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A novel approach for delivery of enzyme drugs: preliminary demonstration of feasibility and utility in vitro

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Abstract

A novel heparin/protamine-based approach for delivery of enzyme drugs without associated toxic effects has been proposed. This approach would allow an enzyme drug to be administered in an inactive (i.e. pro-drug) form and then released at the target site in an active form using protamine as the triggering agent. The pro-drug and the triggered release features of this approach would permit the enzyme drug to act specifically and only on its target substrates while sparing normal substrates, thereby alleviating unwanted toxic effects. The in vitro feasibility of the approach has been successfully demonstrated using trypsin as the model protease drug. In addition, the utility of the approach has also been demonstrated by applying the system in delivering streptokinase, one of the most widely used clinical drugs in thrombolytic therapy. This approach may open up the possibility of developing a wide range of new catalytic drugs that are initially thought to be impossible for therapeutic use due to their potent toxic effects.

Keywords: Heparin; Protamine; Pro-drug feature; Triggered release feature; Enzyme drugs; Streptokinase

1. Introduction

Several attributes of enzymes render them especially suitable as therapeutic drugs; (i) they are very specific and efficient; (ii) they can easily be formulated into liquid dosage forms due to high solubility; and (iii) they are relatively active under physiological conditions. To date, however, only a handful of enzymes including thrombolytic agents (Lijnen and Collen, 1991), L-asparaginase (Ahlke et al., 1997), chymopapain (Tregonning et al., 1991), sutilains (Makepeace, 1983) and DNAse (Falabella et al., 1998) have been approved for clinical uses. The primary reasons for this lack of clinical applications lie in the potential immunogenicity for almost all non-human enzymes, as well as in the indiscriminate nature of an enzyme in its action towards both target and normal substrates. A number of approaches have been attempted to reduce the immunogenicity of an enzyme, and some of them thus far have produced reasonable success (Hershfield et al., 1991; Somack et al., 1991; Mikolajczyk et al., 1996). The inability of an enzyme to distinguish between the

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target and normal substrates for action, however, yields unwanted toxic effects and still stands as the major hurdle to its use as a clinical drug. Take thrombolytic agents as an example; these agents (e.g. streptokinase, urokinase, tissue plasminogen activator (t-PA)) are plasminogen activators that convert inactive plasminogen to active protease plasmin (Lijnen and Collen, 1991; Majerus et al., 1995). Plasmin then degrades fibrin, the principal component of the structural lattice of a thrombus. Plasmin, however, also degrades certain circulating blood clotting factors (e.g. factor VIII) thereby impairing the hemostatic system. While conversion of fibrin-bound plasminogen (i.e. the target substrate) would result in dissolution of the thrombus, conversion of circulating plasminogen (i.e. the normal substrate) would lead to systemic generation of excess plasmin. It is because of this indiscriminate nature of the agent in attacking both target and normal substrates, thrombolytic therapy carries the risk of hemorrhage as a major side effect (Lijnen and Collen, 1991; Majerus et al., 1995). To this regard, a delivery system that could restrict an enzyme drug to act specifically and only on targeted substrates while sparing normal ones would be most ideal and desirable.

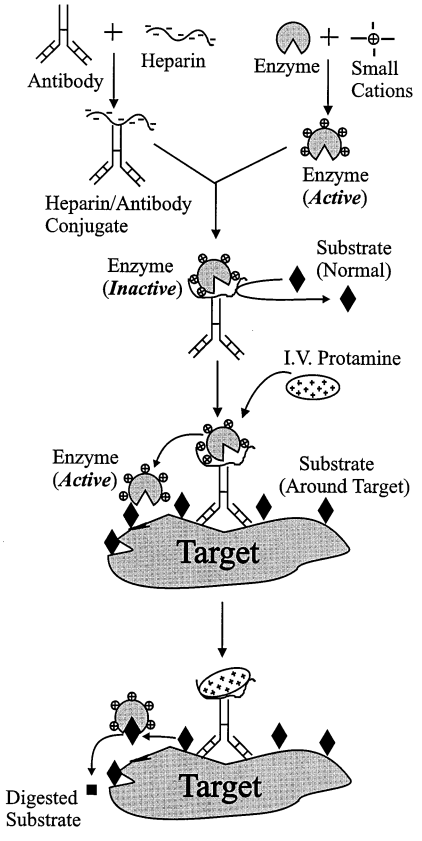

Fig. 1. Schematic diagram of the proposed approach.

We proposed herein a novel delivery system that would permit the administration of an inactive enzyme drug and subsequently a triggered release of the active enzyme at the target site. Fig. 1 presents a mechanistic illustration of the proposed approach. As seen, the system comprises a large protein complex made of two components: (i) a targeting component consisting of an antibody (Ab) chemically linked with an anionic heparin molecule; and (ii) a drug component consisting of the enzyme drug derivatized with cationic species. The two components are attached via a tight but reversible electrostatic interaction. Since the cationic species to be used are relatively small, the modified enzyme is likely to retain a significant level of catalytic activity. This activity, however, would be inhibited after binding with the Ab-heparin counterpart, primarily due to the blockage of the enzyme active site by these appended macromolecules. Thus, similar to a 'pro-drug' type approach, the antibody-bound enzyme will be without its catalytic activity during administration and yet will accumulate at the target site due to the target function of the antibody. Since protamine is an approved clinical heparin antagonist (Jaques, 1973), it can be used safely and effectively as a competing agent to trigger the release of the modified enzyme near the target site. The active enzyme would therefore act selectively and discriminatively on substrates present only around the target site.

In this paper, we utilized trypsin as a model enzyme to demonstrate 'proof of concept' of the proposed delivery system; particularly on the pro-drug and triggered release features. In addition, we also conducted preliminary in vitro studies to demonstrate the 'utility' of the approach in delivering streptokinase, a real and widely used clinical thrombolytic drug in treatment of cardiovascular diseases.

2. Materials and methods

2.1. Materials

Unless otherwise stated, materials were obtained either from Fisher Scientific (Pittsburgh, PA) or Sigma Fine Chemicals (St. Louis, MO). Murine anti-fibrin IgG was purchased from America Diagnostia, Inc. (Greenwich, CT). Water was distilled and deionized.

2.2. Assays

Concentrations of trypsin and azure-A were determined by absorbance at 280 and 620 nm using a molar extinction coefficient of $\varepsilon_{620\ nm} = 2.2 \times 10^4\ cm^{-1}\ M^{-1}$ and $\varepsilon_{280\ nm} = 3.3 \times 10^4\ cm^{-1}\ M^{-1}$, respectively. Activity of trypsin was measured using benzoyl arginine ethyl ester (BAEE) as the substrate, whereas activity of glucose-6-phosphate dehydrogenase was monitored using D-glucose-6-phosphate and β-nicotinamide adenine dinucleotide phosphate (NADP) as the substrate. The aPTT and anti-Xa activities of heparin were measured using the procedures described previously (Byun et al., 1999).

2.3. Preparation of azure-A-modified trypsin

One-half milliliter of a trypsin solution (10 mg/ml; 0.44 mM) was added slowly and dropwise to 2 ml of a well stirred mixture containing 1:1 molar ratio of azure-A:EDAC (1-ethyl-3(3-dimethylaminopropyl)carbodiimide). The molar ratio between trypsin and azure-A (or EDAC) in the final reaction broth was 1:500. The reaction mixture was stirred and incubated at room temperature for 2 h. Unreacted azure-A and EDAC were then removed by passing the reaction mixture through a Sephadex G-25 column (1.5 × 50 cm).

2.4. Measurements of binding constants to heparin

The binding constants of antithrombin III, protamine, and azure-A-modified trypsin to heparin were determined by potentiometric titration using a heparin sensor previously developed in our laboratory (Ma et al., 1993) for end-point detection. The titration data obtained were then recast in a Scatchard form to estimate the heparin-binding constants according to procedures described previously (Yun et al., 1993).

2.5. Heparin-induced inhibition of trypsin activity

Immobilized heparin (the Avidgel-P beads manufactured by BioProbe Inc., Tustin, CA) was used to mimic the macromolecular feature of the heparin-antibody complex. The Avidgel-P beads containing immobilized heparin were added to the above prepared Azure-A-modified trypsin fraction using the ratio of 1 ml of the suction-dried heparin beads per 4 ml of the solution containing the azure-A-modified trypsin. To examine the inhibitory effect of heparin, trypsin activity adsorbed on the heparin beads was compared with that desorbed from the heparin beads. The procedure previously developed in our laboratory for determination of the activity of immobilized enzyme (Yang et al., 1988) was followed to measure the bead-adsorbed trypsin activity. In brief, approximately 1 ml of the suction-dried heparin beads containing the azure-A-modified trypsin was dispersed into 50 mM phosphate buffer to make a final volume of the suspension (termed 'Suspension A') of exactly 3 ml. To 4 ml of the substrate solution containing 1 mM BAEE, 0.8 ml of Suspension A was added. The mixture was well agitated in a 25°C water bath, and after 20 min of incubation, 0.2 ml of 1 N HCl were added to quench trypsin reaction. One milliliter of the supernatant was then withdrawn and assayed for trypsin activity by monitoring the absorbance change at 255 nm. All experiments were conducted in triplicate. To measure the activity of the azure-A-modified trypsin desorbed from the heparin beads, 2 ml of the above Suspension A were withdrawn, suction-dried, and then resuspended into 50 mM phosphate buffer containing 1.2 M NaCl (to completely desorb trypsin from the beads) to make a final volume of exactly 2 ml. After beads separation, 0.8 ml of the supernatant were added to 4 ml of 1 mM BAEE solution, and the same procedure was followed to measure trypsin activity. The degree of inhibition (%) was

then determined by comparing the trypsin activity adsorbed on the heparin beads to that desorbed from the same heparin beads; assuming 100% activity for the latter.

2.6. Protamine-induced reversal of heparin inhibition on trypsin activity

This study was carried out by using the protamine sensor previously developed in our laboratory as the probe (Yun et al., 1995). One milliliter of the suction-dried heparin beads containing azure-A-modified trypsin (prepared according to the procedures described above) was added to a solution containing 5 mg of protamine to make a final volume of exactly 5 ml. Immediately after mixing, 1 ml of the supernatant was withdrawn and diluted with 49 ml of 50 mM phosphate buffer. A protamine electrode sensor was placed immediately in the solution, and the potential change per min was recorded. As a control, the above beads (1 ml) were treated with 1.2 M NaCl solution (4 ml) to completely desorb the azure-A-modified trypsin from the beads, and the supernatant (1 ml) was then diluted with buffer (49 ml) containing 20 μg/ml of protamine and measured immediately for potential change.

2.7. Examination of the utility of the approach in delivering streptokinase

For this study, azure-A was coupled to the β-hemolytic streptokinase (SK) according to the same procedures described above for trypsin. Conversion of the single-chain plasminogen to the two (i.e. heavy and light) plasmin chains was used to monitor the plasminogen-activating activity of the azure-A-modified SK and inhibition by immobilized heparin. In brief, 25 μl of biotinylated human plasminogen (200 μg/100 μl) were added to three 100 μl samples containing: (i) approximately 25 μg of azure-A-modified SK; (ii) a suspension of the heparin beads (1 part of beads: four parts of dH_2O) containing adsorbed azure-A-modified SK; and (iii) the same gel suspension of (ii) plus 50 μg protamine. Three control samples were also prepared similarly by adding 25 μl biotinylated plasminogen to: (a) 100 μl dH_2O; (b) 100 μl dH_2O containing 5 μg of streptokinase; and (c) 100 μl suspension of heparin beads alone (i.e. without any adsorbed azure-A-modified SK). After incubation at 37°C for 15 min, the supernatant of each sample was heated at 100°C for 5 min in the presence of 2% SDS and resolved via SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedures described previously (Yang et al., 1985). The proteins were then transferred to a nitrocellulose membrane and probed with streptavidin conjugated with horse radish peroxidase.

2.8. Preparation of the anti-fibrin antibody-heparin conjugate

Heparin was partially degraded by nitrous acid depolymerization according to a previously described procedure (Olsson and Larm, 1991). To prepare the antibody-heparin conjugate, anti-fibrin IgG (1.0 mg/ml) was oxidized with $NaIO_4$ (20 mM) in 0.05 M sodium acetate buffer at pH 2.7. The reaction mixture was stirred at 4°C for 20 min, and the pH was then adjusted to 7.0 with NaOH to quench the reaction. Unreacted $NaIO_4$ was removed using an Amicon (Beverly, MA) Microconcentrator with a molecular weight cutoff of 30 kDa. To the oxidized anti-fibrin IgG, an excess amount (100:1 in molar ratio) of adipic acid hydrazide was added. The reaction was tumbled at room temperature for 4 h, and unreacted reagents were separated using the same Microconcentrator. The adipic acid hydrazide-activated IgG was then mixed with the above partially degraded heparin (in 0.3 M sodium acetate buffer at pH 4.5) in a molar ratio of 1:100, and after 4 h of incubation at room temperature, excess heparin was removed by using a Sephadex G-100 column. The final anti-fibrin IgG-heparin conjugates were purified using a protamine-Sepharose column and eluted with 1.0 M NaCl.

2.9. Measurements of the anti-fibrin activities

The anti-fibrin activity of the IgG-heparin conjugate was measured using standard ELISA procedures. In brief, the wells in a microplate were coated with fibrinogen as the capturing agent.

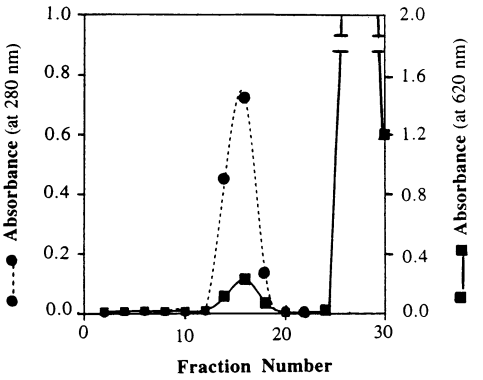

Fig. 2. Sephadex G-25 chromatography. (■), Azure-A, $Abs_{620\ nm}$; (●), Trypsin, $Abs_{280\ nm}$.

Following application of the samples, the wells were treated with anti-murine IgG antibody coupled with alkali phosphatase. The color change of the substrate (*p*-nitrophenyl phosphate) was then monitored at 402 nm. The anti-fibrin activity of the IgG-heparin conjugate was compared with that of the untreated IgG, and the degree of activity retention was estimated by comparing the fold of dilution of the samples that produced the same absorbance at 402 nm.

3. Results and discussion

Trypsin was selected as the model enzyme to examine the in vitro feasibility of the proposed approach. This is because most clinically significant proteases such as coagulation factors are trypsin-like enzymes. Azure-A dye was chosen as the model cation to modify trypsin for several reasons. One is that aside from the positively charged center, azure-A also contains an amino functional group, which can be used for coupling. Another reason is that azure-A is a small (MW: 278 daltons), blue colored dye which absorbs visible light at 620 nm. Coupling of azure-A to trypsin can thus be monitored visually and quantified easily using a spectrophotometer. A third reason is that azure-A is known to possess a strong heparin-binding affinity (Jaques, 1980).

Careful consideration was given to the selection of an appropriate coupling method, with the focus on minimizing both intra- (i.e. within trypsin) or inter-molecular (i.e. between two azure-A or trypsin molecules) crosslinking during the coupling process. Carbodiimide (i.e. EDAC) was selected as the activating agent, since it would allow the initiation of the activation at the –COOH group of an enzyme (Jaques, 1980). Because of this, carbodiimide can be used concomitantly with azure-A (which does not possess the carboxyl group) to activate trypsin without the risk of forming inter-molecular crosslinks between two azure-A molecules. A slow and dropwise addition of trypsin to a vigorously stirred azure-A/EDAC mixture was followed to yield a low local trypsin concentration that would be in favor of azure-A-trypsin coupling as opposed to 'inter- and intra-trypsin' crosslinking.

Fig. 2 shows the elution profile from a Sephadex G-25 column following the conjugation of azure-A to trypsin. As expected, trypsin (monitored at 280 nm) eluted at the void volume whereas azure A (monitored at 620 nm) eluted at the column volume. A small and yet obvious azure-A peak was found to co-elute with the trypsin peak, indicating that certain azure-A had been incorporated onto trypsin. Apparently, this azure-A peak did not result from unreacted azure-A due to poor column resolution, because the absorbance (at 620 nm) between the two azure-A peaks already reached the baseline.

The trypsin fractions between #14 and #18 in Fig. 2 were pooled and further purified by using the Avidgel-P beads containing immobilized heparin. An obvious transfer of the blue color of azure-A from the solution phase to the gel beads was observed, suggesting the binding of the azure-A-modified trypsin to heparin. The bead-adsorbed trypsin appeared to be azure-A-modified species, since the control experiment using untreated trypsin did not show any measurable adsorption on the heparin beads. Because of the requirement of a specific heparin-binding strength for the azure-A-modified trypsin (see below for reasons), only fractions eluted from the heparin beads between 0.8 and 1.2 M NaCl rinsing were collected. Calculation of the protein mass based on absorbance at 280 nm revealed that approximately 31% of trypsin in the initial pooled fractions (i.e.

14– # 18 in Fig. 2) were eluted from the heparin beads using 0.8–1.2 M NaCl wash.

Results from characterization of this azure-A-modified trypsin are summarized in Table 1. Interestingly, the azure-A-modified trypsin retained 80% of the original trypsin activity, when compared to a control sample containing untreated trypsin prepared to the same protein absorbance at 280 nm. This finding suggests that incorporation of azure-A to trypsin does not seem to alter much of trypsin activity. Based on the molar extinction coefficient of $\varepsilon_{620nm} = 2.2 \times 10^4$ cm^{-1} M^{-1} and $\varepsilon_{280nm} = 3.3 \times 10^4$ cm^{-1} M^{-1} for azure-A and trypsin, respectively, the molar ratio of azure-A versus trypsin in the azure-A-modified trypsin was estimated to be about 4.9:1.

To examine heparin-induced inhibitory effect on the azure-A-modified trypsin, trypsin activity 'adsorbed on' and 'desorbed from' the heparin beads were compared. Results (Table 1) show that there was 42% reduction in trypsin activity of the heparin-bound trypsin. It should be noted that this inhibition study was first carried out using the small synthetic substrate BAEE for trypsin. Obviously, for an inhibitory event established on blocking the active site of an enzyme with a macromolecule, such as the mechanism adopted by our proposed approach, the degree of inhibition is dependent on the size of the substrate. While a small substrate can still diffuse through the blockage provided by the macromolecule, a large substrate is likely to be excluded. Therefore, the above 42% inhibition may represent only the minimal degree of inhibition. Since the primary candidates for our proposed delivery system are proteases (e.g. thrombolytic agents) that act on protein substrates, it is necessary to examine the heparin-induced inhibitory effect towards a large substrate. To this regard, the enzyme glucose 6-phosphate dehydrogenase was selected as the trypsin substrate. The reason for utilizing a second enzyme as the substrate is that the degree of inhibition can be easily determined by measuring the activity change of the second enzyme. By using D-glucose-6-phosphate and NADP (β-nicotinamide adenine dinucleotide phosphate) to monitor the activity of glucose 6-phosphate dehydrogenase, it was found that binding of the azure-A-modified trypsin to heparin beads resulted in nearly 83% inhibition in activity towards digestion of glucose 6-phosphate dehydrogenase. Two reasons may attribute to the failure in achieving 100% inhibition. One is that, as shown in Table 1, five azure-A molecules are available in each azure-A-modified trypsin for heparin binding. This number may simply not be enough to yield a complete binding and blockage of the active site of trypsin by heparin. The other reason is that even if five azure-A molecules are sufficient to induce an effective heparin binding, these azure-A molecules may not all be present in the same location or, specifically, in the vicinity of trypsin's active site for heparin binding. Both the number of cations and their locations on the enzyme deem critical in achieving 100% inhibition by heparin interaction.

Previously we reported the development of an electrochemical sensor for protamine measurements (Yun et al., 1995). Since protamine is an excellent substrate to trypsin, it seems possible to use the protamine sensor in monitoring trypsin activity. Indeed, early studies show that the rate of potential decrease measured by the protamine sensor, which reflected the rate of protamine di-

Table 1
Preparation and characterization of the azure-A-modified trypsin

Properties	Azure-A-modified trypsin[a]
Recovery yield (%)	31 ± 3%
Molar ratio (azure-A/trypsin)	4.9:1
Retention of original trypsin activity (%)	80 ± 2%
Heparin-induced inhibition (%)	
Versus. small substrate[b]	42 ± 5%
Versus large substrate[c]	83 ± 3%
Binding constant vs. heparin ($\times 10^7$ M^{-1})	0.74 ± 0.05
Trypsin activity recovered with protamine (%)[d]	100%

[a] This preparation was made following purification using the Avidgel-P heparin beads.
[b] Small substrate: BAEE.
[c] Large substrate: glucose 6-phosphate dehydrogenase.
[d] Heparin beads containing adsorbed azure-A-modified trypsin were treated with a 0.15-M protamine solution (Section 2).

gestion by trypsin, was linearly proportional to the added trypsin activity (Yun et al., 1995). Hence, this protamine sensor was employed in our study to examine the reversal of heparin-induced inhibition on trypsin activity after the azure-A-modified trypsin was desorbed from the heparin beads by a protamine solution. A rate of potential reduction of 5.9 mV/min was observed in the solution where protamine was used both as the eluting agent and substrate, compared to 5.4 mV/min in the solution where 1.0 M NaCl was used as the eluting agent and protamine as the substrate. These results confirm that protamine can be used to completely trigger the release of azure-A-modified trypsin from heparin beads and reverse heparin-induced inhibition.

It should be pointed out that for the proposed heparin/protamine-based delivery system to function, two binding conditions must be met. One is that the binding to heparin of the cation-modified enzyme has to be stronger than that of antithrombin III (ATIII) so that the modified enzyme can remain attached to the heparin-antibody component and be inhibited by heparin during administration. This is because the prelude of all heparin-induced events is on its binding with ATIII. On the other hand, the binding to heparin of the cation-modified enzyme has to be weaker than that of protamine so that protamine can be used effectively to release the modified enzyme, at the target site, from heparin binding and inhibition. The collection of the azure-A-modified trypsin fractions from the heparin beads between 0.8 and 1.2 M NaCl elution (see discussion above) was primarily intended to meet with these binding requirements. It has been reported in the literature that ATIII eluted from the heparin column by 0.8 M NaCl elution whereas protamine by 1.2 M NaCl (Olsson and Larm, 1991). To further validate that such prepared azure-A-modified trypsin indeed possessed the required heparin-binding strength, the binding constants to beef lung heparin of ATIII, protamine, and the azure-A-modified trypsin were determined using the titration method described previously (Ma et al., 1993) and a heparin sensor as the probe. Results (Table 1) show that the heparin binding constant for the azure-A-modified trypsin was 0.74×10^7 M^{-1}; right in-between the values of 0.49 and 24.6×10^7 M^{-1} for ATIII and protamine, respectively.

Due to the lack of a definite target for trypsin delivery, the antibody component of the proposed delivery approach was not examined herein but included in the following utility study. Nevertheless, the above findings did shed light of the feasibility of this delivery approach, particularly in relation to its pro-drug and triggered release features.

To examine the utility of the approach, preliminary studies were conducted by applying the method on delivery of a real enzyme drug such as streptokinase (SK). As discussed previously, SK is one of the most widely used thrombolytic drugs and its clinical use is known to be associated with a high incidence of bleeding complications (Lijnen and Collen, 1991). Unlike other thrombolytic drugs such as t-PA, streptokinase itself is not really a protease with intrinsic catalytic activity. However, it forms a non-covalent complex with plasminogen to produce a conformational change in plasminogen which then becomes an activated protease to convert free plasminogen to plasmin (Lijnen and Collen, 1991). The reason in selecting SK over t-PA to test the approach is that, from a fundamental standpoint, it is far more effective to diminish the binding capability of a protein than to inhibit the activity of an enzyme via the binding of a macromolecule.

By following the same procedures described previously for trypsin, the azure-A-modified SK was successfully produced. This preparation, after purification by the heparin coated beads using the same salt elution (i.e. 0.8–1.2 M NaCl), yielded a molar azure-A/SK ratio of about 5.4:1. These results are consistent with those observed in the trypsin studies (Table 1). Fig. 3 shows the conversion of plasminogen to plasmin by this azure-A-modified SK as resolved by SDS-PAGE; in which the nitrocellulose membrane was treated subsequently with streptavidin-peroxidase and the chemiluminescent peroxidase substrate. As shown in Lane # 1, plasminogen (the band seen between the 106- and 80-K protein markers) itself contained some impurities, as reflected by the presence of some heavy-chain (the band under the

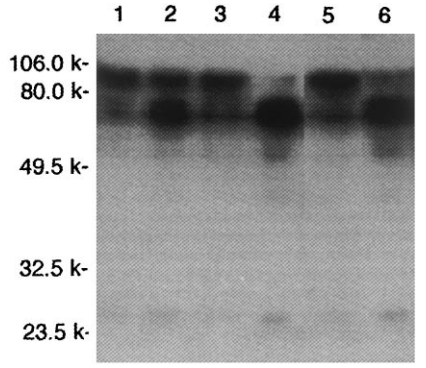

Fig. 3. Function and inhibition of the azure-A-modified SK. The nitrocellulose membrane was treated subsequently with streptavidin-peroxidase and the chemiluminescent peroxidase substrate. For experimental details, please see Section 2. The picture was generated from the original film of the nitrocellulose membrane by computer imaging. Column # 1: plasminogen alone; Column # 2: plasminogen + SK; Column # 3: plasminogen + heparin coated beads; Column # 4: plasminogen + azure-A-modified SK; Column # 5: plasminogen + azure-A-modified SK + heparin beads; and Column # 6: plasminogen + azure-A-modified SK + heparin beads + protamine.

80-K marker) and light-chain (the vague band slightly above the 23.5-K marker) plasmin. Addition of commercial streptokinase (Lane # 2) resulted in a significant conversion of plasminogen to the two plasmin chains; as reflected by the appearance of a much dense band of heavy-chain plasmin at the expense of plasminogen. The appearance of a rather weak band for the light-chain plasmin suggests that most of the biotin labels on plasminogen were attached to the heavy-chain plasmin. Heparin exhibited virtually no effect on plasminogen (see Lane # 3). Results in Lane # 4 clearly demonstrate that the azure-A-modified SK retained a significant level of plasminogen-activating activity, as it converted nearly 90% of plasminogen to the two plasmin chains. Binding of heparin to the azure-A-modified SK was shown clearly to inhibit the plasminogen-activating activity (Lane # 5). In fact, a minimal conversion of plasminogen and more than 90% inhibition of SK activity was observed in Lane # 5, when the results were compared to those seen in Lane # 4 where no heparin was added. When an excess amount of protamine was added to the SK-adsorbed heparin beads, heparin inhibition was reversed and the plasminogen-activating activity of the azure-A-modified SK was restored, as reflected by the significant conversion of plasminogen to the two plasmin chains (Lane # 6). Overall, the utility of the approach in providing a pro-drug and triggered release feature for delivery of real clinical enzyme drugs has been largely confirmed.

The targeting component was examined in our investigation of the utility of the proposed approach. The anti-fibrin IgG-59D8 produced by Haber's group (Bode et al., 1989) was selected because when comparing with other monoclonal antibodies designed to target the thrombus (Lijnen and Collen, 1991), IgG-59D8 yielded the highest specificity to fibrin and was also commercially available. Since the targeting function of this antibody is well established, the only aspects of the targeting component that could possibly affect the overall feasibility of the approach are: (i) whether conjugation of heparin to the antibody will affect the designated functions for both molecules; and (ii) whether the heparin-antibody will possess a similar inhibitory effect as the heparin coated beads on the cation-modified enzyme. To address the first concern, we recently developed a site-directed coupling method that would allow for an end-point attachment of heparin to the F_c region of an antibody. This method would retain an intact heparin molecule for interaction with the cation-modified enzyme, as well as an intact F_{ab} region in the antibody for targeting function. Fig. 4 illustrates the chemical mechanism for this conjugation method. In a brief explanation, this method is based on the fact that immunoglobins usually contain about 3% of the carbohydrate in the F_c region. Under mild and acidic conditions (pH 4–5), sodium periodate ($NaIO_4$) can oxidize the vicinal –OH groups of the carbohydrate to reactive aldehyde residues. Since the reaction is carried out under acidic conditions, the $-NH_2$ groups on the antibody will be protonated. Hence, this $NaIO_4$ activation step will not carry the risk in introducing intra-molecular crosslinking of the antibody. The oxidized aldehyde groups are then converted to highly reactive hydrazide groups by the addition of the

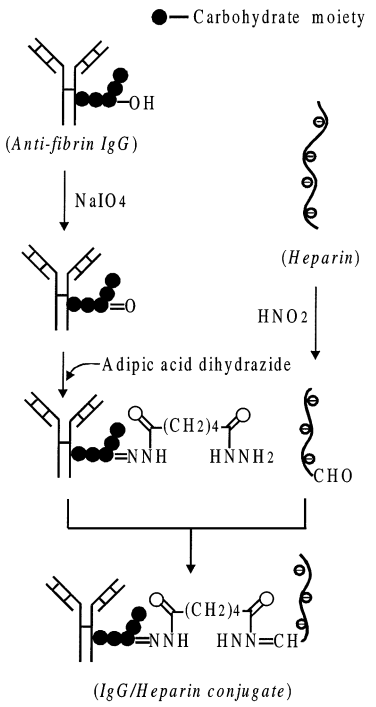

Fig. 4. Schematic illustration of the conjugation between heparin and antifibrin IgG.

adipic dihydrazide crosslinking agent. It should be pointed out that without this extra step of conversion, coupling of heparin to the activated antibody can only take place via the amino groups of heparin and also under basic conditions. As mentioned above, coupling under basic conditions will enhance the risk of forming intra-molecular crosslinking between the activated aldehyde groups and the $-NH_2$ groups of the antibody. With this extra conversion step, however, the HNO_2-treated heparin can be linked via, and only via, its terminal aldehyde group to the antibody. Results obtained from the ELISA assay using fibrin as the capturing agent (Fig. 5) show that the IgG-59D8 on the heparin-IgG-59D8 conjugate maintained over 80% of the original fibrin-targeting ability. In addition, results obtained from the aPTT clotting assay and the anti-Xa chromogenic assay indicated that heparin on the heparin-IgG-59D8 conjugate also retained over 85% of its original anticoagulant activity. Furthermore, heparin on the heparin-IgG-59D8 conjugate also retained its full protamine-binding ability (data not shown), as determined by using the protamine sensor (Yun et al., 1995) and the methodology described previously (Ma et al., 1993). Thus, conjugation of heparin to the antibody using this newly developed method did not alter the designated functions for both molecules.

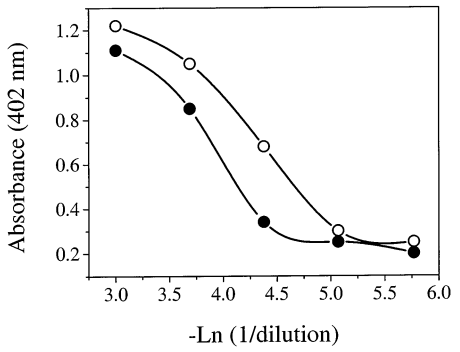

Fig. 5. Fibrin-targeting ability of (○) antifibrin IgG; and (●) antifibrin IgG-heparin conjugates. Reactions were measured by ELISA using fibrin-coated wells in a microplate according to the procedures described in Section 2.

To address the second concern, the inhibitory effect by the heparin-IgG-59D8 conjugate was examined using the same SDS-PAGE method described in Fig. 3. Results show that identical patterns of inhibition and reversal of this inhibition by protamine comparing to those seen in Fig. 3 using the heparin beads were observed. Conversion of plasminogen to plasmin by the azure-A-modified-SK was largely inhibited ($>85\%$) by the heparin-IgG-59D8 macromolecule, whereas this inhibition was completely (100%) reversed after the addition of protamine (Note: figure was not shown to avoid repetition). To this end, the feasibility of the targeting component of our proposed approach is reasonably confirmed.

4. Conclusions

In conclusion, preliminary in vitro studies presented in this paper strongly suggest both the feasibility and utility of the approach in delivering enzyme drugs; particularly related to the pro-drug and triggered release features. Despite promise, however, the current investigation also reveals

limitations of this delivery approach. One limitation is that the recovery yield of the prepared cation-modified enzyme that contains the required heparin-binding strength is relatively low ($\sim 30\%$; Table 1), and the other limitation is the failure in achieving 100% of heparin-induced inhibition (maximum inhibition: $\sim 80\%$; Table 1). Both problems are believed to be attributed to the chemical method employed in coupling of the cations to the enzyme; as it yields a random incorporation of the cations in different locations within an enzyme, as well as an uncontrollable conjugation of various numbers of the cations to each enzyme molecule. To this end, future studies have been focused on the conjugation of a polyarginine peptide containing a specifically defined positive charges to a specific location of an enzyme drug (e.g. t-PA) using the currently evolving recombinant DNA technology.

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