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RESEARCH PAPER

Thrombus-Targeted Nanocarrier Attenuates Bleeding Complications Associated with Conventional Thrombolytic Therapy

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ABSTRACT

Purpose To test the hypothesis that thrombus-specific tissue plasminogen activator (tPA)-loaded nanocarriers enhance thrombolytic efficacy and attenuate hemorrhagic complications.

Methods A series of pegylated and non-pegylated tPA-loaded liposomes were prepared and their surfaces were decorated with the peptide sequence (CQQHHLGGAKQAGDV) of fibrinogen gamma-chain that binds with GPIIb/IIIa expressed on activated platelets. All formulations were characterized for physical properties, stability and *in vitro* release profile. The thrombolytic activities of tPA-loaded liposomes were tested by visual end-point detection, fibrin agar-plate and human blood clot-lysis assays. The thrombus-specificity of the peptide-modified-liposomes was evaluated by studying the binding of fluorescent peptide-liposomes with activated platelets. The pharmacokinetic profile and thrombolytic efficacy were evaluated in healthy rats and an inferior vena-cava rat model of thrombosis, respectively.

Results Both pegylated and non-pegylated peptide-modified-liposomes showed favorable physical characteristics and colloidal stability. Formulations exhibited an initial burst release (40–50% in 30 min) followed by a continuous release of tPA (80–90% in 24 h) *in vitro*. Encapsulated tPA retained >90% fibrinolytic activity as compared to that of native tPA. Peptide-grafted-liposomes containing tPA demonstrated an affinity to bind with activated platelets. The half-life of tPA was extended from 7 to 103 and 141 min for non-pegylated and pegylated liposomes, respectively. Compared to native tPA, liposomal-tPA caused a 35% increase in clot-lysis, but produced a 4.3-fold less depletion of circulating fibrinogen.

Conclusions tPA-loaded homing-peptide-grafted-liposomes demonstrate enhanced thrombolytic activity with reduced hemorrhagic risk.

KEY WORDS localized fibrinolysis · peptide-modified-liposomes · protein delivery · targeted delivery · tissue plasminogen activator

ABBREVIATIONS

AMI	Acute myocardial infarction
ANOVA	Analysis of variance
AUC	Area under the curve
BSA	Bovine serum albumin
CHOL	Cholesterol
DOPE	1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE)
DSPE-PEG-Mal	1,2-distearoyl-*sn*-glycero-3-Phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]
DTT	Dithiothreitol
FITC	Fluorescein isothiocyanate
GPIIb/IIIa	Glycoprotein IIb/IIIa
IVC	Inferior vena cava
PA	Plasminogen activator
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCI	Percutaneous coronary intervention
PDI	Polydispersity index
PEG	Polyethylene glycol
PRP	Platelet rich plasma
RT	Room temperature
SD	Standard deviation

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SPDP	N-Succinimidyl1, 3-(2-pyridyldithio)propionate
TMB	3,3′,5,5′-tetramethybenzidine
tPA	Tissue plasminogen activator

INTRODUCTION

Vascular diseases, including acute myocardial infarction (AMI), stroke and peripheral arterial diseases, continue to be the leading causes of morbidity and mortality for Americans (1). The chief initiating events of AMI involves formation of an occlusive thrombus within the coronary artery that restricts the blood flow to the myocardium (2). The current treatment approaches are aimed towards rapid revascularization of the occluded artery by angioplastic and surgical interventions. Further, fibrinolytic agents such as plasminogen activators (PA), which facilitate rapid restoration of vessel patency, are routinely used along with invasive procedures. Currently used fibrinolytic agents work by converting plasminogen to plasmin and thus trigger body's internal mechanisms to dissolve clot and restore coronary arterial blood flow. However, a major limitation of such agents is their indiscriminate effects on circulating plasminogen, and subsequent generation of off-target plasmin that leads to systemic hemorrhage (3). Indeed, hemorrhagic complications can occur in as many as 20% patients receiving these drugs (4).

The advent of tissue plasminogen activator (tPA) was a major accomplishment in thrombolytic therapy. As the enzymatic activity of tPA increases by 500–1,000 fold in the presence of fibrin (5), it was hypothesized that tPA produces its activity predominantly at the thrombus. However, further investigation revealed that such stimulatory effect was not as significant in humans as that observed in preclinical animals (6). Additionally, the soluble fibrin monomer level is elevated during thrombotic events (7) which could stimulate the lytic activity of tPA, resulting in depletion of critical circulating clotting factors such as fibrinogen, plasminogen and factor V, following intravenous administration of tPA (8). Moreover, a very short half-life (~5 min) and rapid inactivation by circulating inhibitors such as plasminogen activator inhibitor-1 (PAI-1) requires a large dose of tPA that further aggravates the hemorrhagic complications (5,9). Although new generation fibrinolytic agents such as reteplase and tenecteplase, which were developed to overcome these shortcomings, have longer half-lives and enhanced fibrin-specificity, the hemorrhagic incidence continue to exist even with the newer agents (10,11). Thus, there is a need to develop a delivery system that can deliver the drugs and produce thrombolytic action locally at the thrombus site and subsequently attenuate life-threatening bleeding complications associated with current 'clot-busting' agents.

A number of drug delivery approaches have previously been proposed for thrombus specific delivery of PAs including conjugation of fibrin-specific antibody (12), pegylation (13) and use of particulate careers (14). However, these strategies suffered from a number of limitations. First, covalent conjugation of antibodies resulted in reduction in the biological activity of the drug (15). Further, lack of homogeneity in drug-antibody conjugates, instability upon long-term storage and immunogenicity were major concerns for chemically modified PAs (16). While pegylation was successfully used to extend the half-life of PAs, this approach also significantly reduced the enzymatic activity of the thrombolytic agents (13). Use of particulate carrier systems, such as nanoparticles and hydrogels, are reported to improve the delivery of PAs (17,18). But concerns have been raised regarding their mechanisms to modulate drug release at the thrombus site, which restricted their use to treat acute events like AMI when immediate drug action is required. Recently, we and others have proposed chemical and mechanical stimuli based triggered-release delivery systems for tPA (19–21). Although these approaches are considered to be significant advancements towards localized fibrinolytic action, accumulation of therapeutically effective concentration of the drug at the thrombus site prior to the application of triggering agent is one of the major barriers to the success of this delivery strategy. Thus, thrombus targetable delivery system that can achieve a therapeutic concentration of the drug at the clot-site is likely to address some of the limitations of previously used approaches.

In this study, we propose to develop a targetable liposomal delivery system for tissue plasminogen activator (tPA), the most widely used thrombolytic agent. We intend to decorate the surface of the liposomes containing tPA with C-terminal gamma-chain peptide sequence of fibrinogen (CQQHHLGGAKQAGDV) that selectively binds to activated platelets during a thrombotic event (22). We hypothesize that, upon intravenous administration, liposomes containing the drug accumulate over thrombus because of the anchoring moiety and release the drug specifically at the clot-site (Fig. 1). The goal is to achieve a continuous release of tPA thereafter to offer a protection against re-thrombosis, a common phenomenon following reperfusion of occluded arteries. Toward this end, liposomal construct of tPA was optimized to release a substantial amount of drug within 30 min of administration followed by a continuous release over 24 h. The feasibility of this construct was tested by determining the fibrinolytic property of tPA-loaded liposome, stability and *in vitro* drug release profile, binding with activated platelets, pharmacokinetic parameters, clot-lytic efficacy and hemorrhagic safety in a rat thrombosis model.

MATERIALS AND METHODS

Materials

tPA was purchased from Genentech (Genentech Inc, South San Francisco, CA). Cholesterol, phosphatidylcholine (PC),

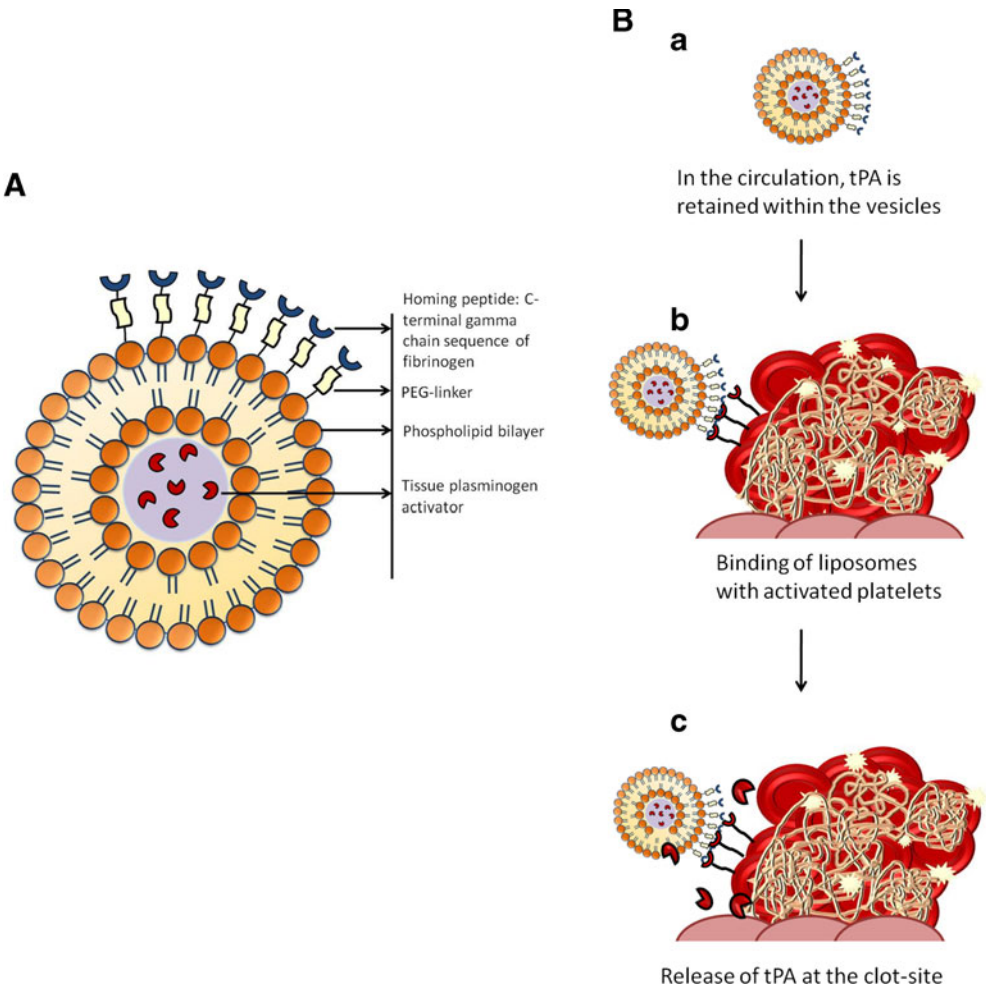

Fig. 1 (**A**) Targetable liposomal construct of tPA. (**B**) The schematic representation of thrombolytic action of liposomal tPA: (**a**) The drug would be within the vesicles while in the circulation, (**b**) accumulate on the thrombus upon interaction with activated platelets, (**c**) active tPA released at the thrombus site.

1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG-Mal) were acquired from Avanti polar lipids (Alabama, USA) and N-succinimidyl1, 3-(2-pyridyldithio)propionate (SPDP) from Molecular Biosciences (Boulder, CO). Plasminogen specific chromogenic substrate S-2251 was obtained from Chromogenix (Lexington, MA) and fluorescein isothiocyanate was from Fisher Scientific (Pittsburgh, PA). The targeting peptide sequence (14-mers of the C-terminal peptide [CQQHHLGGAKQAGDV] from the fibrinogen gamma chain) was custom synthesized by EZBiolab (Carmel, IN). Fibrinogen, plasminogen, thrombin, aldrithiol (2,2′-Dithiopyridine 2,2′-Dipyridyl disulfide), dithiothreitol (DTT), calcium chloride ($CaCl_2$) were procured from Sigma-Aldrich Inc (St. Louis, MO).

Preparation of Peptide-Linked tPA-Loaded Liposomes

tPA-loaded liposomes were prepared in the presence or absence of PEG moiety with varying lipid compositions at different lipid-to-drug ratio. Lipid (μmol) to drug (IU) ratio were 22.5:100K, 45:100K, 90:100K, 135:100K and 180:100K for non-PEGylated liposome (L-1 through L-5) and lipids used were Soy PC, cholesterol and DOPE (Table I). Pegylated liposomes were prepared with the same lipid-to-drug ratio except that DOPE was replaced with DSPE-PEG-Mal (Table I). All liposomal formulations were prepared by solvent evaporation/sonication method and drug was loaded by the freeze-thaw method. Briefly, a stock solution of lipid containing PC, cholesterol and DOPE/DSPE-PEG-Mal, at a ratio of 70:30:5, was prepared in a mixture of chloroform and methanol at 4 to 1 molar ratio (Table I). A lipid film was prepared by placing the lipid mixture in a round bottom flask and subsequent evaporation of the solvent using a Buchi R-114 Rotavapor (Buchi Laboratories AG, Postfach, Switzerland). Residual solvent was completely removed upon application of vacuum and the resulting dry film was hydrated with 2 ml of phosphate buffered saline (PBS). The dispersion thus obtained was vortexed and sonicated for 30 min. An aliquot (200 μl) of tPA solution (100,000 IU) in PBS was added to the freshly prepared liposome and vortexed for 30 s. The liposomal samples were flash frozen under liquid nitrogen and thawed at room temperature. The freeze-thaw cycle was repeated 5 times

Table I Composition of Liposomes (mg/2 ml of PBS)

Formulation code	PC (mg)	CHOL (mg)	DOPE (mg)	DSPE-PEG-MAL (mg)	tPA (IU)
L-1	11.63	2.48	0.796	–	100,000
L-2	23.45	4.97	1.6	–	100,000
L-3	46.5	9.94	3.19	–	100,000
L-4	69.75	14.91	4.8	–	100,000
L-5	93	19.88	6.4	–	100,000
PL-1	11.63	2.48	–	3.15	100,000
PL-2	23.45	4.97	–	6.3	100,000
PL-3	46.5	9.94	–	12.6	100,000
PL-4	69.75	14.91	–	18.9	100,000
PL-5	93	19.88	–	25.2	100,000

PC Phosphatidylcholine; *CHOL* Cholesterol; *DOPE* 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine; *DSPE-PEG-MAL* 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]

for maximum encapsulation of tPA. Large multilameller tPA-loaded vesicles thus produced were extruded through a commercial extruder (Liposofast®, Avestin Inc, Ottawa, Canada) at room temperature and small unilameller vesicles were collected after 11 cycles. Un-entrapped tPA was removed by ultracentrifugation (355,000 × *g*, 1 h, 4°C) in a TL-100 ultracentrifuge (Beckman, USA) using a TLA 120.2 rotor. For grafting of homing peptide (CQQHHLGGAKQAGDV), the amine groups of liposomal (L-1 to L-5) surface were activated by incubation with SPDP (25 μmol) for 30 min. Excess SPDP was removed from the liposome by size-exclusion chromatography and purified liposomes were incubated with the peptide containing a terminal free thiol group. For pegylated liposomes (PL-1 to PL-5), the homing peptide was incubated for 2 h at a peptide to maleimide molar ratio of 1 to 3 and then purified by size-exclusion chromatography. Both peptide-grafted liposomes were stored at 4°C for further experiments.

Physical Characteristics of Liposomal Formulations

The formulations were characterized for particle size, polydispersity index (PDI), zeta potential, stability and entrapment efficiency. To determine particle size and zeta potential, 10 μl of liposomal formulations were dispersed in 1 ml deionized water and analyzed in a Nano ZS90 Zetasizer (Malvern® Instruments Ltd., Worcestershire, UK). The stability of the liposomes was assessed by determining the changes in particle size and drug entrapment efficiency upon storage at 4°C. The particle size and entrapment efficiency was measured every 7 days for 28 days. To determine the entrapment efficiency, an aliquot of liposomes were first diluted twice with 50 mM Tris buffered saline and separated from the dispersion medium by ultracentrifugation (355,000 × *g*, 1 h, 4°C). The pellets were re-suspended, diluted 10 times with the buffer and solubilized by incubating with 1% Triton X-100 at a liposome to detergent ratio of 1:1 for 30 min at 37°C. The amount of tPA was determined using a chromogenic assay (20). Briefly, 1.3 μM of substrate S-2251, 0.24 μM plasminogen in Tris buffered saline (50 mM Tris HCl in 50 mM NaCl with 0.01% Tween 80, pH 7.2) were placed in a 300 μL well of a 96-well plate. An aliquot of lyzed liposomes (34.8 μl) was added to the well. The initial rate of hydrolysis of S-2251 by plasmin, which is produced from plasminogen due to enzymatic activity of tPA, was determined by measuring the absorbance at 405 nm at different time intervals using a micro-plate reader (SynergyMx, Biotek). The slope of the absorbance *versus* time square ($\Delta A/min^2$) curve was expressed as the initial rate of hydrolysis of the chromogenic substrate (23). Finally, the entrapment efficiency was calculated using the following equation: $L/T \times 100$ (*L*: amount of tPA incorporated into liposome, *T*: total amount of tPA).

In Vitro Release of tPA From Liposomal Formulations

In vitro release profiles of representative formulations of plain (L-3) and pegylated (PL-3) liposomes was determined in PBS with continuous stirring (24). Briefly, 1 ml formulations was suspended in 4 ml of PBS and placed in a 37°C temperature-controlled chamber. At certain time intervals, an aliquot of the medium were withdrawn and centrifuged at 355,000 × *g* to collect the supernatant containing tPA. To maintain the sink condition, the amount of medium withdrawn during each sampling was replaced with an equal volume of fresh medium. The amount of released tPA was determined by measuring its concentration in the supernatant using the above described chromogenic assay.

Determination of Fibrinolytic Activity of Entrapped tPA

The fibrinolytic activity of liposomal tPA was determined by two complementary methods: visual endpoint method (21) and agar plate assay (25). For visual end point method, fibrin clot was prepared in glass tubes by mixing 200 μl of 9% fibrinogen, 125 μl of 0.05 units/ml of plasminogen and 50 μl of 250 units/ml of thrombin. An increasing concentration of native tPA (175, 350 and 700 IU/ml), equivalent to the amount in selected lysed liposomal formulations (L-3 and PL-3), were added into three test tubes. Additional formulations without homing peptide were also used as controls. The volume was adjusted to 500 μl by using 0.1 M phosphate buffer at pH 7.3. A glass bead was placed on the top of the clot and the time required for the glass bead to reach the bottom of tube was considered as the time to dissolve the clot by tPAs. This was then converted to the relative rate of fibrinolysis. To maintain the temperature at 37°C, the experiment was carried out in a circulating water bath (Isotemp 2013S, Pittsburg, PA). Since time is a critical parameter in determining the fibrinolytic activity by this method, the liposomes were lysed before adding

to the fibrin clot to eliminate the influence of time that might otherwise be taken by tPA to come out of the lipidic vesicles.

For agar plate assay, 50 mg fibrinogen was dissolved in 5 ml of Tris buffer at pH7.2 and the solution was kept at 37°C. In a separate beaker, 300 mg of low-melting agar was dissolved in 10 ml Tris buffer containing 20 µl of 250 unit/ml of thrombin solution. The melted agar solution was then mixed with the fibrinogen solution and stirred for 1 min. The mixed solution was poured onto a rectangular transparent plastic plate and spread carefully to obtain a homogeneous gel which was then incubated for 2 h at 37°C to form a solidified fibrin gel. On the solidified agar plate, five wells (I.D. 3 mm) were created as sample reservoir and 5 µl of plasminogen (1 mg/ml) was added to each well. Native tPA and the formulations (L-3, PL-3, L-3 without peptide and PL-3 without peptide) containing 100 ng equivalent of tPA were added into respective wells, and incubated overnight at 37°C for complete fibrinolysis. Finally, the fibrinolytic activity of each sample was evaluated by comparing the area of the lysed zone around the wells. To assess the dose-sensitivity of the test, a separate agar plate with varying concentrations of tPA was run following the above mentioned procedure.

Human Blood Clot Lysis Assay

To determine human blood clot dissolving activity of liposomal tPA, a clot lysis assay was performed according to a previously published method (20,21). Briefly, human blood was collected from Coffee Memorial Blood Center (Amarillo, TX) and a blood clot was prepared by incubating 30 µl blood with 13 U/ml of thrombin and 7 mM of $CaCl_2$ at 37°C for 30 min. Following addition of 0.24 µM plasminogen, clots were incubated with 0.05, 0.15 and 0.45 µM tPA or equivalent amount tPA in selected formulations (L-3 and PL-3 with and without peptide) for 2 h at 37°C. The supernatant containing erythrocytes was collected after 2 h to assess the extent of clot lysis. The amount of erythrocytes released from the clot due to the enzymatic activity of tPA was determined indirectly by taking absorbance for hemoglobin at 576 nm (20). The weight of the wet clot was also monitored before and after the treatment with tPA and/or formulations.

Binding of Peptide-Linked Liposomes with Activated Platelets

Binding of liposomes with activated platelets was studied by fluorescence microscopic imaging (26). Platelet rich human plasma (PRP) containing 1.68×10^9 platelets per ml of plasma was collected from Coffee Memorial Blood Center (Amarillo, TX) and platelets were isolated by centrifuging 1 ml of PRP at $400 \times g$ for 15 min at 25°C. The supernatant was removed and the 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 µl, which was then adsorbed onto collagen coated glass coverslips at room temperature and washed thrice with PBS. The adhesion of platelet monolayer on coverslips was confirmed by staining them with FITC-tagged anti-GPIIb/IIIa monoclonal antibody (eBioscience Inc, CA, USA), and subsequently observing under fluorescence microscopy. FITC-labeled peptide-linked formulations (L-3 and PL-3) were prepared for the binding study and the extent of labeling was evaluated by a spectroscopic assay. Fluorescence labeled liposomes were incubated with the adsorbed platelets for an hour in dark at room temperature. Fluorescent liposomes without the homing peptide were also incubated as a control. All incubations were carried out in the presence of 5 mM $CaCl_2$ solution under mild shaking to activate human PRP and facilitate activation of platelet (27). After washing thrice with PBS, the coverslips were fixed with 1% freshly prepared paraformaldehyde for 30 min in the dark at room temperature. The coverslips were then mounted onto glass slides with fluorogel (Electron Microscopy Science, PA, USA) and images were taken with a fluorescence microscope (IX-81, Olympus, Center Valley, PA).

Pharmacokinetic Study

Male Sprague–Dawley rats (Charles River Laboratories, Charlotte, NC), weighing 250–300 g, were used for pharmacokinetic studies. Rats were divided into five groups to receive the following treatments: (i) native tPA, (ii) L-3, (iii) L-3 without homing peptide, (iv) PL-3 and (v) Pl-3 without homing peptide. Briefly, rats were anesthetized by intramuscular injections of a cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg). The formulations were diluted with PBS (pH7.4), and 7,500 IU/kg plain tPA or formulations containing equivalent amount of tPA were slowly administered over 30 s *via* the penile vein. After dosing, 0.3 ml of blood was drawn from tail vein at 1, 5, 10, 15, 30 and 60 min for native tPA and at 1, 2, 4, 6, 9 h for liposomal formulations. Plasma was separated by centrifuging the blood samples at 3,500 × *g* for 10 min. To lyse the liposomes that may possibly be present in the plasma, 150 µl of plasma was placed in a centrifuge tube and treated with an equal volume of 1% Triton-X. For quantitation of tPA, 93.5 µl plasma sample was placed into a 300-µl well containing 200 µl of substrate S-2251 (2 mM) and 6.5 µl of plasminogen (1 mg/ml). Concentration of tPA was determined by the chromogenic assay.

Pharmacological Efficacy in Deep Vein Thrombosis Model

In Vivo Clot Lysis Assay

To study the thrombolytic efficacy, a rat inferior vena cava (IVC) model of deep vein thrombosis was developed

according to a published method (28). Briefly, Sprague Dawley rats (250–300 g) were divided into four groups to receive saline and 1 mg/kg tPA in the form of (i) native tPA, (ii) PL-3 without homing moiety and (iii) PL-3. To induce thrombosis, rats were first anesthetized by ketamine/xylazine cocktail as mentioned above. A midline laparotomy was then performed and the small bowel was pushed slightly to the left of the animal body and the IVC was surgically exposed. A filter paper (1.5 cm) soaked with 35% $FeCl_3$ solution was applied just below the renal veins for 45 min to induce blood stasis and to form consistent platelet rich thrombus (29). After removing the filter paper, the IVC was rinsed with saline and the incision was closed using 3–0 polyglactin 910 sutures (Surgical Specialities Corp, Reading, PA). The formulations were administered *via* the penile vein and 8 h after the treatment, blood samples were collected by cardiac puncture. The IVC was harvested and the thrombus was meticulously collected and the clot was weighed.

Estimation of Fibrinogen

Fibrinogen concentration in rat plasma was determined by an ELISA sandwich assay (23). Briefly, 100 µl of 10,000× diluted plasma sample was incubated with anti-fibrinogen antibody that was pre-coated onto a 96-well plate (Immunology Consultant Lab Inc, OR, USA). After removing any unbound fibrinogen by washing each well thrice, the wells were incubated with 100 µl of anti-rat fibrinogen antibody conjugated with horseradish peroxidase for 30 min at room temperature. After washing three times, the wells were then treated with 100 µl of substrate solution, 3,3′,5,5′-tetramethybenzidine (TMB), for 10 min. All incubations were performed in the dark. After termination of the reaction with 100 µl of 0.3 M sulfuric acid, used as a stop solution, absorbance was measured at 405 nm in a microplate reader (SynergyMx, Biotek, Winooski, VT).

Data Analysis

All data are presented as mean±SD. One way ANOVA was used to determine the statistical significance followed by a *Post hoc* analysis with Turkey's comparison (GraphPad Prism, version 5.0, GraphPad Software, San Diego, CA). Standard non-compartmental analysis (WinNonlin®) was performed to calculate the pharmacokinetic parameters. A p-value less than 0.05 was considered statistically significant.

RESULTS

Physical Properties of tPA-loaded Liposomes

Liposomal formulations were characterized for particle size, polydispersity index (PDI), zeta potential and entrapment efficiency, and the data are presented in Table II. The mean particle size of non-pegylated liposomes was between 151 and 183 nm, whereas that of pegylated liposomes was 170 and 187 nm. No statistically significant differences in the particle sizes of two types of liposomes were observed because both formulations were subjected to extrusion through similar (200 nm) polycarbonate membrane filters. Freeze-thaw cycle, used to encapsulate the drug, has good annealing property, as reflected by reduced particle size and heterogeneity. Conjugation of homing peptides on liposomal surface did not affect the size of the vesicles. The PDI of the formulation was 0.077–0.104, suggesting that the liposomes were monodispersed irrespective of lipid composition and surface modification (24). Zeta potential is a critical parameter for the stability of colloidal preparations such as liposomes. A higher value of zeta potential indicates a higher surface charge which creates repulsive forces for the particles to remain in dispersion. The zeta potential values of the liposomes were between −10.6 and −37.6 mv, pegylated liposomes with more negative values. Negative zeta potential values perhaps stem from the lipid composition used to prepare the liposomes. In fact, pure zwitterionic phosphatidylcholine used to prepare liposomes carries a net negative charge that may contribute to the negative zeta potential of liposomal formulations (30). Pegylated liposomes showed higher negative values perhaps because of the carbamate linkage between PEG and DSPE that is reported to induce a net negative charge on the phosphate moiety at physiological pH (31). Encapsulation of tPA or surface modification with the homing peptide had no significant effect on the surface charge of the liposomes (Table II). Removal of un-entrapped tPA by ultracentrifugation did not cause aggregation of particles, which is evident from the unaltered size distribution of particle following centrifugation. The entrapment efficiency of the non-pegylated liposomes was 12–26%, whereas that for pegylated liposomes was 36–52%. Based on the published report, we hypothesize that enhanced entrapment by pegylated liposomes is related to the increased hydrophiliciy of pegylated lipids compared to that of plain lipids (32). It is possible that a fraction of hydrophilic tPA got entangled within the pegylated arms of liposomes. The grafting efficiency of the peptide was determined spectrophotometrically and was found to be 10.3 and 9.6 µg of peptide per µmol of lipid for non-pegylated and pegylated liposomes, respectively. Based on the physical parameters and entrapment efficiency, L-3 and PL-3 were chosen for further investigation. Soy PC was used to prepare these formulations because of its low phase transition temperature (T_m) (33) that allows efficient drug release at physiological temperature. A 70:30 lipid-to-cholesterol ratio was selected based on an earlier report (24) and our observation that a higher cholesterol level affects the stability of the liposomes (data not shown).

Table II Pysico-chemical Characteristics of Liposomes

		Formulation code								
		L-1	L-2	L-3	L-4	L-5	PL-1	PL-2	PL-3	PL-4	PL-5
Particle size (nm)	−tPA	534 ± 172	1261 ± 141	1508 ± 108	1707 ± 78	3060 ± 1009	503 ± 109	563 ± 84	612 ± 72	699 ± 90	736 ± 78
	+tPA	274 ± 4	388 ± 12	306 ± 46	281 ± 4	561 ± 35	302 ± 36	293 ± 21	287 ± 17	312 ± 35	277 ± 29
	+Extr	167 ± 3	178 ± 2	151 ± 1	173 ± 2	183 ± 2	171 ± 4	168 ± 6	172 ± 2	177 ± 4	186 ± 4
	+pep	168 ± 4	177 ± 5	151 ± 3	174 ± 3	183 ± 3	171 ± 3	170 ± 4	171 ± 2	179 ± 5	187 ± 4
Poly-dispersity index	−tPA	0.86 ± 0.09	0.83 ± 0.15	0.81 ± 0.10	0.98 ± 0.02	0.46 ± 0.08	0.41 ± 0.08	0.40 ± 0.13	0.44 ± 0.14	0.43 ± 0.02	0.50 ± 0.14
	+tPA	0.42 ± 0.02	0.48 ± 0.03	0.51 ± 0.04	0.71 ± 0.06	0.67 ± 0.16	0.24 ± 0.06	0.22 ± 0.09	0.28 ± 0.06	0.27 ± 0.05	0.24 ± 0.04
	+Extr	0.08 ± 0.02	0.07 ± 0.01	0.09 ± 0.01	0.11 ± 0.02	0.08 ± 0.01	0.09 ± 0.03	0.09 ± 0.01	0.10 ± 0.01	0.08 ± 0.03	0.07 ± 0.01
	+pep	0.08 ± 0.03	0.09 ± 0.02	0.10 ± 0.01	0.10 ± 0.03	0.09 ± 0.02	0.07 ± 0.01	0.08 ± 0.03	0.1 ± 0.02	0.07 ± 0.02	0.08 ± 0.02
Zeta potential	−tPA	−28.4 ± 10.2	−8.5 ± 2.9	−14.9 ± 1.8	−12.5 ± 1.1	−14.6 ± 3.6	−32.9 ± 2.4	−42.3 ± 6.7	−33.4 ± 5.2	−46.8 ± 1.6	−26.3 ± 7.3
	+tPA	−15.7 ± 1.1	−29.1 ± 5.1	−15.4 ± 2.2	−13.1 ± 4.9	−8.9 ± 0.1	−28.4 ± 5.6	−45.0 ± 3.9	−36.9 ± 4.1	−33.2 ± 2.9	−34.8 ± 3.5
	+Extr	−16.2 ± 10.2	−10.1 ± 0.6	−16.1 ± 1.4	−13.5 ± 1.4	−17.6 ± 4.8	−33.3 ± 4.3	−38.3 ± 2.2	−28.7 ± 3.9	−38.2 ± 4.9	−32.7 ± 2.6
	+pep	−16.9 ± 1.7	−10.4 ± 0.8	−15.7 ± 3.6	−14.1 ± 6.7	−19.1 ± 11.6	−32.8 ± 2.7	−37.6 ± 3.4	−30.6 ± 2.8	−34.1 ± 3.2	−32.4 ± 3.8
Entrapment efficiency [%]		12.91 ± 2.91	15.23 ± 1.98	20.82 ± 2.45	23.51 ± 3.05	25.63 ± 2.72	36.8 ± 2.87	39.46 ± 3.22	45.08 + 2.08	48.25 + 3.19	51.83 ± 3.63

−tPA Crude liposomes after evaporation & sonication; *+tPA* tPA-loaded liposomes after freeze-thaw cycle; *+Extr* tPA-loaded liposomes following extrusion; *+pep* Peptide-grafted tPa-loaded purified liposomes

Stability of tPA-liposomes

The stability of the formulations was evaluated by monitoring the particle size and tPA-content upon storage at 4°C for 28 days. This storage condition was selected based on a previous report, suggesting that the fluidity and permeability of phospholipid increases at higher temperature (34). In the event of instability, particles undergo agglomeration which is reflected by an increase in particle size. However, there was no significant change in the particle size for any of the formulations (Fig. 2a). A slight sedimentation was observed with L-3 formulations which was readily re-dispersible upon mild shaking. PL-3, however, did not exhibit any signs of sedimentation, which might be attributed to the higher zeta potential value compared to that of L-3. In addition to the particle size, we studied the leakiness of the formulations by assaying the entrapment efficiency under above mentioned storage condition and observed no significant alterations in tPA content (Fig. 2b). This property is important because drug entrapped in the liposomal formulation tends to leak from the bilayer structure upon storage (32).

In Vitro Release Profile

The release of tPA from the liposomes was studied at 37°C for 48 h. Both L-3 and PL-3 showed a faster release of the drug during first 30 min followed by a continuous release for 24 h. In fact, ~50% drug was released from L-3 within the first 30 min with a cumulative release of 92% over a period of 24 h. On the other hand, the initial tPA release from PL-3 was slightly slower (~40%) than that of L-3, and continued to release the drug (up to 80%) for 24 h (Fig. 3). This is consistent with the previous observation that the drug release from pegylated liposomes was slower than conventional liposomes which is linked to the structural integrity of PEG molecule (35). Although no statistically significant differences were observed between two formulations in terms of the amount of drug released in first 1 h, the amount of tPA released from the two formulations became statistically significant starting the 2nd to the 24th hour ($p<0.05$). To test whether Triton X treatment influences the intrinsic activity of tPA, additional control experiments were performed that ruled out the effect of the detergent on the enzymatic activity of tPA (data not shown). The initial burst release from the liposomes can be attributed to the low transition temperature of Soy PC (<37°C) (33) that is likely to disrupt the vesicular structure and release the drug at physiological temperature. It is also possible that a fraction of the drug got adsorbed on liposome surface during the freeze-thaw cycle (36) which might have additionally contributed to the initial rapid release of tPA.

Fibrin Clot-lytic Assay

Protein drugs are likely to undergo denaturation upon exposure to harsh conditions during processing of formulations.

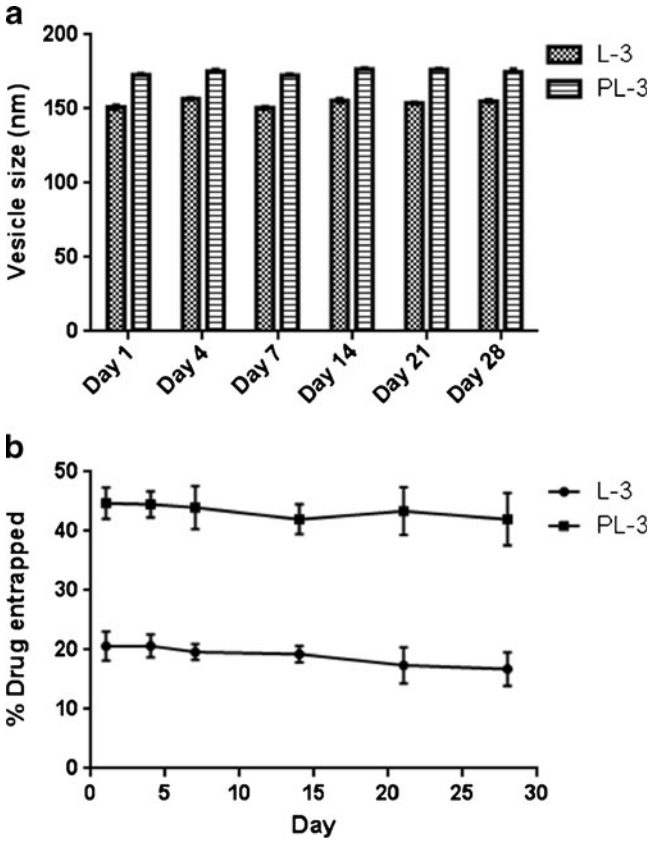

Fig. 2 Stability of the vesicles upon storage at 4°C. No significant changes in (**a**) particle size and (**b**) entrapment efficiency were observed during the storage period. Data represent mean±SD; $n=3$.

Thus, upon development of any formulations containing therapeutic protein, it is important to test their biological activity. Visual end point assay was performed to evaluate the fibrinolytic activity of tPA. The data presented in Fig. 4 suggest that liposomal formulations retained 95–97% of the activity of native tPA. Three different concentrations were used to assess the sensitivity of the assay. No statistically significant differences were observed among various sets of liposomal formulations containing tPA ($p>0.05$), suggesting that tPA entrapped in the liposomes was as active as native tPA.

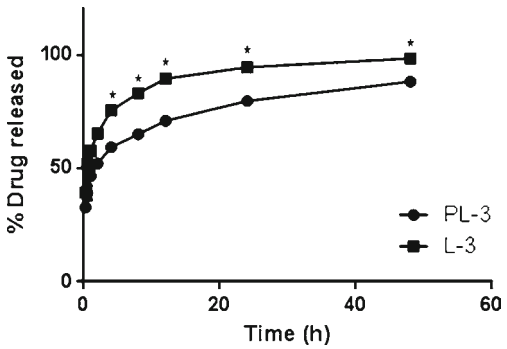

Fig. 3 *In vitro* release of liposomes performed at 37°C in PBS, pH7.4. An initial rapid release is observed within first 30 min which was followed by a continuous release over the period of 24 h. Data represent mean±SD; $n=3$.

A complementary method to study the fibrinolytic property of thrombolytic agents is agar plate assay (37). In this method, the disruption of a fibrin plate is monitored visually against a clear zone created by the enzymatic action of the drug. When L-3 and PL-3 formulations were placed in the sample-well on the agar plate and incubated overnight at 37°C, the size of the clear zone created by the formulations was equal to that created by native tPA (Fig. 5), indicating that the lytic action of liposome encapsulated tPA was unaltered. Liposomes without the homing peptide exhibited a clot-lytic profile similar to that of L-3 and PL-3, suggesting that surface modification with targeting peptide did not adversely affect the enzymatic activity of tPA (well 8–11). Absence of any clear zone around the buffer-containing well confirms that the lysis of agar plate is solely due to the enzymatic activity of the drug.

Human Blood Clot Lysis Assay

Upon establishing the fibrin-clot lysis activity *in vitro*, the activity of liposomal tPA in dissolving human clot was tested. Data suggest that the enzymatic activity of tPA encapsulated in L-3 and PL-3 was about 90% of native tPA (Fig. 6). When the post-treatment clot was weighed to calculate the extent of clot lysis, a similar result was obtained (data now shown). The clot lytic activity of liposomal tPA with peptide was similar to that of without peptide, suggesting that the homing peptide has no influence on the enzymatic activity of tPA (Fig. 6). The incubation time was chosen to be 2 h based on our previous observation that clot-lytic action of tPA levels off within 2 h (20). Compared to native tPA, slightly less activity of the liposomal tPA was observed perhaps because of the incomplete release of tPA from the liposomes. However, fibrinolytic data obtained with lysed liposome suggest that the liposomal tPA retained its enzymatic action in dissolving human blood clot.

Binding Study with Activated Platelets

A critical component of the proposed construct is the homing moiety that is grafted on liposome surface to facilitate accumulation of the vesicles on the thrombus. The C-terminal gamma chain peptide sequence of fibrinogen, which specifically binds with GPIIb/IIIa expressed on the activated platelets of thrombus, should allow the tPA-loaded liposomes to bind with thrombus so that the lytic action of the drug is produced locally at the site of action. In this study, the activation of platelets and the expression of GPIIb/IIIa on their surfaces were first confirmed by staining the platelets with FITC-labeled anti-GPIIb/IIIa antibody. The fluorescence microscopic images revealed that the peptide-linked liposomes, both L-3 and PL-3, showed significantly enhanced staining of the activated platelets compared to the control formulation without homing moiety (Fig. 7). Thus microscopic images exhibit that fluorescent liposomes have efficiently

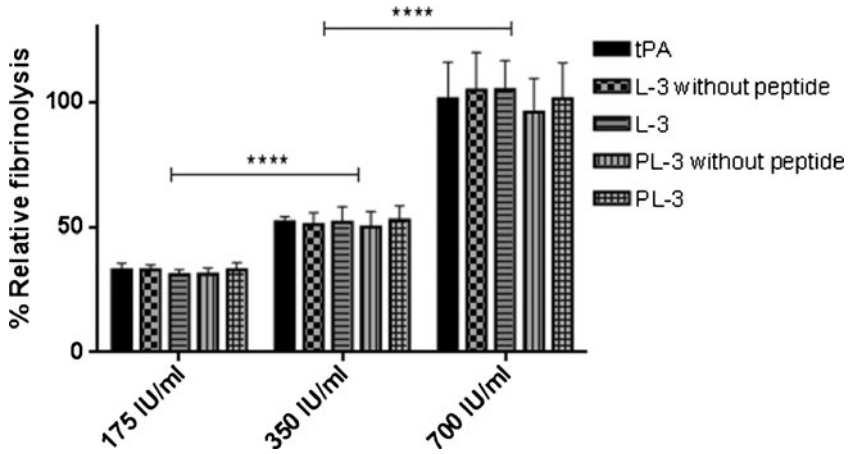

Fig. 4 Fibrinolytic activity of encapsulated tPA as determined by visual endpoint method. The fibrinolytic activity was expressed as% of fully-active unmodified tPA. Data represent mean+SD ($n=3$–6).

interacted with GPIIb/IIIA expressed on the activated platelet surfaces, suggesting that the homing peptide has an affinity to bind with the GPIIb/IIIa.

Pharmacokinetic Studies

The pharmacokinetic profiles of two formulations, L-3 and PL-3, showed a marked improvement in the parameters such as increased half-life and area under the curve (AUC) and reduced clearance (Fig. 8) of tPA. The circulation half-life of tPA encapsulated in L-3 and PL-3 formulations were 103 and 141 min, respectively, which was significantly longer than 7 min half-life of native tPA. Compared to plain tPA, L-3 and PL-3 formulations showed a 5 and 10 fold increase in AUC, respectively. However, the clearance of tPA from L-3

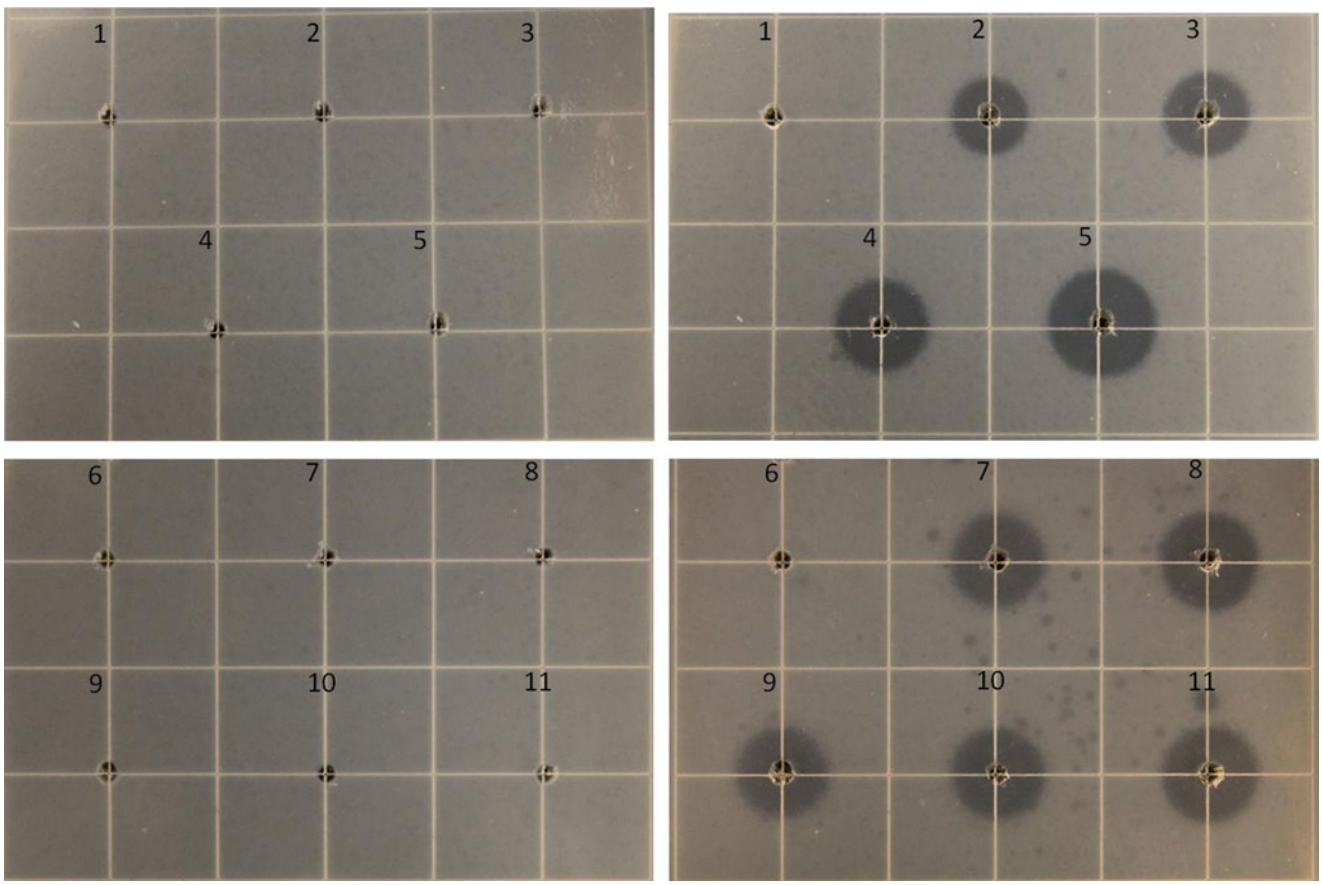

Fig. 5 *In vitro* clot-lysis assay by agar plate method. *Left panel*: Initial state after application of sample; *Right panel*: Fibrinolysis observed after overnight incubation at 37°C; 1. TBS, 2. tPA (25 ng), 3. tPA (50 ng), 4. tPA (100 ng), 5. tPA (200 ng), 6. TBS, 7. tPA 100 (ng), 8. L-3 w/o peptide (100 ng), 9. L-3 (100 ng), 10. PL-3 w/o peptide (100 ng), 11. PL-3 (100 ng) ($n=3$).

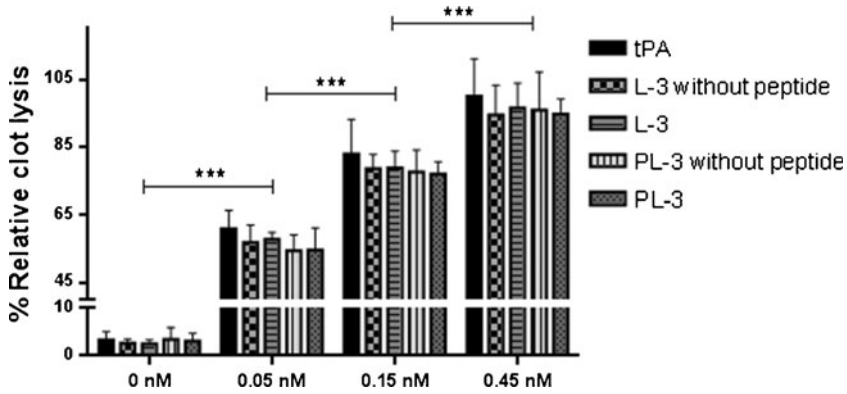

Fig. 6 Human blood clot-lytic activity of the liposomal tPA: experimental condition: human blood sample, 13 U/ml thrombin, 7 mM $CaCl_2$ and 0.24 μM plasminogen. Data represent mean ± SD, $n = 3$.

and PL-3 was several fold less than native tPA (Table III). The improved pharmacokinetic parameter observed with PL-3 may be attributed to the presence of PEG molecules on the liposome that creates a hydration layer on the surface and thus allows the vesicles to avoid uptake by macrophages and reticular endothelial systems (38). This data is consistent with previous studies that reported increase in the circulation life of tPA upon encapsulation in liposomes and polymeric microspheres (4). The presence of homing peptide on the liposome surface had no remarkable effect on the pharmacokinetic profiles of the formulations which might be attributed to the fact that the study was performed in healthy animals without thrombus, which are not expected to alter the distribution profile of the formulations. Based on the favorable pharmacokinetic profiles, we choose PL-3 formulation to evaluate its pharmacological efficacy in a rodent model of deep vein thrombosis.

Thrombolytic Study in a Rat Thrombosis Model

The thrombolytic efficacy of the liposomes was tested in a rat IVC thrombosis model. In this IVC thrombosis model, we have used 35% ferric chloride to induce platelet rich thrombus. A 45-min blotting of the IVC with $FeCl_3$-soaked filter paper produced a large clot with an average weight of 39.4 mg. Upon treatment with native tPA, the clot weight was reduced to 18.9 mg, which was a 50% reduction compared to clot collected from saline treated animals (Fig. 9a). A similar reduction in clot weight was observed when PL-3 without the targeting moiety was administered. However, compared to native tPA treated animals, the peptide-linked pegylated liposome (PL-3) caused 35% enhanced clot-lytic activity. This dramatic increase in clot-lysis suggests that an enhanced amount of therapeutically active tPA interacted with the clot and this was possible because of the presence of the homing moiety on liposome surface. Further, the fibrinogen level observed in native tPA

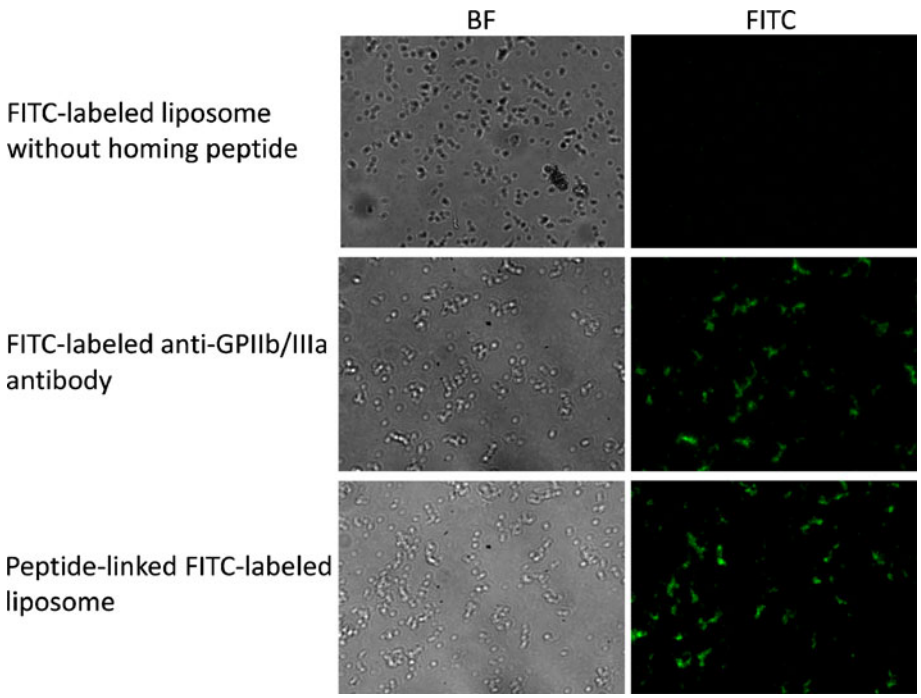

Fig. 7 Binding of peptide-linked liposomes on the surface of activated platelets observed under a fluorescence microscope. The bright field (*BF*) images indicate adsorbed platelets while the FITC images (*green*) demonstrate peptide grafted liposomes. When plain fluorescent liposomes were incubated with activated platelets, no binding was observed (*top panel*). Incubation with fluorescent labeled anti-GPIIb/IIIa antibody (*middle panel*) and peptide grafted liposomes (*lower two panels*) show binding with activated platelets ($n = 3$).

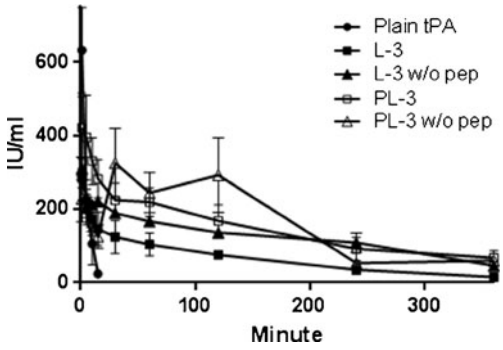

Fig. 8 Pharmacokinetic profile of liposomal tPA after intravenous administration. Results are expressed as mean ± SD ($n = 5–6$).

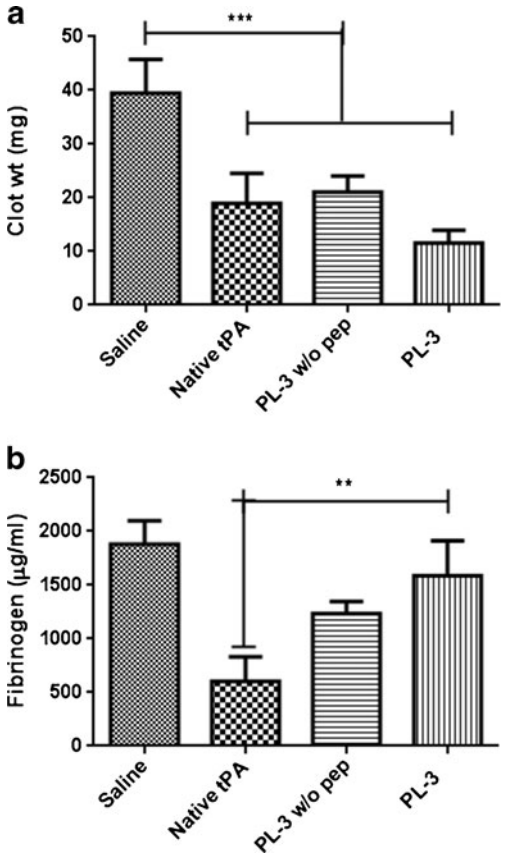

Fig. 9 *In vivo* efficacy and hemorrhagic safety of the liposomal tPA. (**a**) Thrombolyitc action of tPA in dissolving clot, (**b**) effect of liposomal tPA on plasma fibrinogen level. Data represent mean ± SD ($n = 5–6$).

treated animals were remarkably lower than that of the saline treated animals, suggesting that native tPA causes depletion of plasma fibrinogen level, which is in agreement with the published report (23). Importantly, the effect of peptide-linked liposomal tPA on fibrinogen level was negligible, which was about 4.3 fold less than that observed in native tPA treated animals (Fig. 9b). Depletion of fibrinogen by tPA is one of the critical events that increase the risk of systemic bleeding. Pharmacological efficacy data presented in Fig. 9 suggest that targeted liposomal tPA minimally affect the circulating clotting factor and thus has the potential to significantly attenuate hemorrhagic complications.

DISCUSSION

The mainstay of treatment for AMI is rapid recanalization of the coronary artery that could be achieved by percutaneous coronary intervention (PCI). However, such interventions are time sensitive and resource intensive which could potentially be detrimental to patients' health. Treatment with thrombolytic agents plays a critical role for the initial management of the disease because this therapeutic approach is less expensive and simpler than PCI. Therefore, a safer and more efficacious thrombolytic therapy is likely to improve patient outcome. Since a major drawback of the current tPA based thrombolytic agents is undesirable bleeding, we aim to attenuate the bleeding problem by encapsulating tPA into liposomes decorated with a homing peptide that can guide the carrier to accumulate on the thrombus and produce localized action. Liposomes are well established carrier system that can encapsulate both low and high molecular weight therapeutic agents. Because of their ability to encapsulate water soluble drugs, liposomes confer a level of protection to PAs by blocking premature release and/or inactivation by circulating inhibitors, and consequently increase the half-life of the agents (3). Multiple studies demonstrated that liposome-encapsulated PAs are more potent than native PAs as evident by enhanced *in vivo* thrombolysis by liposomal streptokinase, urokinase, and tPA (39). Compared to their native counterparts, liposome-encapsulated PAs have

Table III Pharmacokinetic Parameters of Liposomal tPA

	Control	Formulation			
	tPA	L-3	L-3 w/o pep[a]	PL-3	PL-3 w/o pep[b]
$t_{1/2}$(min)	7.75 ± 0.18	103 ± 34	104 ± 24	141 ± 16	154 ± 31
AUC (IU.hr/ml)	109 ± 28	541 ± 181	879 ± 191	1189 ± 499	1301 ± 172
CL (ml/min/Kg)	1.18 ± 0.31	0.25 ± 0.09	0.14 ± 0.03	0.116 ± 0.04	0.097 ± 0.01

[a] Liposomes with same composition as that of L-3 without having the homing peptide

[b] Liposomes with same composition as that of PL-3 without having the homing peptide

shown significant reduction in reperfusion time along with an increased digestion of thrombus (40,41). We believe that a strategy that can direct the liposomal fibrinolytic agents towards thrombi and facilitate drug release at the target site would maximize drug accumulation on clot site and minimize off-target effects.

The proposed liposomal preparation of tPA showed favorable physical characteristics as a stable vehicle for delivery of tPA. The vesicles were colloidally stable with optimal drug payload. The mechanism of tPA encapsulation by freeze-thaw cycle, still largely unknown, might be attributed to proteins' ability to form amphipathic α-helices and subsequent penetration into phospholipid bilayers (42). tPA, devoid of α-helices at pH 7.4, might take a specific α-helix conformation upon partitioning into lipid bilayers (36). To increase the entrapment efficiency, the number of freeze-thaw cycle was not increased because increasing the cycle over five times does not appear to enhance tPA encapsulation into the lipid bilayers (36). In agreement with previous observation that tPA has a strong propensity to associate with negatively charged liposomes at pH 7.4, it is reasonable to assume that a fraction of entrapped tPA was adsorbed on the surface of the lipid bilayer (36). However, it is also important that the possible surface associated tPA is stable enough so that the drug does not get released instantaneously upon intravenous administration. Since the entrapment was calculated following extensive centrifugation, it can be argued that tPA was stably adsorbed on liposome surface.

The liposomes were optimized to achieve a faster drug release within the first 30 min followed by a continuous release for next 24 h. This feature would allow a single administration of the formulation for the treatment of an acute thrombotic event and prevention of re-thrombosis. However, such release profile might raise concerns regarding the targeting efficiency soon after drug administration. Since it takes about a minute for the blood to circulate in humans, tPA-loaded liposomes would start to accumulate on clot surface soon after administration of the formulations, and will keep accumulating during each circulation. Thus within few minutes, a significant amount of liposomes are expected to accumulate on thrombus surface that can release tPA locally and lyse the clot. We assume that 40–50% of tPA released during the burst phase would dissolve the existing clot, whereas continuous release thereafter would help prevent re-thrombosis. Treatment with fibrinolytic agents is expected to restore blood flow within 90 min in 50% of AMI patients (4). An ideal liposomal delivery can significantly improve this index if tPA is retained within the vesicles and released locally at the thrombus (37). Our proposed system is likely to achieve such goal by means of a clot-specific homing moiety and favorable release profile.

Another unique feature of this construct is the homing peptide, the responsible fraction of fibrinogen gamma-chain, which binds with activated platelets during thrombotic events. Other peptides such as RGD peptides have been used as targeting moiety to bind with platelets (26). However, it is difficult to control non-specific interactions of RGD peptides since they tend to bind with numerous other integrins present throughout the body. In contrast, the peptide used in this experiment utilizes the specificity of circulating fibrinogen towards activated platelets in the body (22). Therefore, we assume that use of this anchoring moiety would reduce non-specific interactions. It is encouraging to observe that liposome encapsulated tPA showed better clot lysis than native tPA. Although the mechanism by which the liposomal formulation is exhibiting such beneficial outcome is not clear, we assume that encapsulation of tPA into liposomes minimizes its inactivation by the circulating inhibitors (PAI-1), prevents from systemic lytic action and hence attenuates depletion of key components of the fibrinolytic system. Moreover, liposome encapsulated tPA are reported to produce enhanced fibrinolysis because of the rupture of the vesicles caused by shear stress that occurs when liposomes come in contact with the clot (39). Such phenomenon, along with anchoring homing moiety and favorable release profile, might facilitate release of drug at the clot site.

However, it is important to discuss that the pharmacological efficacy of the formulations was evaluated in an acute model of thrombosis. Although this is a widely used method, there are concerns regarding the use of this model for clots that develop in the artery during an AMI. One would wonder whether the pharmacological efficacy observed in venous clot would accurately reflect the efficacy in arterial clot. Recognizing the differences between these two types, we used $FeCl_3$ solution to create a platelet rich clot (29), which mimics arterial thrombus. Concerns could also be raised regarding the efficacy of the formulation in an acute clot *versus* the chronic one developed in coronary artery. But we believe that in terms of surface topography, acute clot developed in this model is similar to that observed in coronary artery during AMI. In fact, an acute clot in AMI is formed by tearing of an existing clot that stimulates platelet activation and subsequent thrombus formation, which may lead to an acute ST-elevation myocardial infarction (STEMI) (43). Thus we believe that this model is valid for evaluation of clot lytic activity in clinical situations. All in all, it is reasonable to assume that, following intravenous administration, the targeted liposomes accumulate on the thrombus *via* an interaction between the integrin GPIIb/IIIa on the activated platelets and the homing peptide, and then a rapid release of the drug would facilitate the initial clot-lysis, whereas the continuous release would prevent reformation of thrombus. Since tPA is retained within the vesicles when in the circulation, the critical clotting-factors would be minimally affected. Overall, both reperfusion and prevention of re-occlusion could be achieved by a single bolus dose with minimal systemic hemorrhagic incidence.

CONCLUSION

Data presented in this manuscript suggest that a targetable carrier system for tPA has the potential to minimize the systemic effect of the drug on circulating clotting factors by retaining tPA within the vesicle. The targeting moiety would allow accumulation of the proposed drug containing carrier on the thrombus and thereby facilitate delivery of a larger amount of tPA to dissolve the clot and reduce bleeding risk of plain tPA based therapy. Future studies will be directed to evaluate the distribution of peptide-grafted liposomes in the thrombus microenvironment and optimization of the carrier system for maximal efficacy with minimal off-target effects.

ACKNOWLEDGMENTS AND DISCLOSURES

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REFERENCES

1. Lovlien M, Johansson I, Hole T, Schei B. Early warning signs of an acute myocardial infarction and their influence on symptoms during the acute phase, with comparisons by gender. Gend Med. 2009;6:444–53.
2. DeWood MA, Spores J, Notske R, Mouser LT, Burroughs R, Golden MS, *et al.* Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. N Engl J Med. 1980;303:897–902.
3. Leach JK, O'Rear EA, Patterson E, Miao Y, Johnson AE. Accelerated thrombolysis in a rabbit model of carotid artery thrombosis with liposome-encapsulated and microencapsulated streptokinase. Thromb Haemost. 2003;90:64–70.
4. Chung TW, Wang SS, Tsai WJ. Accelerating thrombolysis with chitosan-coated plasminogen activators encapsulated in poly-(lactide-co-glycolide) (PLGA) nanoparticles. Biomaterials. 2008;29:228–37.
5. Bode C, Runge MS, Haber E. Future directions in plasminogen activator therapy. Clin Cardiol. 1990;13:375–81.
6. Collen D, Stump DC, Gold HK. Thrombolytic therapy. Annu Rev Med. 1988;39:405–23.
7. Wada H, Wakita Y, Nakase T, Shimura M, Hiyoyama K, Nagaya S, *et al.* Increased plasma-soluble fibrin monomer levels in patients with disseminated intravascular coagulation. Am J Hematol. 1996;51:255–60.
8. Stricker RB, Wong D, Shiu DT, Reyes PT, Shuman MA. Activation of plasminogen by tissue plasminogen activator on normal and thrombasthenic platelets: effects on surface proteins and platelet aggregation. Blood. 1986;68:275–80.
9. Chandler WL, Alessi MC, Aillaud MF, Henderson P, Vague P, Juhan-Vague I. Clearance of tissue plasminogen activator (TPA) and TPA/plasminogen activator inhibitor type 1 (PAI-1) complex: relationship to elevated TPA antigen in patients with high PAI-1 activity levels. Circulation. 1997;96:761–8.
10. A comparison of reteplase with alteplase for acute myocardial infarction. The Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO III) Investigators. N Engl J Med. 1997;337:1118–23.
11. Chapman DF, Lyden P, Lapchak PA, Nunez S, Thibodeaux H, Zivin J. Comparison of TNK with wild-type tissue plasminogen activator in a rabbit embolic stroke model. Stroke. 2001;32:748–52.
12. Runge MS, Bode C, Matsueda GR, Haber E. Antibody-enhanced thrombolysis: targeting of tissue plasminogen activator *in vivo*. Proc Natl Acad Sci U S A. 1987;84:7659–62.
13. Berger Jr H, Pizzo SV. Preparation of polyethylene glycol-tissue plasminogen activator adducts that retain functional activity: characteristics and behavior in three animal species. Blood. 1988;71:1641–7.
14. Lippi G, Mattiuzzi C, Favaloro EJ. Novel and emerging therapies: thrombus-targeted fibrinolysis. Semin Thromb Hemost. 2012. doi:10.1055/s-0032-1328935
15. Bos R, Nieuwenhuizen W. The potential improvement of thrombolytic therapy by targeting with bispecific monoclonal antibodies: why they are used and how they are made. Biotherapy. 1992;5:187–99.
16. Harding FA, Stickler MM, Razo J, DuBridge RB. The immunogenicity of humanized and fully human antibodies: residual immunogenicity resides in the CDR regions. MAbs. 2010;2:256–65.
17. Piras AM, Chiellini F, Fiumi C, Bartoli C, Chiellini E, Fiorentino B, *et al.* A new biocompatible nanoparticle delivery system for the release of fibrinolytic drugs. Int J Pharm. 2008;357:260–71.
18. Park Y, Liang J, Yang Z, Yang VC. Controlled release of clot-dissolving tissue-type plasminogen activator from a poly(L-glutamic acid) semi-interpenetrating polymer network hydrogel. J Control Release. 2001;75:37–44.
19. Liang JF, Li YT, Song H, Park YJ, Naik SS, Yang VC. ATTEMPTS: a heparin/protamine-based delivery system for enzyme drugs. J Control Release. 2002;78:67–79.
20. Absar S, Choi S, Yang VC, Kwon YM. Heparin-triggered release of camouflaged tissue plasminogen activator for targeted thrombolysis. J Control Release. 2012;157:46–54.
21. Absar S, Choi S, Ahsan F, Cobos E, Yang VC, Kwon YM. Preparation and characterization of anionic oligopeptide-modified tissue plasminogen activator for triggered delivery: an approach for localized thrombolysis. Thromb Res. 2012. doi:10.1016/j.thromres.2012.11.030.
22. Ware S, Donahue JP, Hawiger J, Anderson WF. Structure of the fibrinogen gamma-chain integrin binding and factor XIIIa cross-linking sites obtained through carrier protein driven crystallization. Protein Sci. 1999;8:2663–71.
23. Naik SS, Liang JF, Park YJ, Lee WK, Yang VC. Application of "ATTEMPTS" for drug delivery. J Control Release. 2005;101:35–45.
24. Kim JY, Kim JK, Park JS, Byun Y, Kim CK. The use of PEGylated liposomes to prolong circulation lifetimes of tissue plasminogen activator. Biomaterials. 2009;30:5751–6.
25. Liang JF, Song H, Li YT, Yang VC. A novel heparin/protamine-based pro-drug type delivery system for protease drugs. J Pharm Sci. 2000;89:664–73.
26. Gupta AS, Huang G, Lestini BJ, Sagnella S, Kottke-Marchant K, Marchant RE. RGD-modified liposomes targeted to activated platelets as a potential vascular drug delivery system. Thromb Haemost. 2005;93:106–14.
27. Eppley BL, Pietrzak WS, Blanton M. Platelet-rich plasma: a review of biology and applications in plastic surgery. Plast Reconstr Surg. 2006;118:147e–59.
28. Downing LJ, Strieter RM, Kadell AM, Wilke CA, Greenfield LJ, Wakefield TW. Low-dose low-molecular-weight heparin is anti-inflammatory during venous thrombosis. J Vasc Surg. 1998;28:848–54.

29. Kurz KD, Main BW, Sandusky GE. Rat model of arterial thrombosis induced by ferric chloride. Thromb Res. 1990;60: 269–80.
30. Natarajan JV, Chattopadhyay S, Ang M, Darwitan A, Foo S, Zhen M, *et al.* Sustained release of an anti-glaucoma drug: demonstration of efficacy of a liposomal formulation in the rabbit eye. PLoS One. 2011;6:e24513.
31. Atyabi F, Farkhondehfai A, Esmaeili F, Dinarvand R. Preparation of pegylated nano-liposomal formulation containing SN-38: *in vitro* characterization and *in vivo* biodistribution in mice. Acta Pharm. 2009;59:133–44.
32. Bai S, Ahsan F. Inhalable liposomes of low molecular weight heparin for the treatment of venous thromboembolism. J Pharm Sci. 2010;99:4554–64.
33. Le UM, Cui Z. Long-circulating gadolinium-encapsulated liposomes for potential application in tumor neutron capture therapy. Int J Pharm. 2006;312:105–12.
34. Toongsuwan S, Li LC, Erickson BK, Chang HC. Formulation and characterization of bupivacaine lipospheres. Int J Pharm. 2004;280:57–65.
35. Gupta Y, Jain A, Jain P, Jain SK. Design and development of folate appended liposomes for enhanced delivery of 5-FU to tumor cells. J Drug Target. 2007;15:231–40.
36. Heeremans JL, Gerritsen HR, Meusen SP, Mijnheer FW, Gangaram Panday RS, Prevost R, *et al.* The preparation of tissue-type Plasminogen Activator (t-PA) containing liposomes: entrapment efficiency and ultracentrifugation damage. J Drug Target. 1995;3:301–10.
37. Gurewich V, Pannell R, Louie S, Kelley P, Suddith RL, Greenlee R. Effective and fibrin-specific clot lysis by a zymogen precursor form of urokinase (pro-urokinase). A study *in vitro* and in two animal species. J Clin Invest. 1984;73:1731–9.
38. Lian T, Ho RJ. Trends and developments in liposome drug delivery systems. J Pharm Sci. 2001;90:667–80.
39. Nguyen PD, O'Rear EA, Johnson AE, Patterson E, Whitsett TL, Bhakta R. Accelerated thrombolysis and reperfusion in a canine model of myocardial infarction by liposomal encapsulation of streptokinase. Circ Res. 1990;66:875–8.
40. Heeremans JL, Prevost R, Bekkers ME, Los P, Emeis JJ, Kluft C, *et al.* Thrombolytic treatment with tissue-type plasminogen activator (t-PA) containing liposomes in rabbits: a comparison with free t-PA. Thromb Haemost. 1995;73:488–94.
41. Perkins WR, Vaughan DE, Plavin SR, Daley WL, Rauch J, Lee L, *et al.* Streptokinase entrapment in interdigitation-fusion liposomes improves thrombolysis in an experimental rabbit model. Thromb Haemost. 1997;77:1174–8.
42. Parente RA, Nadasdi L, Subbarao NK, Szoka Jr FC. Association of a pH-sensitive peptide with membrane vesicles: role of amino acid sequence. Biochemistry. 1990;29:8713–9.
43. Unstable angina and NSTEMI: the early management of unstable angina and non-ST-segment-elevation myocardial infarction. London; 2010.