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Development and characterization of site specific target sensitive liposomes for the delivery of thrombolytic agents

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ABSTRACT

In recent times, search for potent and highly selective thrombolytic agents with minimal side effects has become a major area of research. The aim of the present study was to develop and characterize target sensitive (TS) liposomes encapsulating streptokinase, a thrombolytic agent. The developed TS liposomes were composed of dioleylphophatidyl ethanolamine (DOPE) and dipalmityl-c(RGDfK) (10:1 mol/mol). Dipalmityl-c(RGDfK) was synthesized using typical carbodiimide chemistry using palmitic acid and c(RGDfK), while lysine was used as a spacer. Liposomes were of 100–120 nm size. *In vitro* drug release study showed that nearly 40% drug of the entrapped drug was released in 12 h in the PBS (pH 7.4), however on incubation with activated platelet about 90% of drug was released within 45 min. The results suggested target sensitivity of the liposomes. Further, targeting potential was confirmed using fluorescent microscopy and flow cytometry. Clot lysis study revealed that TS liposomes could not only reduce the clot lysis time but also increase the extent of clot lysis as compared to non-liposomal streptokinase solution. In conclusion, the present liposomal formulation will target the thrombolytic agent to the activated platelets in the thrombus and hence will improve the therapeutic efficacy of the drug.

1. Introduction

Vascular thrombosis is a major clinical problem, particularly in developed Western countries. Indeed, vascular thrombosis accounts for about half of all deaths in these countries as a result of consequential myocardial infarction, stroke, pulmonary emboli and likewise other vascular obstructions. The best way to improve patient survival and decrease morbidity is prompt detection and treatment of thrombosis with thrombolytic agent(s). A variety of thrombolytic agents such as streptokinase (SK), urokinase and tissue plasminogen activator (t-PA) are pharmacological active and available. These are currently in clinical use for dissolving arterial thrombi, particularly in cardiac blood vessels. These thrombolytic agents work by activating the protein plasminogen into plasmin (Kunamneni et al., 2007). However, the systemic side effects of these agents (systemic fibrinogenolysis and bleeding) raise and impose unavoidable clinical difficulties. These side effects are the result, at least in part, of the fact that these therapeutic agents comprise proteolytic components of the blood clotting cascade, and thus their thrombolytic capabilities are also responsible for their capacity to disrupt hemostasis systemically, particularly at dosages which are required and used for therapeutically effective effects of drugs at the thrombus site (Lestini et al., 2002). Thus, the outcome of clinical administration of these agents would obviously be improved if a thrombolytic agent could specifically delivered targeted to the thrombus sites *in vivo*, thereby reducing the incidence of unwanted systemic side effects (Torchilin, 1995).

Site-targeted drug delivery holds promise in the treatment of vascular injury-associated thrombotic and occlusive events caused by cardiovascular diseases (e.g. atherosclerosis) or interventional procedure (e.g. angioplasty and stenting). However, conventional techniques are expensive and require experienced personnel for their use (Huang et al., 2008). Thus, novel carrier based targeted delivery of thrombolytic agents is a desirable promising strategy for the treatment of thromboembolic diseases. Liposomes, a novel vesicular system have been explored by various research groups for the delivery of plasminogen activators (Erdogan et al., 2006; Leach et al., 2003, 2004; Nguyen et al., 1989; Perkins et al., 1997). They reported that vesicular carrier may accumulate in the thrombus and decrease the systemic degradation of the drug as well. For optimal drug delivery, drug should be localized at the site of thrombus and should avoid its non-specific uptake by unwanted tissue. Therefore, a well designed drug delivery system; surface modified with targeting moiety for targeted delivery of thrombolytic agents to the site of thrombus may provide an effective alternative (Vyas and Vaidya, 2009).

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Platelets play a vital role in various thromboembolic diseases. Under normal conditions, platelets circulate freely in blood vessels without interacting with other platelets or the vascular endothelium. However, at the site of endothelial damage, whether from vascular injury or rupture of an atherosclerotic plaque, blood platelets come into contact with the subendothelial extracellular matrix, which leads to a chain of reactions and the formation of a platelet-rich hemostatic plug (Langer and Gawaz, 2008; Xiang et al., 2008). Activation of the GP IIb/IIIa receptor is the common pathway involved in platelet aggregation. GP IIb/IIIa is a heterodimeric platelet membrane receptor and the major integrin on the platelet surface. The GP IIb/IIIa complex is normally present in an inactive state on resting platelets and serves as an adhesion receptor which has low affinity to surface-bound fibrinogen. However, these receptors result in a conformational change on platelet stimulation by physiologic ligands such as thrombin or collagen. This conformational change allows the platelet to bind to fibrinogen in plasma with high affinity (Shah and Goyal, 2004). The bridging of adhered and activated platelets is mediated by the binding of fibrinogen through the RGD (Arg-Gly-Asp) motifs located at the two Aα chains and through dodecapeptide sequences (HHLGGAKQAGDV) located within γ chains to the activated platelets via integrin αIIbβ3 (GP IIb/IIIa) (Huang et al., 2008).

It has been reported that integrin receptor targeted delivery of fibrinolytic agents by conjugation of urokinase with anti-GP IIb/IIIa antibody (7E3) showed higher fibrinolytic (thrombolytic) activity (Bode et al., 1991). Recently, it has also been reported that RGD peptides conjugated liposomes have affinity towards activated platelets and may be useful for the targeted delivery of thrombolytic agents (Gupta et al., 2005; Huang et al., 2008).

Moreover, for the treatment of acute myocardial infarction, rapid clot lysis is required so that tissue damage can be reduced. For increased and instant clot lysis drug concentration should be high in the microenvironment of thrombus. Nevertheless, to achieve such concentration higher amount of drug is required to be administered which may cause other circulatory side effects. In the present investigation, TS liposomes were prepared which not only target the thrombus but also instantly release drug following the interaction with the activated platelets embedded in the thrombus by self-destruction mechanism (contact capping). Thus, higher concentration at the target site may be achieved with reduced side effects.

In 1986, Ho et al., developed TS immunoliposomes composed of primarily an unsaturated PE such as DOPE. As reported earlier that the equilibrium phase of DOPE at the physiological conditions is the inverted hexagonal (H_{II}) phase (Cullis & de Kruijff, 1979), thus stable TS liposomes can be constructed by mixing DOPE with an appropriate amount of amphiphilic molecules i.e. acylated peptide. The acylated peptide not only serves as a bilayer stabilizer for DOPE but also provide the target specificity to the liposomes. After binding with the GP IIb/IIIa receptors on the activated platelets, TS liposomes undergo destabilization and thus release the contents.

2. Materials and methods

Peptides were procured from USV Ltd. Mumbai (India). 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC), N-hydroxy succinimide (NHS), palmitic acid, lysine and dicyclohexyl carbodiimide (DCC) was purchased from Himedia Ltd. Mumbai (India). Di-tertiary butyl pyrocarbonate was purchased from Spectrochem India. Sephadex G-75, dioleylphosphatidyl ethanolamine (DOPE) and D-Val-Leu-Lys-p-nitroanilide dihydrochloride (S-2251) were purchased from Sigma–Aldrich. Di-BOC protected lysine was synthesized by procedure reported elsewhere (Agrawal et al., 2007). Other chemicals unless otherwise stated used were of analytical grade and purchased from Himedia Ltd. Mumbai.

2.1. Synthesis of acylated peptide

2.1.1. Synthesis of NHS-palmitate

N-hydroxy succinimide ester of palmitic acid was synthesized according to the procedure reported earlier, with modification (Lapidot et al., 1967; Shen et al., 2007). Briefly, palmitic acid (PA) was added to the solution of NHS in dry ethyl acetate with continuous vortexing under nitrogen blanket. DCC was dissolved in dry ethyl acetate and mixed with the solution of palmitic acid and NHS. Solution was stirred overnight to complete the reaction. Formation of cloudy dicyclohexyl urea (DCU) confirms the completion of the reaction. DCU was separated from the solution using glass fiber filter pad and vacuum. The solvent was removed from the filtrate using rotary vacuum evaporator under reduced pressure. Obtained crude PA-NHS was purified by recrystallization using hot ethanol. Pure PA-NHS was characterized by FTIR and ^{1}H NMR.

2.1.2. Synthesis of palmitic acid derivatized RGD peptide

Di-tertiary butyl pyrocarbonate (di-BOC) lysine was firstly conjugated to the free amine group of the c(RGDfK) by carbodiimide chemistry. Equimolar quantities of peptide and lysine were reacted in the presence of equimolar quantity of EDC and excess quantity of NHS. Carboxyl group of lysine was conjugated to the amine group of RGD peptide. Subsequently, amino groups of lysine were deprotected using trifluoro acetic acid (TFA). These two free amino groups of the lysine conjugated peptide were used for the further conjugation with the hydrophobic palmitic acid (Fig. 1).

The peptide having an amino-terminal lysine with two free amino groups was added to a 10 mol excess of NHS-palmitate in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4, containing 2% deoxycholate and incubated at 37 °C for 10 h (Hermanson, 1996). Synthesized dipalmityl-RGD was separated by gel filtration chromatography using Sephadex G-75 column. The phosphate buffered saline (PBS), pH 7.4, containing 0.15% deoxycholate was used as an eluant. Final product was characterized by FTIR and mass spectroscopy.

2.2. Activity of acylated peptides

RGD peptide competes for fibrinogen to bind with the integrin receptor on the activated platelets and thus inhibits platelet aggregation. To confirm the activity or binding ability of modified peptide, platelet aggregation study was performed according to the procedure reported earlier by Huang et al. (2008), with modification. Briefly, platelet rich plasma (PRP) was obtained by centrifuging whole blood at 200 g for 15 min at 25 °C. A portion of the PRP was centrifuged at 600 × *g* for another 15 min at 25 °C to precipitate the platelets as a pellet. The supernatant liquid was collected as platelet-poor plasma (PPP). PRP with a platelet count of $2 \times 10^5/\mu l$ was prepared by mixing PRP with PPP and used to prepare the aggregometry samples. PPP was used as a "blank" for obtaining the baseline in the aggregometer. 300 μl of PRP, warmed to 37 °C for 2 min in chrono-log platelet ionized calcium aggregometer (model 600), were incubated with various concentrations of peptide and modified peptide in the presence of agonist thrombin and the maximal aggregation percentage was determined while stirring was continued for 15 min. Half maximal inhibitory concentration (IC_{50}) value for the peptides (RGD and RAD) and modified peptide (acylated peptide) was calculated.

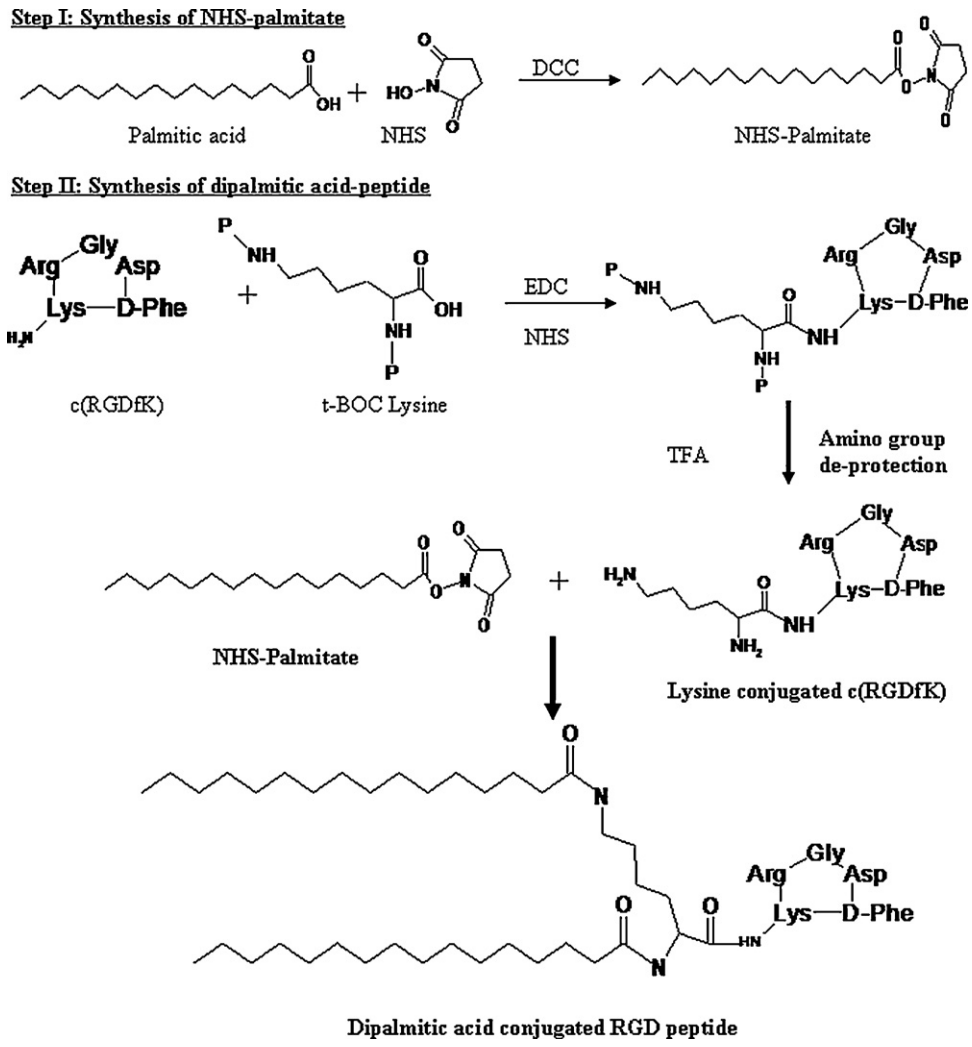

Fig. 1. Schematic presentation showing synthesis steps of dipalmityl-RGD peptide.

2.3. Development of target sensitive liposomes

TS liposomes were prepared using DOPE as a constituting lipid by the procedure reported earlier by Ho et al. (1986). Briefly, DOPE and diacylated RGD were dissolved in chloroform and organic solvent was evaporated using rotary vacuum evaporator (Strike 102, Italy). A thin lipid film was formed which was hydrated using PBS (pH 7.4). Dispersion was vortexed strongly for some times. Dispersed liposomes were sonicated for 3 min to transform them in to small unilammelar liposomes. For the formation of SK encapsulated liposomes, streptokinase solution (1,35,000 IU of SK in 1 ml of PBS) was used as a dispersion media. The unentrapped drug from the prepared vesicular system was separated by gel filtration (Fry et al., 1978) using Sephadex G-100.

2.4. Characterization of developed liposomes

2.4.1. Size, size distribution, shape and entrapment efficiency

The vesicles were characterized for size and polydispersity index by a dynamic light scattering method (Zetasizer nanoZS 90, Malvern Instruments Ltd, UK). Further, their shape and morphology were examined using a transmission electron microscope (TEM) (Hitachi, Japan). For TEM microscopy, specimens were prepared by dropping the dispersion onto carbon-coated EM grids. The grid was held horizontally to allow the molecular aggregates to settle and then tilted to 45° for a while to drain the excess fluid. Then a drop of phosphotungstic acid (pH 4) was added to the grid to impart a negative stain. The grid was then kept aside for 20 s before removing excess stain as above. Specimens were air-dried before examination using a Philips Morgagni, Netherlands transmission electron microscope. Encapsulation efficiency of SK in liposomes was determined by measuring the total protein and SK activity after disrupting the liposomes with 0.1% Triton X-100.

2.4.2. Activity of streptokinase

Activity of SK was measured using the plasmin substrate (S-2251) as reported earlier by Bode et al. (1991) and Couto et al. (2004), with modifications. Briefly, 96-well cell culture plates were coated with TBS-CaCl containing 5% (wt/vol) bovine serum albumin (BSA) to inhibit non-specific absorption. The plates were then extensively rinsed and dried. SK solutions were added to the wells. Then, a substrate solution consisting of (1:3 vol/vol) 4 mM S-2251 in H_2O and plasminogen (0.75 CU/ml) in S-2251 assay buffer (50 mM Tris and 110 mM sodium chloride) was mixed, and 100 μl was added to each well. The plate was immediately placed in the plate reader previously heated to 37 °C. The absorbance of the wells was measured at 405 nm using a SPECTRAmax Microplate Spectrophotometer (Molecular Devices, USA) and the software SOFTmax PRO. Activity of the test samples was determined using calibration curve prepared using standard solutions of SK.

2.4.3. In vitro release study

In vitro release study was performed according to procedure reported earlier by Baek et al. (2009). Briefly, SK entrapped liposomes were placed in a closed vial and immersed in shaking water bath at 37 °C. At predetermined time intervals, *in vitro* release rate of SK from liposomes was determined by separating liposomes from the release medium by ultracentrifugation at 150,000 × *g* for 1 h. SK content in the supernatant was determined by following the procedure as described above.

2.5. Study to confirm the content release at the surface of target (activated platelets) by destabilization of liposomes

Platelets suspension was obtained by the procedure as discussed earlier in text. 100 μl of platelets was incubated in the wells of a collagen coated 96 well microplate. Adhered platelets were activated by incubating with thrombin (1 U/ml). To thrombin activated platelets, 10 μl of RGD- or RAD-anchored liposomes were added and incubated up to 90 min. At different time intervals, S-2251 substrate solution was added to each well and absorbance at 405 nm was recorded using a microplate reader (Molecular Devices, USA). Absorbance after incubation of liposomes with 0.1% Triton X-100 was also observed for total activity and considered as 100. The percent content release was calculated according to the formula

$$\%\,\text{release} = [(A - A_0/A_t - A_0] \times 100$$

where, A and A_t are the absorbance after interaction of formulations with platelets and absorbance after lysis of liposomes with Triton X-100, respectively. A_0 is the absorbance of control. To assess the effects of resting platelets on the release of drug from liposomes, platelets suspension instead of adhered activated platelets was incubated with RGD-liposomes and activity of released drug was determined as discussed earlier.

2.6. Targeting potential of developed liposomal system

To assess targeting potential of liposomes, platelets binding studies were performed using human platelets in a suspension.

2.6.1. Platelets preparation

Platelets were isolated by differential centrifugation from fresh human blood, as already described (Gupta et al., 2007; Shrivastava et al., 2009). Briefly, blood from healthy volunteers was collected in citrate-phosphate-dextrose adenine and centrifuged at 180 × *g* for 10 min. PRP (platelet-rich plasma) was incubated with 1 mM acetylsalicylic acid for 15 min at 37 °C. After the addition of EDTA (ethylenediaminetetraacetic acid) (5 mM), platelets were sedimented by centrifugation at 800 × *g* for 10 min. Cells were washed in buffer A (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 1 mM EGTA (ethylene glycol tetraacetic acid), supplemented with 5 mM glucose, and 0.6 ADPase units of apyrase/mL, pH 6.2). Platelets were finally re-suspended in buffer B (pH 7.4), which was the same as buffer A but without EGTA and apyrase. The final cell counts were adjusted as per the requirement of the experiment. All RGD mediated binding studies were performed in the presence of 5 mM $CaCl_2$.

2.6.2. Microscopy study

The platelets were adsorbed onto collagen coated glass coverslips for 30 min, and then gently washed 3 times with PBS. Thrombin (1 U/ml) was added onto the platelets-adhered coverslips to ensure sufficient activation of adhered platelets. The adhesion and activation state of a platelet was confirmed by phase contrast microscopy. For fluorescent liposome binding studies, coverslip-adhered platelets were co-incubated with FITC labelled RGD, or RAD-modified liposomes for 30 min at room temperature in the dark. Subsequently, the coverslips were washed with PBS, fixed in 1% paraformaldehyde (PFA) for 30 min at 37 °C, and mounted on glass slides to image using fluorescence microscope with phase contrast attachment (Leica, model DM LB2) at 100× in oil.

2.6.3. Flow cytometry study to confirm the platelet targeting potential of liposomes

Platelets suspension with a platelet count of 2×10^8/ml was prepared by mixing platelets pellets with buffer B. Platelets were activated by incubating with thrombin (1 U/ml) for 10 min. Resting platelets or thrombin activated platelets were incubated with FITC labelled RGD-, or RAD-modified liposomes. For gated platelets population, FITC fluorescence was analyzed in a Becton Dickinson FACSCalibur flow cytometer, for 10,000 counts per sample aliquot and data were recorded accordingly. Fluorescence histograms were plotted for both resting and activated platelets populations, which provided quantitative information about the interaction of RGD- and RAD-liposomes with activated platelets.

2.7. In vitro clot dissolving study

In vitro thrombolysis study was performed using method reported elsewhere (Chung et al., 2008; Prasad et al., 2004) with partial modifications. Human whole blood was collected in preweighed sterile microcentrifuge tube and mixed with a buffer solution (10:1 by volume) of 0.9% NaCl and 0.15% $CaCl_2$ at pH 6.6. Thrombin solution (50 units/ml) was added to the blood (0.1:1 in volume), mixed and allowed to form a stable clot at 37 °C for 1–2 h. After clot formation, serum was completely pipetted out without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube − weight of tube alone). 500 μl of fresh human plasma with platelets count of 2×10^8/ml, adjusted by mixing appropriate volumes of PRP and PPP, was mixed with liposomal formulations to obtain equivalent dose of SK (7000 IU/ml) and added to the tubes containing clots. Liposomes without drug were also added to one of the tubes containing clot which served as a negative thrombolytic control. All the tubes were then incubated at 37 °C for 30 min and observed for clot lysis. After incubation, fluid obtained was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. Time to dissolve 50% of the initial clot ($t_{50\%}$) was also determined for various formulations.

3. Results and discussion

The present study is based on the fact that thrombolytic agents act by converting plasminogen to the plasmin in the environment of the thrombus. Thus TS liposomes were developed which target activated platelets and release drug in the thrombus. RGD peptide does not bind with the integrin receptors (GP IIb/IIIa) in the resting platelets while in the activated platelets it has excessively high affinity towards the GP IIb/IIIa receptors. Therefore, RGD peptide grafted liposomes were prepared by incorporation of acylated peptides in the bilayer of DOPE liposomes. It has been reported earlier by Ho et al. (1988) that TS liposomes released the drug in the extracellular environment when acylated antibody attached liposomes bind with the target cells. They suggested that liposomes destabilization at the target site might be due to the mechanism know as 'contact capping'. It was observed that when liposomes come in contact with the target cell, acylated antibody migrated from the bilayer towards the receptor as a result vesicles destabilized due to intrinsic property of DOPE which forms inverted micelles at

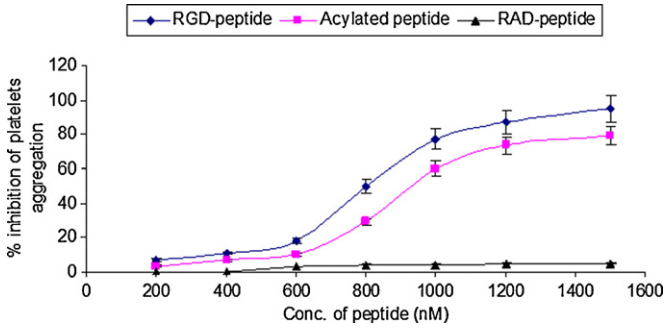

Fig. 2. IC_{50} determination by aggregometry assay for c(RGDfK), c(RADfK) and acylated c(RGDfK) peptide. Data are presented as mean ± SD ($n = 3$).

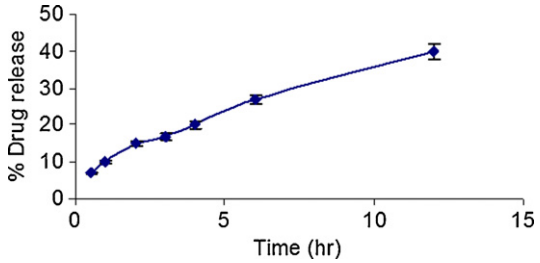

Fig. 3. *In vitro* drug release profile in the PBS, pH 7.4 medium. Data are presented as mean ± SD ($n = 5$).

physiological conditions. At the time of phase transition, liposomal contents released in the environment which may act extracellularly or may diffuse in to the cytoplasm of the cells.

3.1. Synthesis of acylated peptide

Acylated peptide was synthesized in two steps. Firstly, an activated N-hydroxysuccinimidyl ester of palmitic acid was synthesized. The activated NHS ester derivative was subsequently reacted with amino groups of the peptide to form amide bond as presented in Fig. 1. The synthesis of NHS-palmitate was confirmed by FTIR and proton-NMR. IR spectra of activated NHS-palmitate esters distinctly showed that the broad peak of hydroxyl group present in IR spectra of NHS disappeared in the spectra of NHS-palmitate and two carbonyl stretches from NHS at 1742.03 and 1727.89 cm^{-1} further confirmed the synthesis of NHS-palmitate. ^1H NMR spectra showed a broad singlet peaks at approximately 2.8 ppm due to 4-H, triplet resonance at 2.6 ppm due to α-methylene hydrogens (CH_2), multiplet resonances at or around 1.7–1.8 ppm due to terminal methyl hydrogens (CH_3) and broad singlet resonances around 1.2–1.3 ppm due to multiple methylene hydrogens (CH_2) of activated aliphatic NHS esters. The same observations were reported by Shen et al. (2007).

Synthesis of palmitate-RGD conjugate was confirmed by FTIR and mass spectroscopy. In the FTIR spectra, disappearance of two carbonyl peaks of NHS at 1742.03 and 1727.89 cm^{-1} confirmed the synthesis of palmitate-RGD. *m*/*z* value for the conjugate was found to be 1107.6 which was near the expected *m*/*z* value of the theoretical conjugate. *m*/*z* value further confirmed the synthesis of dipalmitic acid-RGD peptide.

3.2. Activity of acylated peptide

IC_{50} value which is the concentration of peptides required to inhibit fibrinogen mediated platelets aggregation in PRP by 50%, was determined to confirm the affinity of the acylated peptides for the GP IIb/IIIa receptor expressed on the surface of activated platelets. Results (Fig. 2) suggest that there was an increase in the IC_{50} value for the acylated peptide, as against 800 nM (unacylated) it was recorded to be 870 nM in case of acylated peptide. This suggests that modification of peptide with dipalmitic acid did not significantly change the affinity of the peptide towards the receptor and subsequent activity thus might be used for the targeting of GP IIb/IIIa receptor on the platelets membrane. Further, it was also observed that peptide having RAD sequence does not or slightly inhibit platelets aggregation.

3.3. Development and characterization of TS liposomes

TS liposomes were prepared by varying the ratio of DOPE and acylated-RGD (10:0–1.5) in order to obtain stable liposomes with different peptide density over the surface. It has already been reported that fatty acid helps in the stabilization of bilayer formed by DOPE (Duzgunes et al., 1985). Characteristics of liposomes formed at various ratios are shown in Table 1. Higher entrapment efficiency of liposomes might be ascribed to higher stability of liposomal bilayer. Liposomes were found to be in the range of 100–120 nm and encapsulation efficiency was found to be in the range of 7–18%. It was found that DOPE: acylated fatty acid, 10:1 is required to stabilize the liposomal bilayer, however, beyond the ratio of 10:1.2 the entrapment efficiency tended to decrease which might be due to aggregation of acylated peptide in the dispersion medium therefore low incorporation in the bilayer (Ho et al., 1986). TEM study suggested that developed liposomes are spherical in shape and unilamellar in nature (photographs not shown).

In vitro drug release study showed that drug released from the liposomes in the PBS (pH 7.4) was nearly 40% in 12 h (Fig. 3) which depicted that liposomes could trap the drug effectively with minimum release in the systemic circulation while *enroute* to the target; however, further *in vivo* studies are needed to be performed to evaluate the effects of various blood components on the stability of the developed liposomes in the blood circulation. Further, PEGylation may also improve the circulation but it needs to be evaluated that PEGylated TS liposomes will retain target sensitivity or not.

Table 1
Characteristics of target sensitive liposomes.

Liposomes composition (DOPE:acylated peptide) mol/mol	% Entrapment efficiency	Vesicles size (nm)		Polydispersity index (PI)	
		Initial	After 24 h	Initial	After 24 h
10:0.1	7 ± 0.53	128 ± 12	145 ± 11	0.25 ± 0.08	0.31 ± 0.09
10:0.2	8 ± 0.72	121 ± 14	142 ± 13	0.24 ± 0.07	0.28 ± 0.05
10:0.5	12 ± 0.41	112 ± 11	125 ± 08	0.21 ± 0.07	0.26 ± 0.06
10:1.0	18 ± 0.35	108 ± 09	115 ± 08	0.18 ± 0.05	0.21 ± 0.07
10:1.2	17 ± 0.28	107 ± 07	116 ± 10	0.17 ± 0.06	0.19 ± 0.07
10:1.5	9 ± 1.15	120 ± 14	143 ± 13	0.25 ± 0.09	0.28 ± 0.08

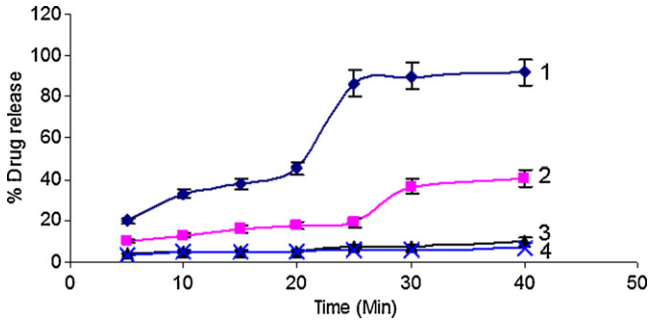

Fig. 4. *In vitro* release profile after incubation of RGD TS liposomes with platelets adhered and activated in the wells of 96-well microplate. RGD-liposomes (1) release drug in higher amount as compared to RAD liposomes (3) after interaction with activated platelets. RGD-liposomes incubated with resting platelets (2) and with RGD peptide pre-incubated activated platelets (4) release low amount of SK. Data are presented as mean ± SD ($n = 5$).

3.4. Drug release at the target site due to self-destruction of liposomes

TS liposomes have the property of not only binding with the target cells but also subsequent self-destabilization property. The entrapped drug released at the target site i.e. after binding with the activated platelets is shown in Fig. 4. It was found that about 90% of entrapped drug is release within 45 min of incubation. Drug release after interaction with the platelets adhered on collagen coated microplate showed that when RGD-conjugated liposomes come in contact of the integrin receptors on the activated platelets, the liposomes destabilization occurs. Destabilization of the liposomes undergoes due to inherent property of DOPE which forms inverted micelles thus content of the liposomes released at the surface of platelets. However, the mechanism of destabilization of these liposomes is yet to be explored. It may be either due to migration of acylated peptide from the bilayer and its subsequent accumulation at the surface of platelets or by the contact between the liposomes binds to the same surface of the target cell. However, drug release was inhibited by pre-incubation of platelet surface with an excess free RGD peptide that indicated that drug release is induced by interaction of the acylated RGD with integrin GP IIb/IIIa on the activated platelets leaving liposomes destabilized. Further, it was recorded that liposomes incubated with resting platelets did not release the content as efficiently as with activated platelets, as the former do not express integrin receptor which corresponds to RGD hence it remains associated with liposomal bilayer imparting needed stability. Drug release after interaction with resting platelets might be ascribed to some extent of activation of platelets during isolation procedure.

3.5. Targeting potential of the developed system

3.5.1. Microscopic observation

In order to confirm the binding of TS liposomes on to the activated platelets, fluorescence microscopy study was conducted using FITC labelled RGD-liposomes. The TS liposomes were incubated with platelets adhered to the surface of coverslips. Platelets adherence at the surface of coverslips was confirmed by phase contrast microscopy. Fluorescence photographs (Fig. 5) showed that platelets incubated with RGD-liposomes showed higher fluorescent intensity as compared to RAD-liposomes. The same fields of the coverslips were observed under the phase contrast mode which showed that activated platelets were present at exactly the same position where fluorescent spots were located.

3.5.2. Flow cytometry to confirm the targeting potential of liposomes:

Platelets in suspension were used to assess the binding ability of the developed liposomes. From Fig. 6 it may be observed that in the resting stage of platelets, RGD- and RAD-liposomes bind to the platelets equally. This binding may be ascribed to the non-specific binding of modified liposomes to the surface of resting platelets or may be due to some extent of activation of platelets during isolation procedure. However, when platelets were activated by thrombin, RAD-liposomes showed same fluorescence whereas RGD-liposomes bind more specifically and high

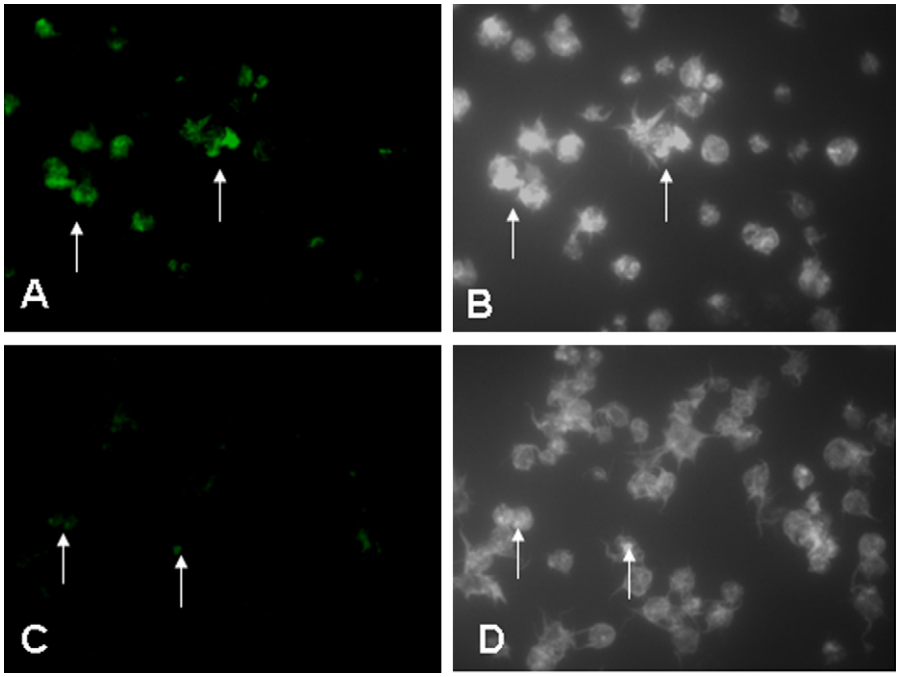

Fig. 5. Microscopic observation of the platelets incubated with FITC labelled RGD- (A) and RAD-liposomes (C) under fluorescence microscope. Same field was also observed under phase contrast mode (B) and (D), respectively. Figures show that RGD liposomes bind more efficiently to the activated platelets as compared to RAD liposomes.

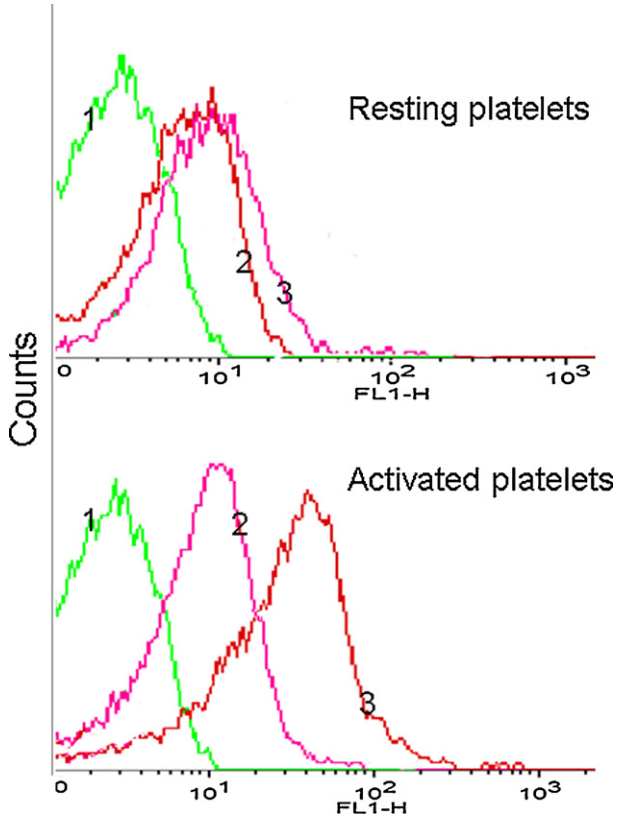

Fig. 6. Flow cytometric data after incubating liposomes with platelets suspension *in vitro*. In the resting state RGD (3) and RAD (2) liposomes show equal fluorescence, however, after activation with thrombin, platelets incubated with RGD liposome showed higher fluorescence, over unlabelled platelets (1).

fluorescent shifting was observed as shown in the fluorescence histogram. Results confirmed that in resting state integrin receptors (GP IIb/IIIa) are not appropriately conformed to bind RGD peptide, however, on incubation with thrombin; receptors are turn to be active form leading to the conjugation of RGD liposomes which in turn bind more efficiently as compared to RAD liposomes.

3.6. *In vitro* blood clot dissolving study

The main objective of the present study was to increase the accumulation of SK in the thrombus and to increase the clot lysis activity of the SK. As it is reported earlier that SK is deactivated in the plasma due to the presence of anti-streptokinase antibodies and plasminogen activator inhibitors-1 (PAI-1). Encapsulation in the liposomes increases the stability of SK in the plasma during *enroute* circulation by preventing their exposure to degrading substances. RGD modified TS liposomes will release the drug at the target site (thrombus) after binding with the platelets embedded in the thrombus matrix while drug release in the circulation will be retarded. RGD peptide based targeted t-PA delivery to the thrombus for the increased clot lysis was performed by Chung et al. (2008). They reported that after modification of PLGA nanoparticles, higher intra-clot thrombolysis was observed. They also described that higher clot lysis was due to interaction of RGD-PLGA nanoparticles with the platelet embedded in the thrombus. Pores in the fibrin matrix were also reported to exist in the size of below one micron. Thus nanocarriers have seen the better options for the targeted delivery of t-PA to the thrombus interior. Based on this hypothesis nanosized RGD modified TS liposomes were developed for the site directed higher thrombolysis as well as to reduce the side effects due to systemic release of SK.

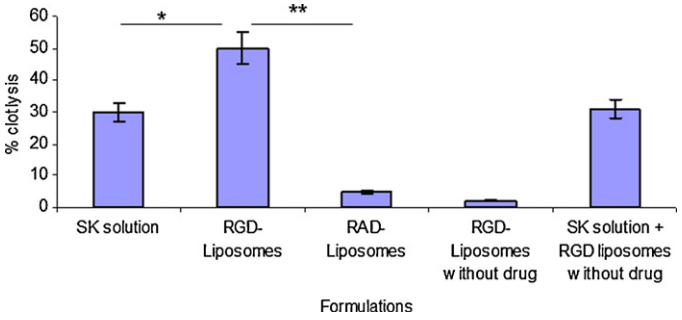

Fig. 7. Graphs showing % clot lysis with various SK formulations. % clot lysis for RGD liposomes are significantly higher ($^{*}p < 0.01$, $n = 3$) than that of SK solution. % clot lysis for RAD liposomes are significantly lower than that of SK solution ($p < 0.001$, $n = 3$) and RGD liposomes ($^{**}p < 0.001$, $n = 3$).

Thrombolysis study with human blood clot was performed *in vitro* in microcentrifuge tubes.

Clot lysis study conducted in the microcentrifuge tubes showed that RGD-liposomes took less clot lysis time and demonstrated high clot dissolving capacity as depicted from the weight of residual clot after treatment with various formulations. It was found that after 30 min of treatment, 50% clot lysis was obtained with RGD-liposomes as compared to 30% with SK solution. However, with RAD-liposomes and control liposomes, negligible clot lysis was recorded. Clot lysis with SK solution + RGD-liposomes without drug was found to be near about to plain SK solution that further confirmed the receptor mediated interaction of RGD-liposomes to the thrombus (Fig. 7). To measure the efficacy of formulations to impart reperfusion of the occluded vessels, time required to dissolve 50% (t_{50}) was also measured. It was found that RGD-liposomes dissolve 50% clot in 30 min, whilst SK solution could dissolve the same in 55 min (Fig. 8). Less clot lysis time (t_{50}) recorded for RGD-liposomes may be due to inhibition of premature inactivation of the SK during incubation with plasma and at the same time TS liposomes could have released drug in higher concentration in the vicinity of thrombus.

As reported by early investigators that front edge of thrombus is composed of fibrin network thus SK may relatively adsorbed at the outer surface of thrombus resulting in low degree of intra-clot lysis (Leach et al., 2003, 2004). However, in case of nanosized TS liposomes, it may be hypothesized that they can move in to the interior of the thrombus through the pores where they will interact with the activated platelets and release the drug owing to self-destruction mechanism. Thus, total thrombolytic activity of RGD-liposomes was found higher than free drug. Less clot lysis time of RGD-liposomes may be attributed to the higher amount of SK at the thrombus site while in case of SK solution low amount of SK may present at the surface of thrombus. Thus, nanosized RGD-liposomes

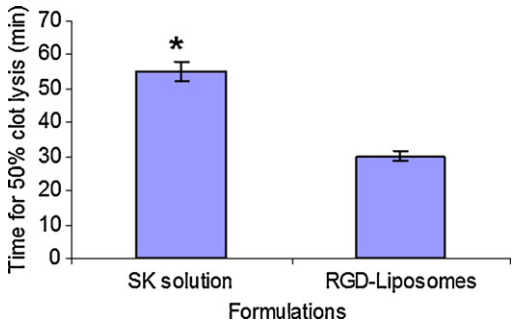

Fig. 8. Graphs showing clot lysis time (t_{50}), time to dissolve 50% of clot wt, with various SK formulations. Clot lysis time for RGD liposomes are significantly shorter ($^{*}p < 0.01$, $n = 3$) than that of SK solution.

may be helpful to accelerate the intra-clot lysis as well as to reduce the clot lysis time.

It has already been reported by Bode et al. (1991) that urokinase conjugated with monoclonal antibody (7E3) that selectively binds to platelets membrane glycoprotein (GP IIb/IIIa), caused accelerated clot lysis in platelet rich thrombus. The same results were obtained in our experiments, however, we used RGD conjugated TS liposomes to deliver SK to the site of platelets rich thrombus.

4. Conclusion

Various strategies have been used to increase the thrombolytic activity and to reduce the systemic side effects of the plasminogen activators. However, liposomes are highly promising carriers for the delivery of drug through systemic route. As compared to particulate carriers, liposomes are flexible vesicular system thus drugs at the site of action may be released more precisely. In addition bilayer membrane could protect the drug from circulating PAI-1 as well. Thus, streptokinase encapsulated RGD peptide conjugated TS liposomes are specific to the site of thrombus. It was found that TS liposomes released the drug following their binding with the activated platelets more effectively than that with resting platelets. Fluorescence microscopy and flow cytometry study further confirm the binding of liposomes to the activated platelets. Clot dissolving study revealed that RGD conjugated TS liposomes could not only reduce the clot lysis time but also increased the total clot dissolution. Further, studies are needed to be performed to evaluate *in vivo* biodistribution and targetability of the developed system. However, based on these preliminary studies it can be concluded that RGD modified TS liposomes hold promise to be a pragmatic carrier for the treatment of arterial thrombosis, clinically.

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References

Agrawal, P., Gupta, U., Jain, N.K., 2007. Glycoconjugated peptide dendrimers-based nanoparticulate system for the delivery of chloroquine phosphate. Biomaterials 28, 3349–3359.

Baek, S.H., Park, S.J., Jin, S.E., Kim, J.K., Kim, C.K., Hwang, J.M., 2009. Subconjunctivally injected, liposome-encapsulated streptokinase enhances the absorption rate of subconjunctival hemorrhages in rabbits. Eur. J. Pharm. Biopharm. 72, 546–551.

Bode, C., Meinhardt, G., Runge, M.S., Freitag, M., Nordt, T., Arens, M., Newell, J.B., 1991. Platelet-targeted fibrinolysis enhances clot lysis and inhibits platelet aggregation. Circulation 84, 805–813.

Chung, T.W., Wang, S.S., Tsai, W.J., 2008. Accelerating thrombolysis with chitosan-coated plasminogen activators encapsulated in poly-(lactide-co-glycolide) (PLGA) nanoparticles. Biomaterials 29, 228–237.

Couto, L.T., Donato, J.L., de Nucci, G., 2004. Analysis of five streptokinase formulations using the euglobulin lysis test and the plasminogen activation assay. Braz. J. Med. Biol. Res. 37, 1889–1894.

Cullis, P.R., de Kruijff, B., 1979. Lipid polymorphism and the functional roles of lipids in biological membranes. Biochim. Biophys. Acta 559, 399–420.

Duzgunes, N., Straubinger, R.M., Baldwin, P.A., Friend, D.S., Papahadjopoulos, D., 1985. Proton-induced fusion of oleic acid-phosphatidylethanolamine liposomes. Biochemistry 24, 3091–3098.

Erdogan, S., Dr. Özer, A.Y., Volkan, B., Caner, B., Bilgili, H., 2006. Thrombus localization by using streptokinase containing vesicular systems. Drug Deliv. 13, 303–309.

Fry, D.W., White, J.C., Goldman, I.D., 1978. Rapid separation of low molecular weight solute from liposomes without dilution. Anal. Biochem. 90, 809–815.

Gupta, A.S., Huang, G., Lestini, B.J., Sagnella, S., Kottke-Marchant, K., Marchant, R.E., 2005. RGD-modified liposomes targeted to activated platelets as a potential vascular drug delivery system. Thromb. Haemost. 93, 106–114.

Gupta, R., Chakrabarti, P., Dikshit, M., Dash, D., 2007. Late signaling in the activated platelets upregulates tyrosine phosphatase SHP1 and impairs platelet adhesive function: regulation by calcium and Src kinase. Biochim. Biophys. Acta 1773, 131–140.

Hermanson, G.T., 1996. Bioconjugate Techniques. Academic Press, San Diego, CA.

Ho, R.J.Y., Rouse, B.T., Huang, L., 1986. Target-sensitive immunoliposomes: preparation and characterization. Biochemistry 25, 5500–5506.

Ho, R.J.Y., Ting-Beall, H.P., Rouse, B.T., Huang, L., 1988. Kinetic and ultrastructural studies of interactions of target-sensitive immunoliposomes with Herpes Simplex Virus. Biochemistry 27, 500–506.

Huang, G., Zhou, Z., Srinivasan, R., Penn, M.S., Kottke-Marchant, K., Marchant, R.E., Gupta, A.S., 2008. Affinity manipulation of surface-conjugated RGD peptide to modulate binding of liposomes to activated platelets. Biomaterials 29, 1676–1685.

Kunamneni, A., Taleb, T., Abdelghani, A., Ellaiah, P., 2007. Streptokinase—the drug of choice for thrombolytic therapy. J. Thromb. Thrombolysis 23, 9–23.

Langer, H.F., Gawaz, M., 2008. Platelet–vessel wall interactions in atherosclerotic disease. Thromb. Haemost. 99, 480–486.

Lapidot, Y., Rappoport, S., Wolman, Y., 1967. Use of esters of N-hydroxysuccinimide in the synthesis of N-acylamino acids. J. Lipid Res. 8, 142–145.

Leach, J.K., O'rear, E.A., Patterson, E., Miao, Y., Johnson, A.E., 2003. Accelerated thrombolysis in a rabbit model of carotid artery thrombosis with liposome-encapsulated and microencapsulated streptokinase. Thromb. Haemost. 90, 64–70.

Leach, J.K., Patterson, E., O'rear, E.A., 2004. Improving thrombolysis with encapsulated plasminogen activators and clinical relevance to myocardial infarction and stroke. Clin. Hemorheol. Microcirc. 30, 225–228.

Lestini, B.J., Sagnella, S.M., Xu, Z., Shive, M.S., Richter, N.J., Jayaseharan, J., Case, A.J., Kottke-Marchant, K., Anderson, J.M., Marchant, R.E., 2002. Surface modification of liposomes for selective cell targeting in cardiovascular drug delivery. J. Controlled Release 78, 235–247.

Nguyen, P.D., O'rear, E.A., Johnson, A.E., Lu, R., Fung, B.M., 1989. Thrombolysis using liposomal-encapsulated streptokinase: an in vitro study. Proc. Soc. Exp. Biol. Med. 192, 261–269.

Perkins, W.R., Vaughan, D.E., Plavin, S.R., Daley, W.L., Rauch, J., Lee, L., Janoff, A.S., 1997. Streptokinase entrapment in interdigitation-fusion liposomes improves thrombolysis in an experimental rabbit model. Thromb. Haemost. 77, 1174–1178.

Prasad, S., Kashyap, R.S., Deopujari, J.Y., Purohit, H.J., Tori, G.M., Daginawala, H.F., 2004. Development of an in vitro method to study clot lysis activity of thrombolytic drugs. Thromb. J. 4, 1–4.

Shah, H.D., Goyal, R.K., 2004. Glycoprotein IIb/IIIa receptor and its inhibition: a platelet-directed therapeutic strategy. Ind. J. Pharmacol. 36, 133–139.

Shen, S.I., Kotamraj, P.R., Bhattacharya, S., Li, X., Jasti, B.R., 2007. Synthesis and characterization of RGD-fatty acid amphiphilic micelles as targeted delivery carriers for anticancer agents. J. Drug Target. 15, 51–58.

Shrivastava, S., Bera, T., Singh, S.K., Singh, G., Ramachandrarao, P., Dash, D., 2009. Characterization of antiplatelet properties of silver nanoparticles. ACSNano 3, 1357–1364.

Torchilin, V.P., 1995. Targeting of drugs and drug carriers within the cardiovascular system. Adv. Drug Deliv. Rev. 17, 75–101.

Vyas, S.P., Vaidya, B., 2009. Targeted delivery of thrombolytic agents: role of integrin receptors. Exp. Opin. Drug Deliv. 6, 409–508.

Xiang, Y.Z., Kang, L.Y., Gao, X.M., 2008. Strategies for antiplatelet targets and agents. Thromb. Res. 123, 35–49.