utilizing these approaches to develop a clinically usable GPIIb/IIIa bioreactor for the treatment of immune thrombocytopenic purpura (ITP)—an autoimmune disease.


*Keywords:* Protamine bioreactor; Heparin adsorption; Langmuir adsorption isotherm; Poly(ethylene glycol) modification; Polylysine amplification

## 1. Introduction

Extracorporeal blood circulation (ECBC) has become one of the most frequently used clinical procedures in recent years. It is used in many surgical procedures including cardiopulmonary bypass, hemodialysis, blood oxygenation, and implantation of artificial organs. It is estimated that approximately 20 million ECBC procedures are employed each year (Yang et al., 1993). In all of these applications, systemic level of the anticoagulant heparin is required to prevent the clotting process that is initiated when blood comes into contact with the ECBC device. This high level of heparin, however, often leads to a high incidence (7–30%) of bleeding complications (Hirsh, 1984). To prevent this bleeding risk, protamine is normally administered to the patient at the conclusion of the ECBC procedure to reverse the anticoagulant effects of heparin. Unfortunately, protamine administration at times can lead to serious or even fatal adverse side effects (Weiler et al., 1990).

We, previously, reported a novel approach that could simultaneously eliminate heparin- and protamine-induced toxic effects. The approach consisted of placing a hollow fiber-based filter device containing immobilized protamine (termed a "protamine bioreactor) at the distal end of the ECBC circuit (Teng et al., 1988). This protamine bioreactor would specifically remove heparin after heparin served its anticoagulant purpose and before heparin was returned to the patient, thereby eradicating heparin-induced bleeding risk. Alternatively, this protamine filter would also restrict protamine from entering the patient, thereby aborting protamine-associated toxic effects. Results from in vivo studies demonstrated that application of this protamine bioreactor not only effectively removed heparin from the blood circuit but also did not elicit any protamine-associated hypotensive responses in dogs (Yang et al., 1991).

Despite promises, early studies also revealed two major shortcomings that must be overcome in order for the protamine bioreactor to be successfully applied clinically. The first challenge came from the difficulty in producing a stable linkage between protamine and the hollow fiber surface. Protamine represents a truly unique protein molecule with ∼67% of its content being arginine residues (Ando et al., 1973). Thus, protamine lacks any usable functional groups (e.g. –OH, $-NH_2$) for conjugation. While the cyanogen bromide (CNBr) activation method employed previously is suitable to create a linkage on the rather inactive $NH_2$ side group of arginine, the produced cyanate ester linkage was nevertheless unstable and prone to hydrolysis. It has already been reported in literature, and further confirmed by us (Yang and Teng, 1990), that more than 50% of the ester linkages are hydrolyzed within a short period of several hours. This significant amount of protamine, leached from the bioreactor into the circulation, could potentially present the risk of inducing protamine-associated toxic effects. The second drawback was concerning the relatively low capacity of surface areas provided by hollow fibers for protamine immobilization and subsequent heparin adsorption. Since the protamine bioreactor would be used in an ECBC procedure under an extremely high blood flow rate (the blood flow rate for cardiopulmonary bypass is

about 2–4 L/min), a hollow fiber-based device became essential due to its nonresistant flow properties. Unfortunately, hollow fibers also yields the lowest surface area to volume ratio compared to other, and particularly the particulate, configurations. Previous animal studies showed that the hollow fiber-based protamine bioreactor could, at best, remove 50% of the heparin administered at a clinically relevant dose (Yang et al., 1991).

In this paper, we present novel strategies to overcome these two limitations. To attenuate protamine leaching from the bioreactor, a new coupling method based on the use of 4-(oxyacetyl)phenoxyacetic acid (OAPA) as the activating reagent was employed to create stable amine linkages via the formation of a Schiff base with the abundant arginine residues in protamine. To significantly increase the capacity of the protamine bioreactor, we took the same concept of constructing tall buildings to resolve the over-crowded living space in highly populated areas by creating multiple layers of surface areas for protamine immobilization and subsequent heparin adsorption via the coating of the hollow fiber surface with the long-chain, high molecular weight poly-lysine polymer prior to protamine conjugation (see Fig. 1B for details). In addition, we also took advantage of the well-known low interfacial energy and unparalleled molecular flexibility of the poly(ethylene glycol) (PEG) polymer (Greenwald et al., 2000) to tailor the immobilized protamine with three-dimensional, completely free dynamic mobility for heparin binding (see Fig. 1C for details). Promising results were attained by utilizing these new strategies.

## 2. Experimental methods and materials

### 2.1. Materials

Cyanogen bromide (CNBr), poly-L-lysine (PLL; MW 84 kDa), and protamine sulfate (Grade III from herring), glutaric anhydride, *N*-acetyl-L-arginine, 1-ethyl-3-(3-dimethylamino propyl)carbodiimide hydrochloride (EDAC); 2,4,6-trinitrobenzenesulfonic acid (TNBS; 95%); *O*-phenylene diamine (PD), and sodium borate were obtained from Sigma (St. Louis, MO). Triethylamine (TEA; 99%), and barbituric acid were from Aldrich (Milwaukee, WI). Heparin (sodium salt from porcine intestinal mucosa; 165 USP U/mg) was from Pharmacia (Peapack, NJ). The *O*,*O*′-bis(2-aminoethyl)polyethylene glycol (termed "dPEG" or "PEG"; MW 3.4 kDa) and Azure II were from Fluka (Milwaukee, WI). Water was distilled and deionized (dd$H_2O$).

The compound, 4-(oxyacetyl)phenoxyacetic acid (OAPA, MW 208 Da; see Fig. 2 for chemical structure), was synthesized according to the method of Duerksen (Duerksen and Wilkinson, 1987). Cellulose fibers were chosen since they are already used clinically in hemodialysis. They were obtained by disassembling the Travenol (Deerfield, IL) Model 1500 CF hemodialyzer. Fine cellulose particles were prepared by freezing the whole fibers in liquid nitrogen, grinding with a mortar and pestle until fine, and then sifting through a 200 nm mesh to attain fine particles with a diameter less than 75 μm. The surface area of the ground cellulose fiber, measured using BET analysis, was found to be 0.4 $m^2$/g.

### 2.2. Methods

#### 2.2.1. Assays

Heparin and protamine concentrations were precisely determined, utilizing a well-established titration method (Ramamurthy et al., 1999), using the heparin or protamine sensors previously developed in our own laboratory (Ma et al., 1993; Yun et al., 1995). These sensor-based methods have been shown to detect heparin or protamine at their respective clinically relevant concentration ranges (1–10 U/mL for heparin and 3 μg/mL for protamine) in both buffer and undiluted plasma/blood samples.

Activation of the cellulose hollow fibers by 4-(oxyacetyl)phenoxyacetic acid (OAPA) was monitored by measuring the OAPA substitution on fibers using phenylene diamine, according to the method described by Duerksen and Wilkinson (1987). Briefly, to determine OAPA on the fibers, 5–10 mg of the OAPA-activated fibers and 100 μL (5 mg/mL) phenylene diamine were added to a test tube and allowed to react for 3 h. The sample was then diluted by 100-fold and the absorbance was measured at 289 nm.

To determine the degree of poly-lysine or PEG conjugation, the levels of primary amines were quantified by adding saturated sodium borate solution to the poly-lysine or PEG-linked fibers, followed by the addition of

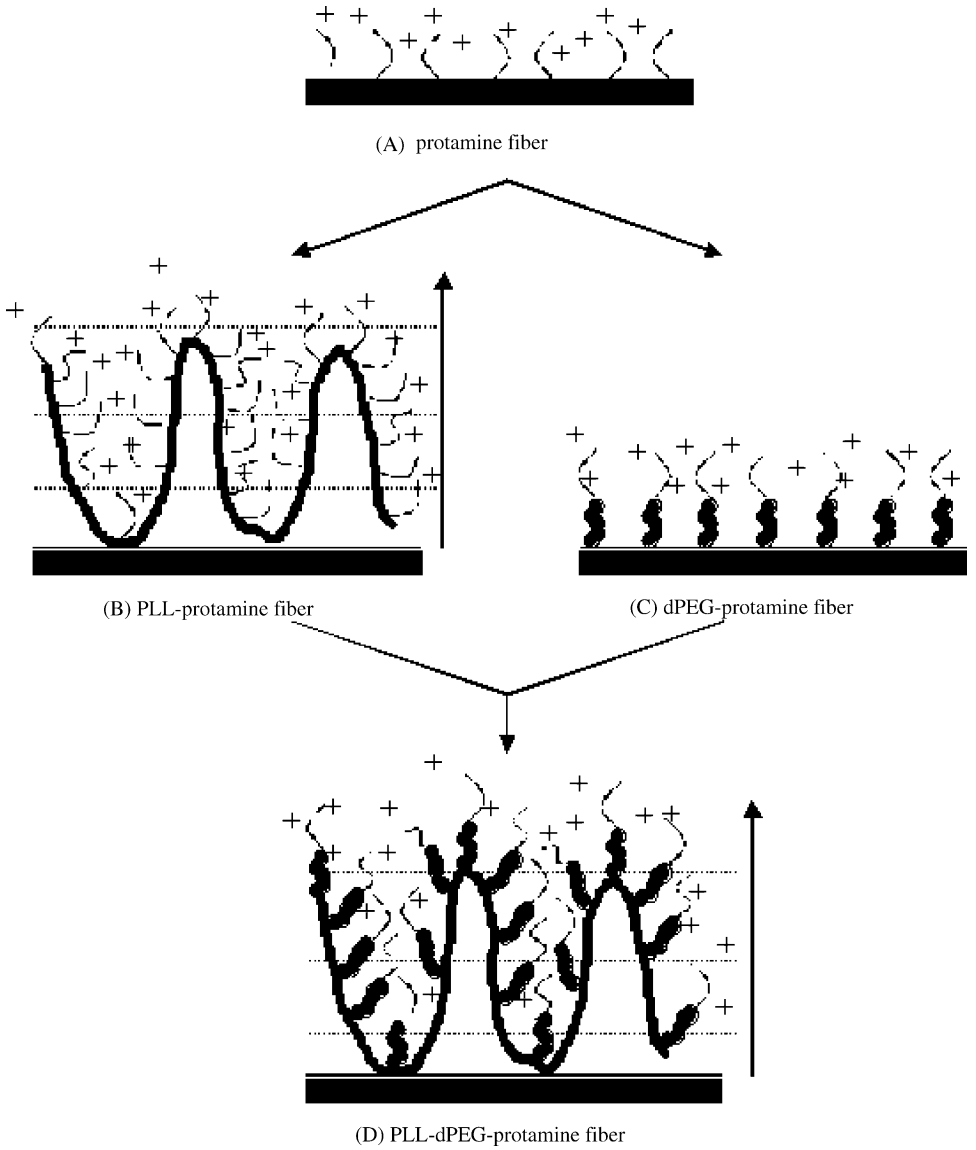


Fig. 1. Schematic illustration of new strategies to improve the capacity and functionality of protamine-immobilized hollow fibers: (A) previous method for protamine ( ) immobilization, (B) new strategy of surface amplification by poly-L-lysine (PLL) ( ), (C) new strategy of functionality enhancement by incorporating polyethylene glycol spacer arm ( ) and (D) future direction of concurrent application of both the PLL- and PEG-modification methods.

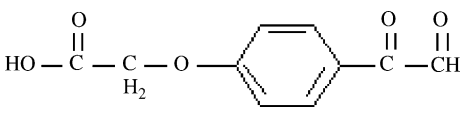

Fig. 2. Chemical structure of OAPA.

three to five drops of the 2,4,6-trinitrobenzenesulfonic acid (TNBS) as an indicator. A yellow color would correspond to the presence of a low concentration of primary amines whereas an orange color would correspond to the presence of a high concentration of amines (Inman et al., 1973).

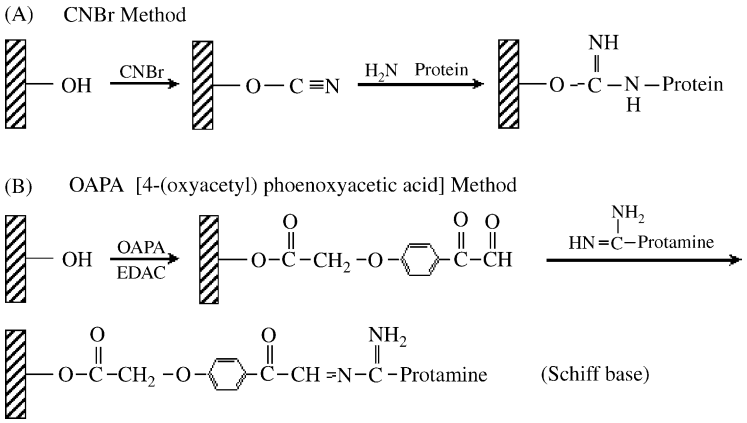


Fig. 3. (A) Mechanism of CNBr-activated protamine immobilization on cellulose fibers and (B) mechanism of OAPA-activated protamine immobilization on cellulose fibers.

### *2.2.2. Cyanogen bromide (CNBr) activation method for protamine immobilization*

Protamine immobilization, via the use of the CNBr activation method, was performed according to a modified procedure of Wilchek et al. (1984). A schematic illustration of the reaction mechanism is depicted in Fig. 3A. In brief, fine fiber particles (200 mg) were thoroughly wetted with ddH2O (2 mL) at 4 °C. Cyanogen bromide in acetone (0.4 g/mL) was then added to the stirred fiber suspension, followed by the dropwise addition of triethylamine (TEA; 1.5 mL) to stimulate the coupling reaction. The CNBr-activated fibers were then rinsed subsequently with 2 mL of 50% acetone/0.05 M HCl, 0.05 M HCl, and water. A small portion of the fibers was withdrawn to determine the degree of CNBr activation using the method by Kohn and Wilchek (1978). To the remaining fibers, 2 mL of protamine solution (10 mg/mL in 0.5 M NaCl, pH 9.0) was added and the fibers incubated at 4 °C for 24 h. The amount of protamine coupled to the fibers was determined by measuring the protamine concentration in the coupling solution before and after the immobilization process.

### *2.2.3. OAPA activation method for protamine immobilization*

To immobilize protamine with the OAPA activation method, a modified protocol of Duerksen and Wilkinson (1987) was employed. A schematic illustration of the reaction mechanism is depicted in Fig. 3B. In brief, 0.55 g OAPA was added to a suspension containing 1.5 g of the fiber particles at pH 5. EDAC (2.5 g) was then added to the fibers and the reaction allowed to continue for 2 h. The OAPA-activated fibers were then vacuum filtered and rinsed with 15 mL 0.2 M NaCl thrice. Ten milliliters of the washing solution were withdrawn and tested for OAPA using phenylene diamine according to the procedures described previously. If the sample showed positive in OAPA, another rinsing with 15 mL of 0.2 M NaCl was followed. After complete rinsing, a small sample of the OAPA-activated fibers was withdrawn to test for the degree of OAPA activation. The remaining OAPA-activated fibers were then reacted with 15 mL of the protamine solution (5 mg/mL in 50 mM $NaHCO_3$ at pH 9.0) for 13 h at room temperature. Afterwards, the protamine-linked fibers were vacuum filtered and washed several times with 100 mL of 0.12 M NaCl solution. The supernatant was retained for protamine concentration measurement to assess the degree of protamine immobilization. A small sample of the protamine-linked fibers was also reacted with TNBS to further validate the degree of protamine immobilization.

### *2.2.4. OAPA method for the preparation of PLL- or PEG-linked protamine fibers*

To amplify the surface of the cellulose fibers with poly-L-lysine (PLL) or PEG, the hydroxyl groups on cellulose fibers were reacted with glutaric anhydride to produce active carboxyl groups. Glutaric anhydride was chosen due to its high solubility in water. To perform the experiments, ground fibers (1.5 g) were placed in 6 mL of 0.1 M NaCl. One gram of glutaric anhydride

was then added to the fiber suspension and the pH was adjusted to pH 4.0 (optimum range pH 3.7–4.3) using 1 M NaOH. After 20 min of incubation, the period that the anhydride had either completely reacted or hydrolyzed, the fibers were vacuum filtered and washed thrice with 15 mL of 0.2 M NaCl. A small sample of the activated fibers was withdrawn and titrated to determine the number of carboxyl groups. The remaining activated fibers were suspended in 6 mL of 0.2 M NaCl, followed by the addition of either 200 mg of PLL or 300 mg of PEG. EDAC (2.5 g) was then added to the fiber suspension and the pH was maintained in the range of pH 4.5–6.0. After 1 h, the fibers were vacuum filtered and washed thrice with 15 mL of 0.2 M NaCl. The washes were tested with TNBS for residual PLL or PEG concentrations. A small sample of the PLL- or PEG-linked fibers was withdrawn and tested for primary amine groups according to the procedures described previously.

#### 2.2.5. Hydrolysis measurements

Both the OAPA- and CNBr-activated, protamine-linked fibers were tested for hydrolysis. In brief, the protamine fibers (0.65 g in dry weight) were washed with 100 mL of 1 M NaCl followed by 100 mL of 0.12 M NaCl. Protamine fibers produced by each activation method were then suspended in PBS buffer and incubated at either room temperature or 37 °C. At time intervals of 4, 24, 48, and 72 h, an aliquot of buffer was withdrawn and measured for protamine leaching, by hydrolysis, using the previously described sensor method. An equal volume of fresh buffer was added to the incubation broth to keep the total volume constant during the hydrolysis studies.

#### 2.2.6. Adsorption isotherms of the protamine fibers

*2.2.6.1. Langmuir isotherm.* Investigation of the adsorption isotherm of an affinity type bioreactor is most commonly done by applying the non-linear Langmuir adsorption isotherm (McCabe et al., 1985):

$$C_b = \frac{K_{EQ} C_S C_f}{1 + K_{EQ} C_f} \quad (1)$$

where $C_b$ corresponded to the amount of heparin adsorbed to immobilized protamine, $C_f$ the equilibrium heparin concentration in the solution, $K_{EQ}$ and $C_S$ are constants representing the equilibrium adsorption constant and saturation capacity (i.e. the maximum concentration of surface-adsorbed heparin), respectively. For small values of $K_{EQ}$ and/or $C_f$, the denominator is approximately equal to 1, and thus the Langmuir equation asymptotically approaches the linear isotherm:

$$C_b = (K_{EQ} C_S) C_f \quad (2)$$

Hence, at low heparin concentrations, such as those encountered clinically in extracorporeal heparin therapies, the Langmuir and linear isotherms, and their constants, should be similar. Heparin adsorption data was fitted to the Langmuir isotherm equation using the non-linear fitting program in Origin 5.0. Graphing $C_b$ versus $C_f$ would yield a slope equal to $K_{EQ}$ multiplied by $C_S$ (Eq. (2)), whereas the $x$-intercept of the double reciprocal plot, $1/C_b$ versus $1/C_f$, of the Langmuir isotherm would yield the value of $1/C_S$, as indicated by Eq. (3).

$$\frac{1}{C_b} = \frac{1}{K_{EQ} C_S C_f} + \frac{1}{C_S} \quad (3)$$

## 3. Results and discussions

### 3.1. Protamine leaching from the bioreactor

CNBr activation is the method most widely used in conjugation of proteins to biomaterial surfaces possessing abundant –OH groups, such as those of the cellulose hollow fibers. As shown in Fig. 2A, activation of the –OH groups with CNBr results in the formation of active cyanate ester derivatives, which can then be readily linked to the $-NH_2$ groups on the lysine residues of a protein molecule. Yet, protamine is a unique small protein with two-thirds of its amino acid composition being arginine residues, and it contains either no or a single lysine residue (Ando et al., 1973). Furthermore, the N-terminal of protamine, which in most proteins would yield an additional $-NH_2$ group for conjugation, is a proline residue that offers virtually no usable functional group. In our previous investigation (Kim et al., 1992), protamine was linked to the cellulose fibers via CNBr activation of the $-NH_2$ groups on arginine. Despite success, CNBr reacts favorably with the primary amine groups on lysine (Wilchek et al., 1984). Technically speaking, the $-NH_2$ group on arginine is not really a primary amine but a guanidine group that

Table 1
Hydrolysis of protamine from cellulose fibers activated by the CNBr or OAPA methods

| Incubation time (h) | Amount of protamine hydrolyzed (mg) after each incubation | | | |
|---|---|---|---|---|
| | CNBr | | OAPA | |
| | 25 °C | 37 °C | 25 °C | 37 °C |
| 4 | 0.31 | – | 0.00 | 0.01 |
| 24 | 0.60 | 1.53 | 0.00 | 0.01 |
| 48 | 0.57 | 0.61 | 0.02 | 0.02 |
| 72 | 0.44 | 0.27 | 0.02 | 0.04 |
| Total amount of protamine (mg) hydrolyzed after 72 h incubation | 1.91 | 2.42 | 0.04 | 0.08 |
| Total amount of protamine (mg) initially immobilized on fibers | 7.69 | 7.68 | 7.38 | 7.52 |
| Degree of protamine leaching after 72 h incubation (%) | **24.9** | **31.5** | **0.54** | **1.1** |

The amount of hydrolyzed protamine (mg) was measured at each designated time point. For experimental details, please see Section 2.

is far less reactive toward CNBr. Aside from the formation of weak linkages with the arginine residues on protamine, it was also reported that the isourea bonds formed via CNBr activation was labile and susceptible to hydrolysis, rendering the immobilized ligands to be slowly detached from the biomaterial surface (Wilchek et al., 1984; Yang and Teng, 1990). This could pose concerns during clinical applications of the protamine bioreactor, as the leached protamine would re-enter the circulation, causing protamine-associated toxic effects.

To address this issue, we explored the possibility of activating the arginine residues on protamine with 4-(oxyacetal)phenoxyacetic acid (OAPA) (see Fig. 3 for chemical structure). OAPA is a modified version of phenylglyoxal, a compound, which is commonly used in the elucidation of the role of arginine in the active site of an enzyme (Duerksen and Wilkinson, 1987). In general, OAPA chemically and specifically binds to arginine and subsequently inactivates the biological function of arginine. It was demonstrated that phenylglyoxal formed a Schiff base or *N*-substituted imine with the guanidino group; the side chain of an arginine residue (Duerksen and Wilkinson, 1987). Since protamine was known to exert its heparin binding via the positive charges (i.e. on arginine residues) rather than biological functions, it was hypothesized that modification of a small portion of the abundant arginine residues in protamine would not alter much of the heparin-adsorbing activity or capacity of immobilized protamine.

Table 1 provides a comparison of the hydrolysis rate of immobilized protamine by these two coupling methods (i.e. CNBr versus OAPA). As shown, there was virtually no hydrolysis, at both room temperature and 37 °C, when the OAPA method was applied. A mere 1.1% of protamine leaching, which was statistically insignificant and likely due to an inaccuracy in sensor measurement of the low protamine concentrations, was estimated following 72 h incubation of the protamine-immobilized fibers in buffer at 37 °C. On the other hand, a significant protamine leaching was observed when the CNBr-activated protamine fibers were incubated for 72 h either at room temperature (25% leaching) or 37 °C (32% leaching). Based on these findings, clinical application of the OAPA-prepared protamine bioreactor deems to be safe, because none of the existing extracorporeal heparin therapies is expected to proceed over a period of 72 h. In addition, even if the calculated protamine-leaching rate (i.e. 1.1% after 72 h incubation at 37 °C) is both real and accurate, this low level of protamine in the circulation is not likely to trigger any toxic side effects; as clinical reports have suggested that protamine toxicity is dose-dependent (Weiler et al., 1990).

Results also showed that the amount of protamine immobilized by using the OAPA method (i.e. 7.45 mg of protamine/g of fibers) was comparable to that (7.69 mg/g fibers) by the CNBr method, despite the fact that the OAPA method was presumably more capable of utilizing the arginine residues on protamine. Nevertheless, this observation was somewhat anticipated

Table 2
Summary of results of CNBr- or OAPA-produced protamine fibers, as well as further PLL- or PEG-modified protamine fibers

| Types of protamine fibers tested | Amount of immobilized protamine (mg/g fibers) | Saturation capacity ($C_S$) (mg heparin/g fibers) | Langmuir constant($K_{EQ}$) (mL/mg heparin) | $K_{EQ}C_S$ calculated |
|---|---|---|---|---|
| CNBr-produced | 7.69 + 0.83 | 6.5 + 1.4 | 0.42 + 0.08 | 2.73 |
| OAPA-produced | 7.45 + 0.21 | 5.7 + 1.1 | 0.37 + 0.06 | 2.11 |
| PLL-modified | 20.21 + 6.14 | 11.5 + 1.8 | 0.98 + 0.30 | 11.27 |
| PEG-modified | 12.96 + 6.25 | 13.2 + 1.3 | 0.64 + 0.18 | 8.45 |

For experimental details, please see Section 2.

since the amount of protamine that could be coupled to the fibers was governed primarily by the available surface area of these cellulose fibers. Because of a similar protamine loading, bioreactors prepared by either activation method yielded a similar heparin adsorption capability (6.5 mg/g versus 5.7 mg/g for CNBr- and OAPA-activated fibers, respectively; see the "$C_S$" column in Table 2). These results suggested that the adsorption capacity of the protamine fibers was still governed primarily by the surface area of the fibers. The presence of an almost identical $K_{EQ}$ (~0.4 mL/mg; see the "$K_{EQ}$" column in Table 2) further indicated that neither conjugation method would yield any negative effect with regard to heparin adsorption by the immobilized protamine.

### *3.2. Amplification of the capacity and functionality of the hollow fiber-based protamine bioreactor*

The protamine bioreactor is expected to be used in clinical situations of extracorporeal therapies to remove heparin, thereby eliminating heparin-induced bleeding complications. As known, all ECBC procedures demand a very high blood flow rate (note: even in kidney dialysis, which requires the least vigorous blood flow, the blood flow rate is about 250 $cm^3$/min). To meet such needs, a hollow fiber-based device becomes absolutely essential. Among all types of material geometries and configurations employed in constructing a bioreactor for toxin removal, including the most widely used particulate-based systems, hollow fiber remains to be the only system that is completely non-resistant to flow and also does not require any separation of plasma from blood components. Yet, a hollow fiber-based bioreactor encounters the most severe shortcoming of lacking sufficient surface areas for agent immobilization. Our previous assessment based on in vitro results (Kim et al., 1992) suggested that with the current protamine loadings (i.e. 7–8 mg protamine/g of fibers) and a total heparin dose of 3000–10,000 units employed clinically in ECBC situations, approximately 250 g of hollow fibers would be required to fully reverse the anticoagulant activity of heparin. Such fiber weight would be equivalent to the size of three presently used hemodialyzers; too large to be realistically usable. Consistent with this assessment, our in vivo studies in dogs also indicated that the prototype protamine bioreactor removed <50% of the administered heparin before it became completely saturated (Yang et al., 1991). In view of such shortcomings, it is obvious that both the capacity and functionality of the current protamine bioreactor must be significantly improved in order for it to be possibly applied under clinical situations.

To overcome such limitations, we attempted two innovative strategies to upgrade both the surface area of the hollow fiber-based device and the adsorption efficiency of the immobilized protamine. To drastically increase the available surface area of the fibers for protamine immobilization, adopting the commonsense principle of constructing tall buildings in resolving the over-crowded living space in highly populated areas, we created multiple layers of surface areas for protamine immobilization (and subsequent heparin adsorption) via the coating of the fiber surface with long-chain poly-L-lysine (PLL) polymer prior to protamine conjugation (see Fig. 1B for mechanism). To significantly increase the heparin-adsorbing efficiency of immobilized protamine, we took advantage of the unparalleled molecular flexibility of the poly(ethylene glycol) (PEG) polymer to tailor the immobilized protamine with a three-dimensional, dynamic mobility for heparin binding (see Fig. 1C for mechanism). To precisely assess the effects of these two amplification methods, heparin adsorption on the un-modified, PLL- or PEG-modified protamine fibers was analyzed by construction of the adsorption isotherms. Fig. 4

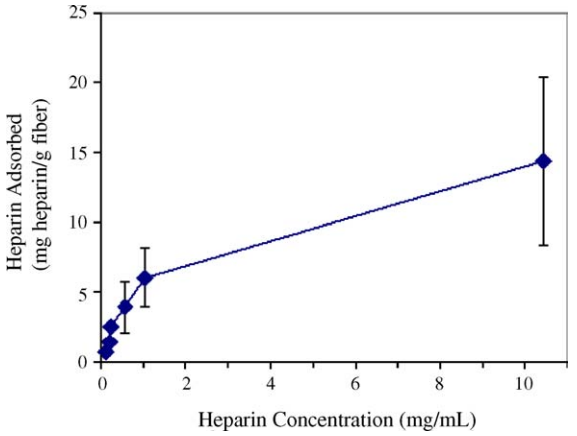


Fig. 4. Representative adsorption isotherm of heparin adsorption on protamine-linked fibers.

provides a representative $C_b$ versus $C_f$ heparin adsorption isotherm made on OAPA-activated and otherwise unmodified protamine fibers. The Langmuir adsorption constants, $K_{EQ}$ and $C_S$, can be determined from this curve and the related double-reciprocal graph accordingly.

Table 2 summarizes the results obtained by utilizing these two novel approaches. As seen, PLL modification resulted in an increase of protamine loading by nearly 3-fold over that of the un-modified fibers (20.21 mg/g versus 7.45 mg/g), suggesting the formation of three equivalent surface layers for protamine immobilization. Similarly, but at a smaller scale, the use of PEG also yielded a significant effect on the amount of immobilized protamine, as a 1.7-fold increase in protamine loading over that of the OAPA-protamine fibers was observed (from 7.45 to 12.96 mg/g). This magnification in protamine loading by PEG was probably due to an enhanced ease in conjugating protamine to the three-dimensionally floating PEG chains rather than to the two-dimensionally oriented fiber surfaces.

Accordingly, there was also a significant enhancement of both the Langmuir adsorption constant (i.e. $K_{EQ}$) and saturation capacity (i.e. $C_S$) on heparin adsorption of protamine fibers amplified with both methods. As shown in Table 2, the $K_{EQ}$ value was increased by 2.7- and 1.7-fold for the PLL- and PEG-modified protamine fibers, respectively, over the unmodified, OAPA-produced protamine fibers. In addition, the $C_S$ value was increased from 5.7 mg/g fibers for the unmodified protamine fibers to 11.5 and 13.5 mg/g for PLL- and PEG-treated protamine fibers, respectively.

As discussed previously, the principle of PLL-induced amplification on loadings was by the creation of multiple surface layers for protamine conjugation, whereas the theory of PEG-induced magnification on adsorption functionality was by the enhancement of motion freedom of immobilized protamine for heparin binding. Based on these hypothesized mechanisms, the conventional wisdom would suggest that the former approach would lead to a more dramatic change in the saturation capacity (i.e. $C_S$) whereas the latter in the adsorption constant (i.e. $K_{EQ}$) of the protamine immobilized fibers. Nevertheless, results in Table 2 displayed a somewhat reversed pattern. When comparing to PLL-modified protamine fibers, the PEG-modified fibers yielded a comparable or slightly higher $C_S$ (13.2 mg/g versus 11.5 mg/g fiber) but much lower $K_{EQ}$ (0.64 mL/mg versus 0.98 mL/mg heparin). A possible explanation for this contradiction, although purely based on speculation, is that the long-chain PLL, with a consistent and repeating sequence of positive charges (due to the amine group on lysine), could also possess a certain degree of spatial freedom for dynamic motion; although this flexibility would certainly be weaker than that of PEG due to the exceptionally low interfacial energy of the latter. On the other hand, the unparalleled spatial mobility of PEG could render the PEG-linked protamine to be far more available and accessible to heparin interaction than the protamine molecules that were tightly fixed to the PLL chain. A delicate combination of these two effects could eventually cause the PLL-modified protamine to display a higher $K_{EQ}$ but a relatively close overall $C_S$.

Also as discussed previously, at low heparin concentrations such as those encountered clinically in extracorporeal therapies, the Langmuir adsorption isotherm would asymptotically approach to the linear isotherm. To this regard, the $K_{EQ}C_S$ should provide a better evaluation of how the protamine fibers would perform under low heparin concentrations. Compared to the OAPA-produced protamine fibers, both modification methods resulted in a remarkable enhancement in the functionality of the unmodified protamine fibers as a 5.2- and 4.0-fold increase of the $K_{EQ}C_S$ value was obtained for the PLL- and PEG-amplified protamine fibers, respectively. Overall speaking, the PLL method appeared to

yield a slight advantage (by ∼20%) over the PEG-based amplification method, based on the total amount of heparin these two types of modified protamine fibers could adsorb under the same initial heparin dose. Further studies are currently in progress with the plan of integrating the two modification methods concurrently (see Fig. 1D for this new strategy) so that a highly effective and reasonably sized (e.g. <50% of the size of an existing hemodialyzer) protamine bioreactor, that is suitable for clinical applications, can be developed to abort the toxic effects associated with ubiquitous uses of heparin and protamine in cardiovascular operations. In a broader view, the two amplification methods could be applied generically to any affinity type bioreactor to improve its functionality. Indeed, efforts have already been initiated, in our laboratory, integrating these two new strategies to develop a GPIIb/IIIa-immobilized bioreactor for the removal of anti-platelet auto-antibodies for the treatment of immune thrombocytopenic purpura (ITP); a commonly encountered autoimmune disease.

## Acknowledgments

This study was partially supported by NIH Grants R41 HL59705 and R01 HL67347. The financial support by the Whitaker Foundation Biomedical Engineering Graduate Fellowship, to which Tanya Wang was the graduate recipient, is also acknowledged.

## References

Ando, T., Yamasaki, M., Suzuki, K., 1973. In: Kleinzeller, A. (Ed.), Molecular Biology, Biochemistry, and Biophysics. Springer-Verlag, pp. 1–109.

Duerksen, P.J., Wilkinson, K.D., 1987. Anal. Biochem. 160, 444–454.

Greenwald, R.B., Conover, C.D., Choe, Y.H., 2000. Crit. Rev. Therap. Drug Carrier Syst. 17, 101–161.

Hirsh, J., 1984. Nouv. Rev. Fr. Hematol. 26, 261–266.

Inman, J.K., Merchant, B., Tacey, S.E., 1973. Immunochemistry 10, 153–163.

Kim, J.S., Yang, A.J., Yang, V.C., 1992. Biotechnol. Bioeng. 39, 450–456.

Kohn, J., Wilchek, M., 1978. Biochem. Biophys. Res. Commun. 84, 7–14.

Ma, S.C., Yang, V.C., Fu, B., Meyerhoff, M.E., 1993. Anal. Chem. 65, 2078–2084.

McCabe, W.L., Smith, J.C., Harriott, P., 1985. Unit Operations of Chemical Engineering. McGraw-Hill Publishing Company, New York.

Ramamurthy, N., Baliga, N., Wakefield, T.W., Andrews, P.C., Yang, V.C., Meyerhoff, M.E., 1999. Anal. Biochem. 266, 116–124.

Teng, C.L., Kim, J.S., Port, F.K., Wakefield, T.W., Till, G.O., Yang, V.C., 1988. Trans. Am. Soc. Artif. Org. 34, 743–746.

Weiler, J.M., Gellhaus, M.A., Carter, J.G., Meng, R.L., Benson, P.M., Hottel, R.A., Schillig, K.B., Vegh, A.B., Clarke, W.R., 1990. J. Allergy Clin. Immun. 85, 713–719.

Wilchek, M., Miron, T., Kohn, J., 1984. Method Enzymol. 104, 3–55.

Yang, V.C., Ma, S.C., Meyerhoff, M.E., 1993. Chemtech 23, 25–32.

Yang, V.C., Port, F.K., Kim, J.S., Teng, C.L.C., Till, G.O., Wakefield, T.W., 1991. Anesthesiology 75, 288–297.

Yang, V.C., Teng, C.L., 1990. In: Gebelein, C. (Ed.), Biomimetic Polymer. Plenum Press, New York, pp. 175–190.

Yun, J.H., Meyerhoff, M.E., Yang, V.C., 1995. Anal. Biochem. 224, 212–220.