

# Serum albumin–protamine conjugate for biocompatible platform for targeted delivery of therapeutic macromolecules

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Received 28 June 2013; revised 31 July 2013; accepted 9 August 2013

Published online 27 August 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.34916

**Abstract:** A well-defined, one-to-one conjugate between human serum albumin (HSA) and protamine was synthesized and characterized as a biocompatible carrier for macromolecules. In circulation, the conjugate will camouflage drug molecules upon complex formation, while liberating free drug at the desired location using a triggering mechanism. The N-terminus of protamine was thiolated and conjugated with the unpaired Cysteine-34 of HSA, and was purified by ion-exchange chromatography. The molecular weight of the conjugate was 70.8 kDa, confirming one-to-one conjugation between HSA (66.6 kDa) and protamine (4200 Da). Superimposed fluorescence spectra of native HSA and HSA–protamine conjugate indicated no conformational change around the Trp-214. The conjugate had marked reduction in hemolytic and cytotoxic properties compared to protamine. When

therapeutic potential was tested using tissue plasminogen activator as a model drug, HSA–protamine conjugate suppressed the enzymatic activity by 65%, which was fully recovered by a triggering agent, heparin. The construct showed binding characteristics with activated platelets upon conjugation with a targeting peptide, demonstrating flexibility to introduce suitable homing moiety on the surface. The camouflaged construct retained triggered release property in human plasma condition. Overall, the conjugate has a good potential to serve as a biocompatible platform for macromolecular drugs. © 2013 Wiley Periodicals, Inc. *J Biomed Mater Res Part A: 102A: 2481–2490*, 2014.

**Key Words:** albumin-based delivery, localized thrombolysis, prodrug strategy for macromolecules, albumin as a carrier

**How to cite this article:** Absar S, Nahar K, Choi S, Ahsan F, Yang VC, Kwon YM. 2014. Serum albumin–protamine conjugate for biocompatible platform for targeted delivery of therapeutic macromolecules. *J Biomed Mater Res Part A* 2014;102A:2481–2490.

## INTRODUCTION

The use of human serum albumin (HSA) as a carrier for therapeutic molecules has been under extensive investigation. HSA itself serves as a therapeutic agent in the events of severe burns, hypovolemia, surgical blood loss, acute or chronic liver failure, cardiopulmonary bypass, nutrition support, and so forth.<sup>1</sup> Furthermore, the ability of HSA to bind with multiple ligands, and consequently serve as a depot for numerous exogenous and endogenous substances translated the use of HSA as a drug carrier from bench to bedside. An example includes development of insulin Levemir® which contains myristic acid at the ε-amino group that binds with HSA after administration into the body, resulting in a 50- to 70-fold increase in the half-life of the drug.<sup>2</sup> The albumin-paclitaxel nanoparticle, Abraxane®, becomes soluble upon intravenous administration, mimicking endogenous albumin. These nanoparticles are

transported into the interstitium of a solid tumor through the interaction of albumin with the glycoprotein gp60 that is expressed on the endothelial cell surface.<sup>3–5</sup> Recently, cell penetrating albumin conjugates have also been reported for intracellular delivery of doxorubicin.<sup>6</sup> Furthermore, Kratz and co-workers proposed an albumin-based pro-drug approach by conjugating doxorubicin to cysteine-34 of endogenous HSA.<sup>7,8</sup> The prodrug reduced the systemic toxicity with a favorable cytotoxic profile when the active drug was released from albumin, either in the mild acidic environment of the tumor extracellular compartment or intracellularly in the acidic lysosomal compartment.<sup>9</sup> Compared to the promising outcome of this approach with small molecules, there are rooms for advancement in the development of albumin-based drug carrier for macromolecules, such as enzyme drugs that are administered directly into the vascular compartment.

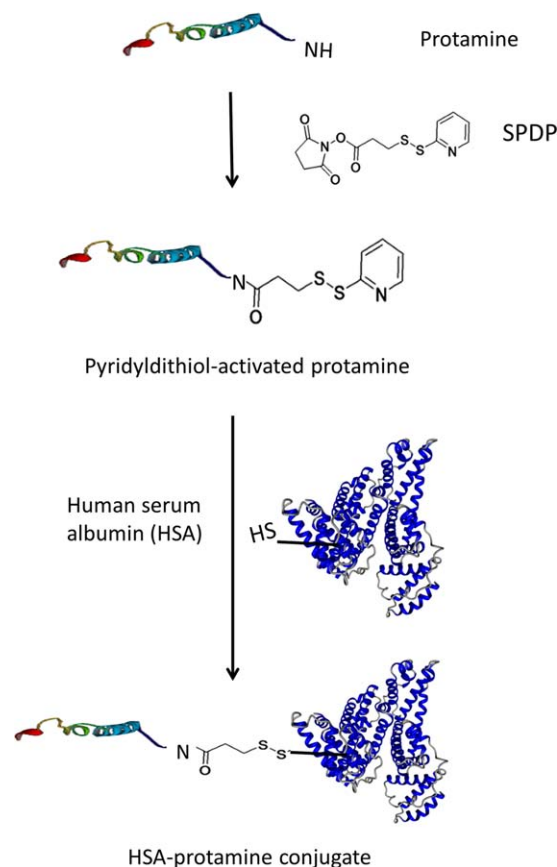
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Therapeutic proteins, enzymes, oligonucleotides, and monoclonal antibodies have gained significant attention because of their highly specific action on the respective substrates. However, in many cases, the molecular targets are present throughout the circulation causing the macromolecules unable to distinguish the targets in the diseased site from those in general tissues generating off-target effects. An effective way to overcome this shortcoming could be targeted delivery, which, however, still carries the potential for acting on off-target substrates while traveling in the circulation. Thus, a pro-drug approach for these biomacromolecules is warranted to suppress the activity of the drugs in the systemic circulation, and regenerate at the target site. Being the most abundant plasma protein, HSA can serve as a camouflage for therapeutic agents in the systemic circulation.<sup>10,11</sup> To that end, we propose an albumin-based pro-drug construct for enzyme drugs in which the drug will be carried by albumin via electrostatic interaction that will be disrupted by a triggering agent upon reaching the target. As a model macromolecule, we have used tissue plasminogen activator (tPA) that produces enzymatic action by converting plasminogen to plasmin, which further degrades fibrin mesh to restore blood flow to an occluded vessel. However, tPA possesses life-threatening bleeding risk because of its indiscriminate action on plasminogen.<sup>12</sup> Therefore, a prodrug approach to minimize ubiquitous plasmin generation while maximizing tPA's exposure at the clot site could significantly attenuate bleeding complication and enhance therapeutic outcome. Whereas the accumulation of the agent at the clot site can be achieved by using a suitable homing moiety, regeneration of activity remains a critical challenge because of lack of triggering environment, that is, pH, temperature, or reducing agent, during a thrombotic event. As heparin is commonly used in the therapeutic management of thrombotic events, we have designed the construct such that the regeneration of activity can be achieved by heparin administration. Hence, a one-to-one conjugate between albumin and protamine was developed (Fig. 1). On the other hand, tPA, the most common thrombolytic agent, was modified to introduce a series of negative charges so that it forms an electrostatic complex with the protamine moiety of the albumin-protamine conjugate. We chose the homing moiety based on the interaction of fibrinogen with integrin GPIIb/IIIa that is expressed on activated platelets, the underlying factor of aggregation and formation of occlusive thrombus. Ware et al. reported the C-terminal gamma chain peptide sequence of fibrinogen, CQQHHLGGAK-QAGDV, as the central structural segment responsible for the binding,<sup>13</sup> and hence was used as targeting moiety. Thus, the construct will accumulate on the thrombus following which therapeutically active tPA will be released upon interaction with subsequently administered heparin. In the current work, we have synthesized and purified albumin-protamine conjugate, characterized for vascular and hemo-compatibility and demonstrated the feasibility to be used for a targeted prodrug construct for thrombolytic enzyme.

## MATERIALS AND METHODS

### Materials

HSA (from human blood source), protamine, fibrinogen, plasminogen, aldrithiol (2,2'-Dithiopyridine 2,2'-Dipyridyl-di-



**FIGURE 1.** Graphical representation of one-to-one conjugate preparation via Cys-34 of HSA and N-terminus (secondary amine) of protamine. The three-dimensional structure of HSA was generated by PPG: *the Protein Picture Generator* <http://bioserv.rpbs.jussieu.fr/PPG>; DINO: *Visualizing Structural Biology (2002)* <http://www.dino3d.org>.<sup>21</sup> Atomic coordinates were downloaded from the Protein Data Bank, accession number 4iw2. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

sulfide), dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO). N-Succinimidyl-1,3-(2-pyridyldithio) propionate (SPDP) was obtained from Molecular Biosciences (Boulder, CO). tPA was purchased from Genentech (Genentech, South San Francisco, CA). LMWH was obtained from Eisai (Woodcliff Lake, NJ). The plasmin specific chromogenic substrate S-2251 was obtained from Chromogenix (Lexington, MA). Fluorescein isothiocyanate (FITC) was purchased from Fisher Scientific (Pittsburgh, PA). The 7-mers of glutamate oligopeptide (CEEEEEEE) and the targeting peptide sequence 14-mers of the C-terminal peptide (CQQHHLGGAK-QAGDV) from the C-terminus of fibrinogen gamma chain were obtained from EZBiolab (Carmel, IN). All the cell culture related materials were obtained from American Type Cell Collection (ATCC; Manassas, VA). All other buffers and chemical reagents were purchased from Sigma-Aldrich and of reagent grade.

### Activation of N-terminus proline of protamine

Protamine (10 mg) was taken in a scintillation vial into which 770  $\mu$ L of 2M NaCl dissolved in 20 mM phosphate

buffer was added. The mixture was gently stirred at room temperature for 10 min. Separately, 120 mg of polyethylene glycol-600 was dissolved in 770  $\mu\text{L}$  of 100 mM phosphate buffer and added to protamine solution. Once protamine is completely dissolved, SPDP, dissolved in 550  $\mu\text{L}$  dimethylformamide (DMF), was added drop-wise at a feed-ratio of 5, 10, 15, and 20-fold excess of protamine. The reaction was continued for 4 h at room temperature, followed by quenching with 100  $\mu\text{L}$  of 100 mM glycine. Excess SPDP was purified by Fast Protein Liquid Chromatography (FPLC, Biologic Duo Flow, Biorad) using ion-exchange CM-Sepharose column (GE Healthcare) with elution buffer (A) 20 mM phosphate buffer and (B) 2 mM NaCl + 20 mM phosphate at pH 7.4. To quantify the yield of activated protamine, 900  $\mu\text{L}$  of the collected sample was taken in a quartz cuvette and absorbance was measured at 343 nm by Diode array spectrophotometer (HP 8453A, Olis). Then, 100  $\mu\text{L}$  of 0.5M DTT solution was added and, following 15 min of incubation, the absorbance at 343 was again measured. The release of pyridyl-2-thione upon treatment of DTT was calculated to determine the extent of protamine activation.

#### Synthesis and purification of HSA-protamine conjugate

HSA (10 mg) was dissolved in 20 mM phosphate buffer at pH 7.2 from which 500  $\mu\text{L}$  was taken and added drop wise to 500  $\mu\text{L}$  of activated protamine (protamine-PDP) solution. The reaction kinetic was monitored by Diode array spectrophotometer at 343 nm by measuring the release of pyridine-2-thione with time. Once the reaction progression was confirmed, the feed-ratio was optimized by introducing 1, 2, 3, and 4-fold molar excess of HSA to the activated protamine and incubating at room temperature for 2 h with mild shaking. The HSA-protamine conjugate was then purified in one-step using ion-exchange CM-Sepharose column with the same elution buffer A and B as mentioned in "Activation of N-terminus proline of protamine" section. The yield of the conjugate was determined by UV spectroscopy at 280 nm.

#### Determination of molecular weight and fluorescence spectra of HSA-protamine conjugate

The molecular weight of the prepared HSA-protamine conjugate was determined by electrospray mass spectrometry (Agilent 6520 Q-TOF). An aliquot of 40  $\mu\text{L}$  sample at a concentration of 1 mg/mL was loaded into the autosampler vial from which 5  $\mu\text{L}$  was injected into a C-8 poroshell column. Buffer A, composed of 5% acetonitrile with 0.1% formic acid / 95% water with 0.1% formic acid, was run through the sample loaded column for 2 min at a flow rate of 0.5 mL/min. Then a gradient to 100% acetonitrile with 0.1% formic acid was run over 10 min. The mass spectrometer was run in positive ion mode, with reference mass correction on a dual ESI source. The data were processed with Agilent's MassHunter software. To compare the prepared HSA-protamine conjugate with native HSA (1 mg/mL), a similar run was performed keeping all the parameters same as that of the sample. For fluorescence spectroscopic study, a sample (1 mg/mL) of HSA and HSA-protamine conjugate

was taken in a cuvette with 1-cm path length and measurement was performed on Hitachi F-2500 fluorescence spectrophotometer. The spectrum was recorded in the range of 300–400 nm using excitation wavelength of 295 nm.

#### Hemolysis of protamine-conjugated HSA carrier

Hemolysis of the prepared HSA-protamine was monitored, which could be originated from the cationic protamine moiety. Human blood sample was collected from Coffee Memorial Blood Center (Amarillo, TX). An aliquot of 100  $\mu\text{L}$  of blood cells were taken and diluted to 1 mL with saline. The cells were washed three times using centrifugation (Legend Micro 17R, Thermo Scientific) and the final volume was made 1 mL. The cells were further diluted 10 times from which 100  $\mu\text{L}$  was taken in each eppendorf tube into which protamine or HSA-protamine conjugate was added at a final protamine concentration of 10  $\mu\text{g}/\text{mL}$ . The final volume of 1 mL was incubated at 37°C for different time points. Deionized water and saline was used as positive and negative controls, respectively. Following incubation, the samples were centrifuged at 800g for 2 min and the supernatants were analyzed for hemolysis by measuring absorbances at 540 nm on a spectrophotometer.

#### Cytotoxicity study of prepared carrier system

The cytotoxicity of HSA-protamine conjugate was tested with arterial (pulmonary) smooth muscle cells using MTT assay.<sup>14,15</sup> Cells were cultured in Dulbecco's Modified Eagle's Medium containing Fetal Bovine Serum and penicillin/streptomycin. 50,000 cells were seeded on 96-well flat bottom plate and kept overnight for settling down. On the following day, protamine or HSA-protamine conjugate was separately added to the wells at a concentration range from 0 to 1 mg/mL equivalent of protamine, and incubated for 4 h at 37°C. The culture media was then replaced by 100  $\mu\text{L}$  of fresh media containing 0.5 mg/mL MTT solution, and incubated for another 4 h. After gently removing the MTT solution, the crystalline precipitate was dissolved in 100  $\mu\text{L}$  of dimethylsulfoxide (DMSO; molecular biology grade) and absorbance was measured at 570 nm. The percent of viable cells were calculated by the following equation:

$$\% \text{ of Viable cells} = (A_{\text{treated}} - A_{\text{MTT}}) / (A_{\text{control}} - A_{\text{MTT}}) \times 100$$

#### HSA-protamine conjugate in the triggered-release approach for thrombolytic macromolecules

The proof-of-principle triggered-release system for macromolecules with our prepared HSA-protamine conjugate was tested using tPA as a model drug. To introduce electronegative functionality, tPA was conjugated with low molecular weight heparin (LMWH) by previously described method.<sup>10</sup> HSA-protamine conjugate at 50-fold excess of tPA was incubated with tPA for 30 min at room temperature to facilitate electrostatic interaction between LMWH and protamine moieties. The amidolytic activity of tPA upon complex formation with HSA-protamine was measured by Chromogenic assay.<sup>16</sup> Briefly, 1.3  $\mu\text{M}$  of substrate S-2251, 0.24  $\mu\text{M}$

plasminogen and 0.5  $\mu\text{g}/\text{mL}$  tPA dissolved in 50 mM Tris HCl containing 50 mM NaCl with 0.01% Tween 80 at pH 7.2 was taken in a 300  $\mu\text{L}$  well of 96-well plate. The initial rate of hydrolysis of S-2251 by plasmin generated from plasminogen by tPA was determined by measuring the absorbance at 405 nm at different time intervals using a micro-plate reader (SynergyMx, Biotek). The slope of absorbance against square of time ( $\Delta A \text{ min}^{-2}$ ) was expressed as the initial rate of S-2251 hydrolysis. To assess heparin-mediated triggered release, the assay was also performed in the presence of 0.4  $\mu\text{mL}$  of heparin.

### **Binding study of homing-peptide decorated HSA-protamine conjugate with activated platelets**

The homing peptide, CQQHHLGGAKQAGDV, was conjugated to the primary amines of HSA of the conjugate. Briefly, SPDP, 20-fold excess of HSA, was dissolved in DMF and added drop-wise to the HSA-protamine conjugate. Following 30 min incubation at room temperature, excess SPDP was removed by size-exclusion chromatography, and the resulting purified thiol-reactive conjugate was incubated with the homing peptide for 30 min at room temperature. Finally, the homing-peptide decorated HSA-protamine was again purified by size-exclusion chromatography. The binding characteristic of the conjugate with activated-platelets were tested by fluorescence microscopy.<sup>17,18</sup> Human platelets ( $1.68 \times 10^9$  platelets/mL of plasma) were collected from Coffee Memorial blood center (Amarillo, TX) and were isolated by centrifuging 1 mL of platelet-rich plasma at 400g for 15 min at 25°C. After removing the supernatant, platelets were re-suspended in PBS containing 1% Bovine Serum Albumin (BSA). The sample was diluted to make a suspension containing 250,000 platelets per  $\mu\text{L}$ , which was then adsorbed onto collagen-coated glass cover-slips at room temperature for 1 h and washed thrice with PBS. To confirm the adhesion of platelet monolayer on cover-slips, cells were stained with FITC-tagged anti-GPIIb/IIIa monoclonal antibody (eBioscience, CA, USA), and subsequently observed under fluorescence microscopy (with magnification of  $10 \times 60 \times 1.5$  (overall 900 $\times$ ). FITC-labeled peptide-linked camouflaged tPA construct was incubated with the adsorbed platelets for 1 h in dark at room temperature. Fluorescent construct without the homing peptide was also incubated as a control. All incubations were carried out in the presence of 5 mM  $\text{CaCl}_2$  solution under mild shaking to facilitate activation of platelet.

### **Stability of the construct in human plasma and blood environment**

Fresh human plasma and blood samples were collected from coffee memorial blood center (Amarillo, TX). To test the stability of the electrostatic interaction between anion-modified tPA and HSA-Protamine conjugate, albumin-camouflaged tPA was incubated with plasma for 30 min at 37°C. An aliquot of 50  $\mu\text{L}$  of the incubated plasma sample was then taken and added to a well (96-well plate) to perform Chromogenic assay as described previously (section HSA-protamine conjugate in the triggered-release approach

for thrombolytic macromolecules). To evaluate the triggered release profile, a heparin (0.4  $\mu\text{mL}$ ) treated group was also tested with the camouflaged tPA group.

### **Statistical analysis**

All data were presented as the mean  $\pm$  SD. One-way ANOVA was performed to compare among the groups and analyze the statistical significance. Post-hoc analyses were conducted using Tukey's comparison (GraphPad Prism, version 5.0, GraphPad Software, San Diego, CA). *p*-values less than 0.05 were considered statistically significant.

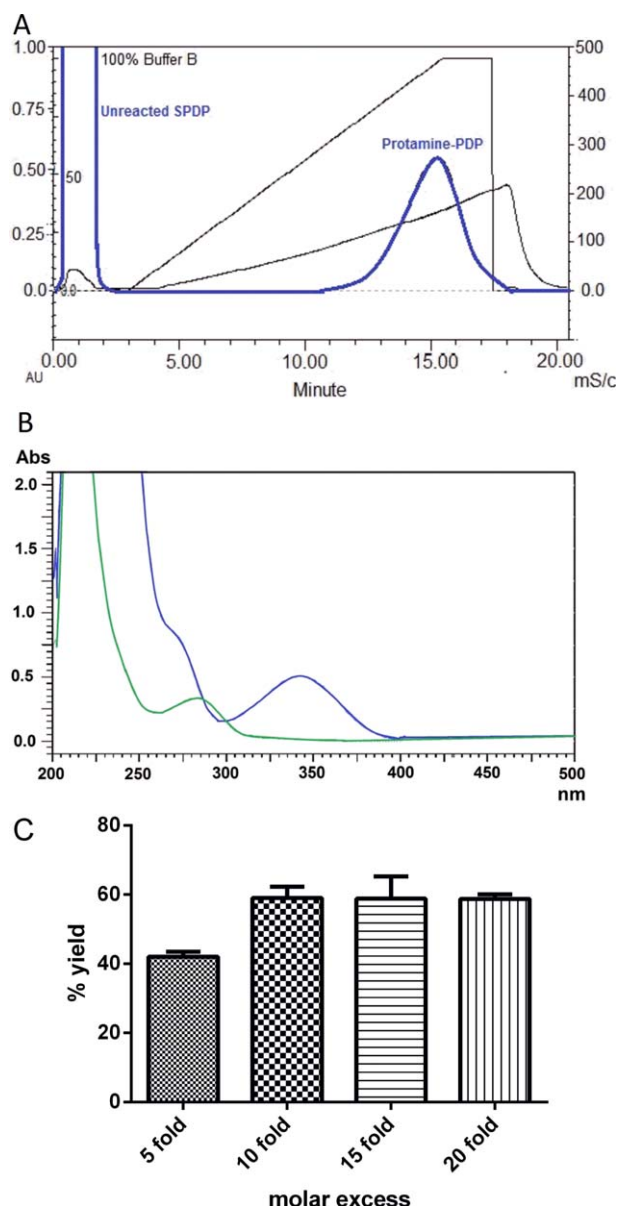
## **RESULTS**

### **N-terminal thiolation of protamine**

As protamine does not contain any lysyl residues, the only site to introduce the SPDP linker at neutral to slightly alkaline condition is the N-terminal proline, a secondary amine.<sup>19</sup> However, addition of SPDP in either ethanol or DMF caused precipitation of protamine when the reaction media was phosphate buffered saline, which was later solved performing the reaction in 2M NaCl stabilized with PEG-600. FPLC chromatographic data demonstrate that the purification method effectively removed unreacted SPDP [Fig. 2(A)]. Protamine activation was qualitatively confirmed by the release of pyridine-2-thione at 343 nm upon treatment with DTT [Fig. 2(B)]. Quantification indicates that as much as 41% of protamine was activated by SPDP when 5-fold excess of SPDP was used. The extent of activation increased to 63% when amount of SPDP was increased by 2-fold. However, further increase in SPDP mole% did not produce any increase in protamine activation [Fig. 2(C)]. Hence, a 10-fold excess of SPDP over protamine was selected as the optimum feed ratio.

### **Preparation of HSA-protamine conjugate**

Addition of HSA solution into activated protamine spontaneously released pyridine-2-thione [Fig. 3(A)], indicating the reaction between the Cys-34 of HSA and activated protamine. When protamine solution, without SPDP modification, was reacted with HSA, no reaction was evident by spectroscopy. The kinetics of the reaction revealed that it reaches a plateau at around 10 min [Fig. 3(B)]. The one-step purification method effectively separated unreacted HSA, unreacted activated protamine, and the HSA-protamine conjugate. Because of no affinity towards CM-sepharose column, unreacted HSA elutes immediately after being injected, which was confirmed by separate injection (data not shown). The reason that HSA-protamine conjugate elutes separately from unreacted activated protamine may be attributed to the slight decrease in binding affinity of the bulky HSA-protamine to CM-Sepharose resin. With the increasing feed-ratio of HSA over protamine-PDP, an increase in the peak for HSA-protamine conjugate was observed with corresponding decrease in the protamine-PDP peak, suggesting the depletion of protamine with increasing production of the conjugate [Fig. 3(C)]. A 4:1 molar ratio of HSA:protamine resulted with almost complete depletion of protamine. Quantification of HAS-protamine



**FIGURE 2.** (A) Purification of PDP-activated protamine by ion-exchange chromatography using CM-sepharose column. The 1 mL reaction mixture was eluted using buffer A: 20 mM phosphate, pH 7.4; B: 20 mM phosphate pH 7.4 + 2M NaCl. (B) Release of pyridyl-2-thione measured at 343 nm upon treatment of PDP-activated protamine with DTT. The green and blue lines represent chromatographic traces of PDP-activated protamine before and after addition of DTT, respectively. (C) % Yield of PDP-activated protamine with increasing mol:mol feed ratio of SPDP to protamine. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

conjugate also demonstrated that the yield of the conjugate increased with increasing HSA molar ratio from 1:1 to 4:1 as that of protamine [Fig. 3(D)]. Thus, a 4-fold excess of HSA was selected for further preparation of the conjugate.

#### Characterization of HSA-protamine conjugate

The goal of this conjugation reaction is the formation of one-to-one conjugate between HSA and protamine to avoid

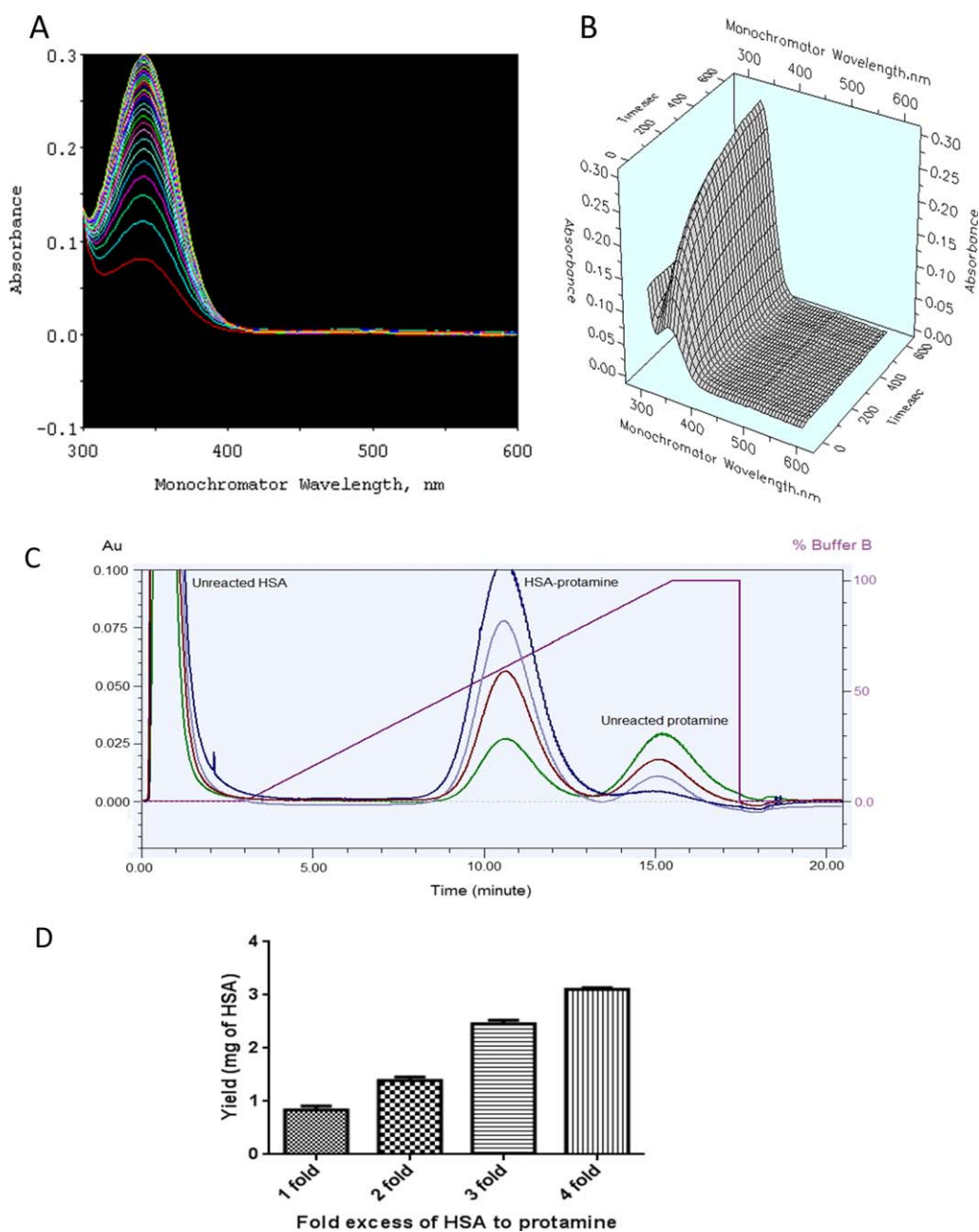
heterogeneity in drug delivery application. The electrospray mass spectroscopy data revealed that the molecular weight of the conjugate was around 70.8 kDa [Fig. 4(B)]. As the molecular weight of native HSA was found to be 66.6 kDa [Fig. 4(A)] and that of protamine was 4.2 kDa, the weight of the conjugate demonstrates a 1:1 conjugation. Furthermore, the intrinsic fluorescence spectrum of HSA-protamine was analyzed to determine if protamine conjugation had altered the conformation of HSA around the tryptophan residue (Trp 274). Upon excitation at 295 nm, both native HSA and the HSA-protamine conjugate demonstrated maximum emission intensity at 340 nm. Moreover, the emission profile was nearly identical and resulted in superimposed spectra between native HSA and HSA-protamine conjugate [Fig. 4(C)], indicating no quenching of the intrinsic fluorescence of Trp-214 residue of HSA. This indicates that conformation of the hydrophobic binding pocket in the second  $\alpha$ -helix domain of HSA remained unaffected after conjugation with protamine.<sup>20</sup>

#### Biocompatibility of HSA-protamine conjugate

The potential hemolysis, if occurs because of a high positive charge density of protamine, can limit the application of the proposed conjugate as a drug delivery tool. The hemolysis study demonstrated that protamine, in the form of HSA-protamine conjugate, had produced no lytic effect and was comparable to that of saline at equivalent protamine concentration of 10  $\mu\text{g}/\text{mL}$ , whereas native protamine, at the same concentration, caused considerable hemolysis [Fig. 5(A)]. As the maximum concentration of protamine in human blood with our proposed delivery system would be  $\leq 4 \mu\text{g}/\text{mL}$ , we claim the conjugate hemocompatible. Further, the compatibility study in the vasculature revealed that the cell viability was almost unaffected with HSA-protamine conjugate as demonstrated by  $>90\%$  survival at a concentration equivalent to 1 mg/mL of protamine. However, cell viability was significantly compromised (only 20% survival) at similar concentration of native protamine [Fig. 5(B)]. Thus both the hemocompatibility and cellular toxicity studies indicate that the proposed conjugate is unlikely to exert any deleterious effect at a concentration that will be applied for its use as a therapeutic cargo.

#### Proof-of-principle application of HSA-protamine conjugate as a carrier for therapeutic macromolecule

The sole purpose of this conjugate is to mask the enzymatic activity of a macromolecule, tPA in this case, by HSA, and to regenerate the activity using therapeutic steady-state concentration of heparin. In order to form the electrostatic complex between tPA and HSA, we have previously functionalized tPA with electronegative charge.<sup>10</sup> When the HSA-protamine conjugate was introduced to tPA with electronegative functionality,  $\sim 65\%$  of tPA's activity was suppressed (Fig. 6). However, when heparin (0.4  $\mu\text{g}/\text{mL}$ ) was added to the "camouflaged" tPA construct, the activity was fully regenerated, suggesting that while HSA masked tPA's activity by forming electrostatic complex with

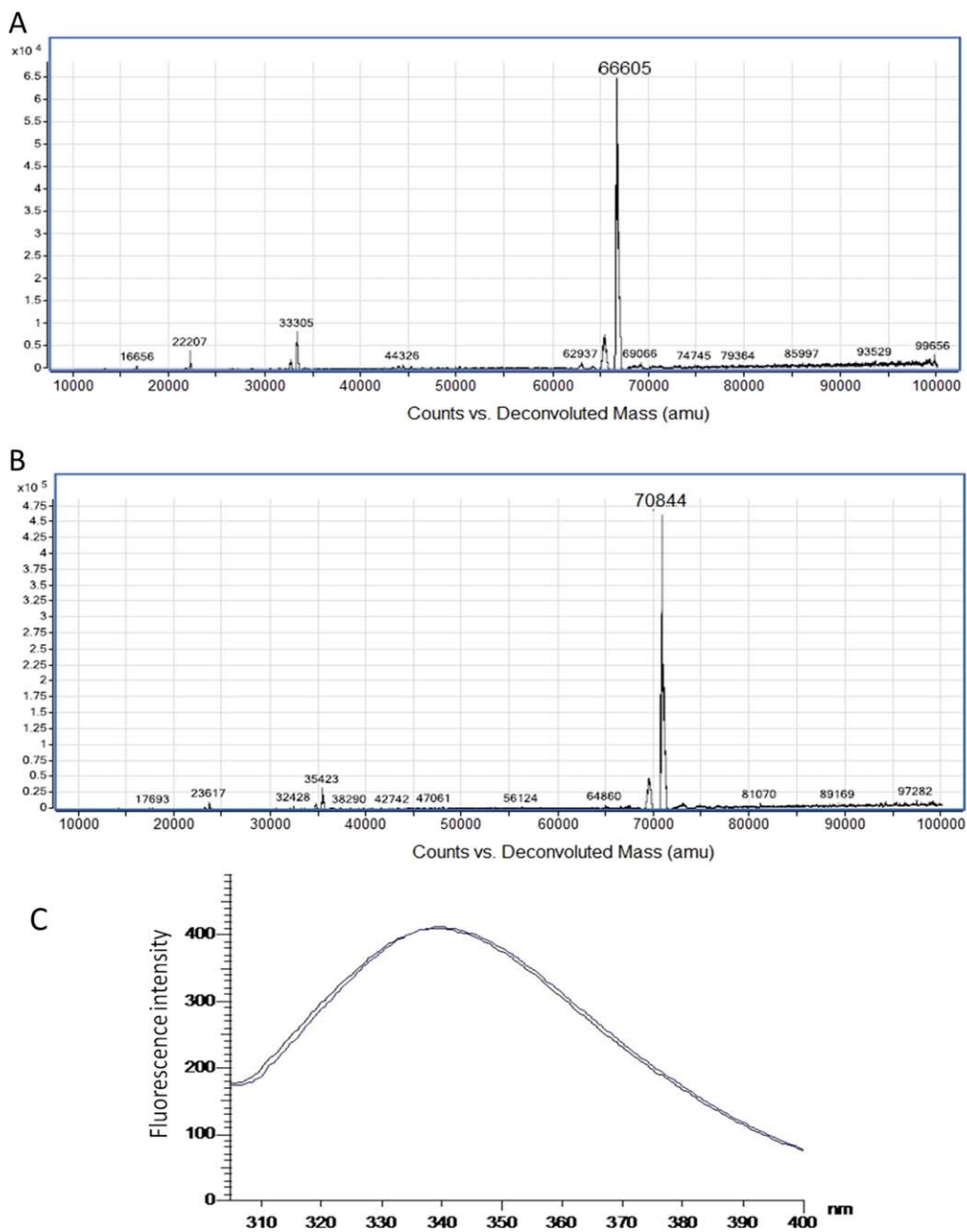


**FIGURE 3.** (A) Spontaneous reaction between HSA and PDP-activated protamine as monitored by the release of pyridyl-2-thione at 343 nm. The absorbance at 343 increased with increasing time. (B) Time course of HSA–protamine–PDP reaction: a 3D spectroscopic trace of the reaction from diode-array spectrophotometer demonstrating a saturation point at around 10 min. (C) One-step purification of HSA–protamine conjugate from unreacted HSA and protamine-PDP using ion-exchange chromatography. The 1-mL reaction mixture was eluted on a CM-sepharose column with elution buffer A: 20 mM phosphate, pH 7.4; B: 20 mM phosphate pH 7.4 + 2M NaCl. The green, red, violet, and blue lines represent 1, 2, 3, and 4-fold molar excess of HSA to protamine, respectively. (D) Yield of HSA–protamine conjugate with increasing molar excess of HSA to that of protamine. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

electronegative tPA surface, administration of heparin disrupted the construct and liberated free tPA because of its strong affinity towards protamine. This observation indicates that if the camouflaged structure can be directed towards thrombus using a homing mechanism, the systemic “lytic” effect of tPA can be attenuated and the thrombolytic action can potentially be regenerated by heparin, which is commonly administered during thrombosis management.

#### Binding of homing peptide-decorated HSA–protamine conjugate with activated platelets

The proposed targeted/triggered delivery system using HSA–protamine conjugate is contingent upon the accumulation of the macromolecule on its site of action, which in this case is thrombus. Hence, the surface of HSA–protamine conjugate was decorated with the homing peptide that binds with GPIIb/IIIa present on activated platelets during

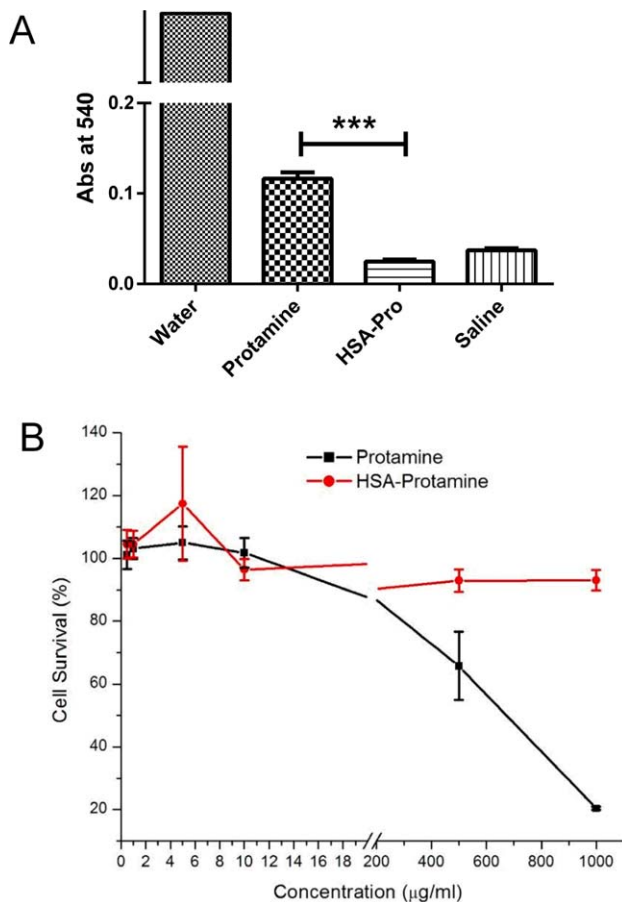


**FIGURE 4.** Electro spray mass spectrometry of (A) native HSA and (B) HSA–protamine conjugate, indicating molecular weight of each species. (C) Overlapping intrinsic fluorescence emission spectra of HSA and HSA–protamine conjugate. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

thrombotic event. Fluorescence microscopic image revealed that FITC-labeled peptide-grafted conjugate bound to the activated platelet surface as demonstrated by the staining of almost all the cells (Fig. 7). However, fluorescent conjugate without the homing peptide did not exhibit any binding with platelets. In the control experiment, activation of platelets was confirmed by the staining of the cells with FITC-labeled anti-GPIIb/IIIa antibody. Thus the binding study suggests that HSA–protamine conjugate can be decorated with a suitable homing peptide to direct the cargo at a desired site.

#### Stability of the HSA-mediated construct in human plasma

The electrostatic interaction between HSA–protamine conjugate and anion-functionalized tPA was stable enough to mask the enzymatic activity of tPA in human plasma (Fig. 8). Moreover, the characteristic regeneration of activity was retained upon the treatment with heparin. The activity of the camouflaged tPA after heparin addition was ~3-fold higher than that of plain tPA. As heparin stimulates tPA activity both *in vitro* and *in vivo*,<sup>21</sup> results were compared with the control group that was also treated with heparin.

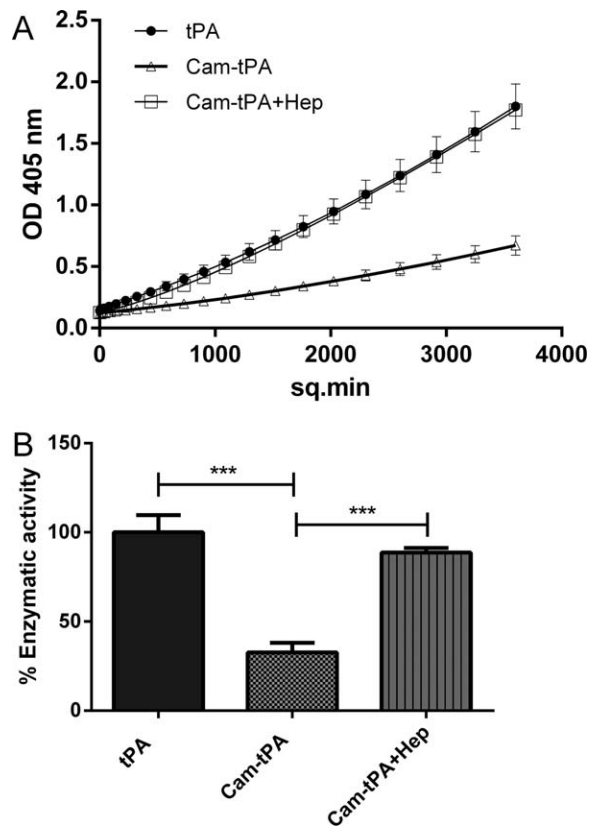


**FIGURE 5.** (A) Hemo-compatibility of HSA-protamine conjugate. The extent of hemolysis was determined by measuring the absorbance of released hemoglobin at 540 nm. An equivalent protamine concentration of 10 µg/mL was used for both protamine and HSA-protamine groups ( $n = 3$ ). (B) Cell viability of HSA-protamine conjugate as determined by MTT assay using arterial (pulmonary) smooth muscle cells ( $n = 8$ ). Data represent mean  $\pm$  SD. \*\*\* $p < 0.001$ . [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

Thus, it was confirmed that the higher enzymatic activity of tPA in the heparin-triggered group was not only because of heparin stimulation but also the removal of the albumin camouflage.

## DISCUSSION

Use of HSA as a delivery tool has made seminal contribution in the field of controlled drug delivery. Albumin microspheres, for instance, have shown potential to deliver both small and large therapeutic molecules.<sup>7</sup> Because of its natural abundance in blood, albumin-mediated delivery system can be ideal in designing a triggered release system for therapeutic macromolecules where the activity of the therapeutic cargo should be protected in blood. The key challenge in developing such delivery systems is masking the activity of the drug in the systemic circulation in a reversible manner so that pharmacologic action can be triggered at the disease site. As, unlike intracellular environment, blood environment lacks environmental triggers, such as pH or

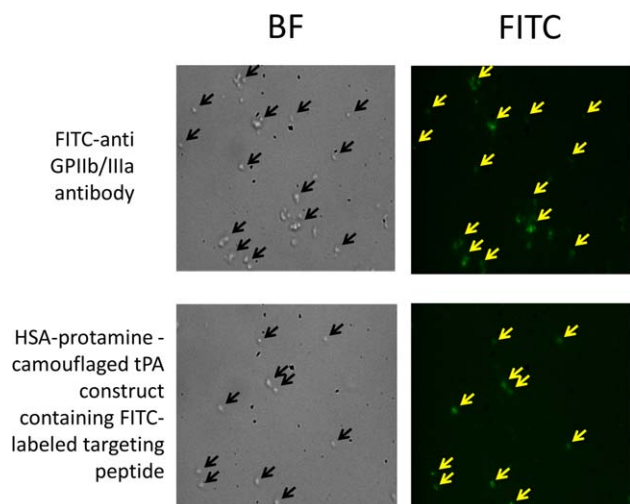


**FIGURE 6.** HSA-protamine conjugate in suppression and regeneration of activity of thrombolytic agent, tPA. (A) Chromogenic assay determining enzymatic action of tPA using plasmin-specific substrate S-2251. Experimental condition: HSA-protamine 0.2 µM, S-2251 1.2 mM, plasminogen 0.24 µM, tPA 0.5 µg/mL, and heparin 0.4 µg/mL. (B) Enzymatic activity of tPA upon complex formation with HSA-protamine and subsequent regeneration with heparin. The activity was calculated from the slope of the enzymatic assay. Data represent mean  $\pm$  SD ( $n = 3$ ). \*\*\* $p < 0.001$ .

reducing environment, regeneration of activity is often difficult. A classic example of protecting a protein in blood includes PEGylation of proteins to increase circulation half-life. However, covalent attachment of PEG on the protein surface, ironically, often results in diminished drug activity because of steric hindrance created by PEG molecules that prevent the target molecule to interact and produce pharmacological action, as was the case with tPA.<sup>22</sup> Our proposed HSA-protamine conjugate is designed to address the above mentioned limitations in which the bulky albumin moiety will serve as a "camouflage" for the drug in the systemic circulation, whereas the positively charged protamine will facilitate formation of an electrostatic complex with the drug. Depending on the therapeutic application, a triggering agent can be chosen to disrupt the construct and liberate free drug at the site of action.

Preparing a one-to-one conjugate between two different proteins is desirable to avoid potential heterogeneity that might result from numerous reactive functional groups present on their surface. Considering the presence of the single, relatively reactive secondary amine group on the N-terminus (proline) of protamine, and the free thiol group





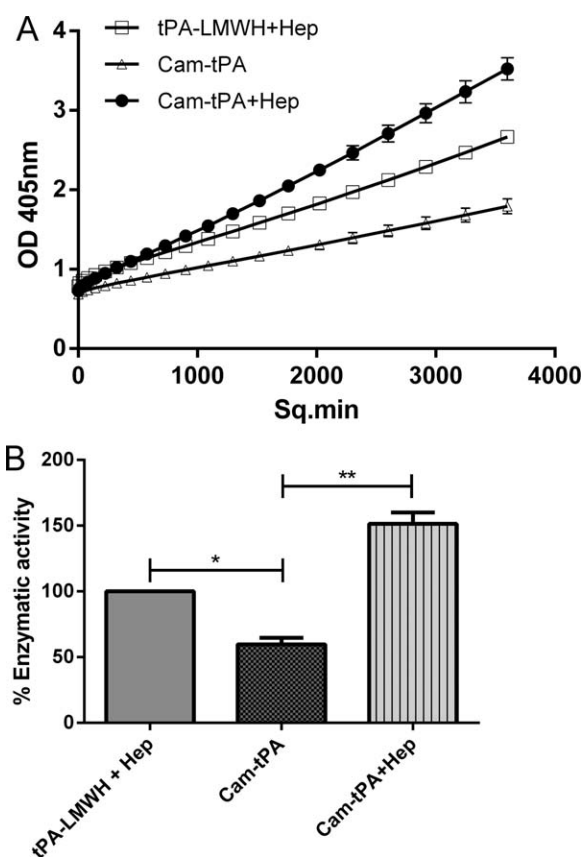
**FIGURE 7.** Binding of surface decorated HSA-protamine conjugate with activated platelets observed under fluorescence microscope (900 $\times$  magnification). The bright field images indicate the adsorbed platelets while the FITC images staining with peptide-linked HSA-protamine conjugate. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

on Cys-34 of HSA, reaction scheme was designed to link these two so that any heterogeneity can be avoided. However, a critical challenge was activation of the N-terminal proline of protamine to make it thiol-reactive. Upon stabilization of protamine solution with high concentration of NaCl and PEG-600, a significant amount (63%) of protamine was linked to SPDP that eventually demonstrated a spontaneous reaction with the Cys-34 of HSA. The chromatographic method employed to purify protamine from unreacted SPDP could not distinguish between unreacted and reacted protamine. However, it is important to note that, following conjugation with HSA, only the HSA-protamine conjugate was collected leaving the activated and non-activated protamine that was eluted later [third fraction in the chromatogram; Fig. 3(C)]. The second fraction was the desired HSA-protamine conjugate, as evident by the electro-spray mass spectrometry. The selection was initially based on partial charge screening because of the presence of albumin that would allow the conjugate to elute relatively earlier than native protamine, and also from the observation that upon increasing concentration of albumin, the second fraction reaches a higher peak with consequent reduction of the later fraction, which is assumed to be the unreacted protamine. Moreover, the collected fraction was finally confirmed as HSA-protamine conjugate by determining the molecular weight that was found to be consistent with the sum of the weights of HSA and protamine.

The hemolysis and cell viability study further suggest that the possible deleterious effect of cationic protamine is diminished by the presence of albumin. We argue that the immunogenicity associated with protamine use would also be attenuated upon conjugation with albumin. This argument is supported by earlier observation that conjugation of PEG molecules to protamine attenuates complement activation and subsequent immunogenic response<sup>19</sup> because of

the shielding effects created by PEG. As albumin, being several-folds larger than protamine, is conjugated to N-terminus of protamine, a similar nonimmunogenic profile is expected. It should also be noted that albumin is the most abundant protein in human plasma that, along with its size, makes it an excellent choice as a “camouflaging moiety”. Moreover, HSA offers a high degree of flexibility for surface modification that allows introducing homing moieties to direct the construct towards any desired site of action.

The proof-of-concept study using tPA showed convincing outcome in camouflaging tPA's enzymatic action upon complex formation with HSA-protamine conjugate, followed by regeneration of activity using therapeutic steady state heparin concentration. Therefore, the lytic action of tPA will be impeded in the systemic circulation but will be potentiated at the clot site, and hence will attenuate bleeding complication that can occur with conventional tPA therapy. The construct was designed such that tPA's action can be triggered using a triggering agent that is already included in the therapeutic modality of thrombosis management. Therefore, unlike other triggered-release systems, any external trigger, for example ultrasound, is not required. It should be noted that in the plasma condition, activity of the camouflaged tPA



**FIGURE 8.** Stability of HSA-protamine mediated camouflaged tPA in human plasma. (A) Amidolytic activity of tPA determined after incubation in human plasma. (B) Enzymatic activity of tPA upon camouflaging action of HSA-protamine and triggered release by heparin. Data represent mean  $\pm$  SD ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ .

after regeneration in human plasma was even higher than that of native tPA. Based on the concept of steric stabilization, the shielding effect created by the HSA moiety can potentially attenuate PAI-1 mediated inactivation of tPA in plasma, while no such protection is available for the native tPA. This observation is in accordance with the hypothesis that, using HSA-protamine, the macromolecules will be protected not only from producing systemic action, but also from circulating inactivating molecules which eventually could offer a larger amount of active drug in the site of action, thereby minimizing the dose.

Along with thrombolytic agents, a potential application for this construct could be the delivery of anticancer drug for which minimization of systemic exposure is warranted. Moreover, the utility of albumin carrier to solid tumor through endothelial binding with GP60 is well documented.<sup>3-5</sup> In this case, regeneration of activity can be achieved by reduction of disulfide bond between HSA and protamine at the intracellular compartment, offering active chemotherapeutic agent at the cancerous site. Similar approach can also be applied to intracellular delivery of genetic materials by protecting them in the systemic circulation, making this system a potentially widely applicable, biocompatible carrier of therapeutic macromolecules.

## CONCLUSION

In this study, we have proposed a novel albumin-based delivery system for therapeutic macromolecules. A one-to-one conjugate between HSA and protamine was synthesized and characterized for physical properties and biocompatibility. A proof-of-principle study using thrombolytic protein, tPA, as a model drug demonstrated that HSA-protamine conjugate can form a stable electrostatic complex with functionalized tPA and significantly mask its enzymatic activity. A suitable triggering agent, in this case heparin, disrupts the construct and regenerates tPA's activity. Hence, the proposed HSA-protamine conjugate holds the potential towards a newer triggered release approach for therapeutic proteins.

## ACKNOWLEDGMENT

The authors would like to thank Mr. George Littlejohn at the Coffee Memorial Blood Center (Amarillo, TX) for providing us with human plasma sample. The authors are also thankful to Mr. Jim Windak at University of Michigan for the electrospray mass spectrometry work. This work was supported in part by the internal fund of TTUHSC and in part by Health Professions Division Research Grant from Nova Southeastern University.

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