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Review

Functionalized carriers for the improved delivery of plasminogen activators

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ABSTRACT

Various plasminogen activators have been routinely used for the treatment of thrombotic diseases. However, these agents possess various problems *e.g.* short half life and other bleeding complications. To improve the effectiveness as well as to reduce the side effects of these drugs, various modifications have been made. For example, fibrin specific plasminogen activators have been developed. However, these agents also demonstrated various bleeding complications, clinically. Nowadays, so many carrier systems have been explored to improve the activity of these agents. Novel carriers not only improve the effectiveness of these drugs but also reduce the side effects. In the present review, we discuss novel carrier based strategies to improve the delivery of the plasminogen activators to site of thrombus.

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Contents

1. Introduction

Cardiovascular diseases are typically localized to discrete vascular regions, affording great opportunity for targeted pharmacological treatment. Vascular thrombosis is a major clinical problem, particularly in developed Western countries. Indeed, vascular thrombosis is a major reason for about half of all deaths in these countries as a result of consequential myocardial infarction, stroke, pulmonary emboli and similar other vascular obstructions.

When a blood vessel suffers an injury, the body uses platelets and fibrin to form an immediate blood clot, as the first step of repairing process so that any possible loss of blood can be prevented. Thrombosis may obstruct the normal blood flow in blood vessels of the circulatory system. The formation of thrombus plays a critical role in the progression of a number of cardiovascular pathologies (Fuster et al., 1992). The cardiovascular pathologies mainly include: (i) atherosclerotic heart disease (myocardial infarction); (ii) cerebrovascular disease (stroke); and (iii) venous thromboembolism (VTE) (deep vein thrombosis (DVT) and pulmonary embolism (PE). Primary cause of myocardial or cerebral infarction is the atherosclerotic degeneration of the vessel wall. However, thrombotic occlusion of major essential blood vessels is the key event that triggers and as a result presents the clinical syndrome. Venous thromboembolism is a silent yet potentially fatal disease that affects over 2 million Americans annually (Collen et al., 1988; McGuire and Dobesh, 2004).

The best way to improve patient survival and to decrease rate and extent of morbidity is prompt and early detection and

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also the treatment of thromboembolism using an effective thrombolytic therapy. A variety of physiological plasminogen activators belonging to a class of serine proteases of the tissue (tissue plasminogen activator, tPA) or urokinase (urokinase, UK) types and bacterial proteins (streptokinase, SK) are available and clinically being used. These agents catalyze the hydrolysis of plasminogen at the Arg_{561}–Val_{562} bond which results in the formation of active plasmin. Plasmin acts by degrading insoluble fibrin clot that constitutes the clustered nuclei of thrombi. Fibrin serves not only as a substrate for plasmin but also as a surface for the specific adsorption of plasminogen as well as tPA (Vyas and Vaidya, 2009).

2. Plasminogen activators: a comparison

SK was the first clinically used thrombolytic agent (Kunamneni et al., 2007). It is obtained from Streptococci and is relatively inexpensive compared to other fibrinolytic agents. SK as such possesses no intrinsic enzymatic activity, however after interaction with the plasminogen it is converted in to an activator complex. The SK-plasminogen complex catalyzes the conversion of another plasminogen molecule into plasmin. After that SK-plasminogen complex is converted into SK-plasmin. It should be noted that plasmin, in contrast to the SK-plasminogen complex, is rapidly inhibited by α_2-antiplasmin and cannot activate plasminogen in the blood. Therefore, the SK-plasmin complex (not inhibited by α_2-antiplasmin) can generate plasmin in the thrombus as well as in the blood flow, with a danger of hyperlytic state development (Maksimenko and Tischenko, 1999).

Another plasminogen activator, UK, activates plasminogen directly. It is extracted from urine. It is non-antigenic or pyrogenic, however very expensive (Matsuo, 2005; Kim et al., 2009). UK can alternatively be produced using recombinant DNA technology in microorganism, however the therapeutic efficacy of such product may remain lower than that of tPA.

SK and UK are first-generation thrombolytic agents which do not exhibit an affinity for fibrin (thrombus), therefore generate plasmin in the systemic circulation also. This leads to serious side-effect, *e.g.*, systemic bleeding. Development of such haemorrhagic manifestations and other complications like allergic reactions necessitate the search for new plasminogen activators with increased selectivity (thrombospecificity) (Table 1).

Fibrin-specific plasminogen activators appear to be the preferred means of thrombolytic therapy as compared to fibrin-nonspecific agents. These agents are sufficiently inert and act on the plasminogen adsorbed at the surface of thrombus, thus localize the activity near or around the clot. A series of fibrin-specific derivatives have been developed. tPA is an example of such agents that possesses higher affinity for fibrin and proved to be highly effective thrombolytic agent, clinically (Banerjee et al., 2004; Ouriel, 2004; Szemraj et al., 2005; Baruah et al., 2006; Rivera-Bou et al., 2010).

The complicated administration regimen and relatively high cost of the preparation are the major disadvantages of tPA therapy. At the same time, these preparations are not significantly superior to SK in term of activity. This entailed the need for development of novel strategies which can improve the effectiveness of conventional thrombolytic agents.

3. Novel approaches to enhance therapeutic activity of plasminogen activators

Plasminogen activators have extremely short half-life (18–60 min for SK, 15 min for UK and 4–6 min for tPA), which requires their higher doses in order to obtain desired therapeutic effects that, however may lead to haemorrhagic complications. Short half-lives are generally due to fast renal clearance owing to the hydrophilic properties of these agents and their small size or due to enzymatic degradation in blood, liver and kidney. Strategies to prolong plasma half life time may lead to improved pharmacokinetic profiles of conventional drugs. Therefore, it is required that a pertinent drug carrier should be developed that prolongs the biological half-life of plasminogen activators *in vivo* and hence reduces the total administered dose of the drug necessary for the treatment *vis-a-vis* minimizes its side effects.

Various strategies to improve the half-life of proteins have been reported. These include chemical modification of the protein drugs, such as modification of N- and C-terminals of protein, replacement of labile amino acids, cyclization and increment of molecular mass by PEGylation or oligomerization, enzyme inhibition and introduction of drug delivery systems, such as liposomes, biodegradable particles *etc.* (Heeremans et al., 1995a,b; Leach et al., 2003, 2004; Werle and Schnurch, 2006; Chung et al., 2008).

3.1. Strategies to prolong the half life of protein drugs (plasminogen activators)

The rapid and strong immune response associated with intravenous SK (foreign protein) infusion frequently limits the duration of therapy. Therefore, various chemical modifications have been made in the SK to produce the derivatives which are non-antigenic. These derivatives could be utilized in patients with high antibody titer. PEGylation, covalent attachment of methoxypolyethyleneglycol (mPEG) to protein significantly increases circulating time, reduces immunogenicity and antigenicity of the proteins and at the same time retains bioactivity (Kodera et al., 1998; Veronese, 2001).

Table 1
Characteristics of different plasminogen activators.

S.no.	Plasminogen activators	Ml. wt. (KD)	Fibrin selectivity	Half-life (min)	Immunogenicity	Remarks
1	SK	47	No	10/90	Yes	It is an indirect plasminogen activator
2	UK	32	No	2	No	It is a direct plasminogen activator
3	APSAC	131	No	40–90	Yes	It does not require free circulating plasminogen to be effective
4	ProUK	49	No	9	No	It is a prodrug of UK and has high clot selectivity
5	SAK	16.5	Yes	6	Yes	In contrast to SK, it possesses high fibrin specificity
6	t-PA/rt-PA	39/68	Yes	15/5	No	These convert plasminogen to plasmin in the presence of fibrin and activate clot bound plasminogen 100 fold more effectively than circulating plasminogen

Abbreviations: APSAC, acylated plasminogen-streptokinase activator complex; SAK, staphylokinase; rt-PA, recombinant t-PA.

The polyethyleneglycol (PEG) has been approved by FDA for use as a vehicle or base in foods, cosmetics and pharmaceuticals, including injectable, topical, rectal and nasal formulations (Greenwald et al., 2003). PEG shows little toxicity, and is eliminated from the body intact through either the kidneys (for PEGs < 30 kDa) or in the faeces (for PEGs > 20 kDa) (Yamaoka et al., 1994). PEG lacks immunogenicity, and antibodies to PEG are rarely generated in rabbits only if PEG is combined with highly immunogenic proteins (Harris and Chess, 2003).

Koide et al. (1982) derivatized SK with PEG_{5000} which was activated by the cyanuric chloride method. These preparations did not react with anti-streptokinase sera; however, SK lost 67% of its activity. Later, a novel method was used for PEG activation in which 1,l′-carbonyldiimidazole was used to form an imidazole carbamate derivative of the polymer (Beauchamp et al., 1983). This activated PEG couples to lysine residues in proteins. Proteins derivatized with 1,l′-carbonyldiimadazole-activated PEG demonstrated significantly long plasma half-lives while activity was only minimally decreased. Thus, PEGylation can be considered as one of the possible approaches to improve pharmacokinetic and therapeutic efficacy.

Prolonged plasma half-life can also be achieved without chemical modification of the drug by using properly designed and developed delivery systems. Among various novel carriers, liposomes are widely explored as drug carrier for oral, parenteral and topical administrations and also to avoid undesired metabolism both *in vitro* and *in vivo* (Kim et al., 2009).

It was demonstrated by Kim et al. (1998) that liposomes composed of distearoylphosphatidyl ethanolamine-N-poly (ethylene glycol) 2000 (DSPE-PEG_{2000}) offer prolonged circulating half-life and improved area under the plasma concentration–time curve from time zero to infinity (AUC_∞) to the SK, *i.e.*, 16.3- and 6.1-fold, respectively in comparison to those of SK alone after femoral administration in rats. Recently, Kim et al. (2009) have also used PEGylated liposomes to prolong the circulation half-life of tPA. In this study, liposomes were prepared using egg phosphatidylcholine, cholesterol, cholesterol-3-sulfate and DSPE-PEG_{2000}. It was found that conventional as well as PEGylated liposomes could increase the half-life of tPA by 16- and 21-folds, compared with free tPA, respectively. These studies suggested the successful use of PEGylated liposomes to increase the half-life of plasminogen activators. Thus, higher retention of the drug in the circulation may be achieved by using such PEGylated liposomes. At the same time, long circulatory (PEGylated) liposomes may accumulate in the thrombus area owing to the nanosized liposomes.

3.2. Targeted delivery of thrombolytic agents

The basic problems associated with conventional systemic drug administration are even biodistribution throughout the body and lack of specificity towards the target site. Therefore, large doses are required to achieve high local concentration. The high dose may further increase the adverse side effects and non-specific toxicity. Therefore, drug targeting is considered to be the best approach to obviate or resolve these dose-related problems (Torchilin, 2000; Vyas et al., 2001).

The targeting moiety (antibody or ligand) can be attached directly to an active moiety (therapeutic or diagnostic unit) or to the surface of soluble or insoluble carriers loaded with therapeutic molecules (Fig. 1). Different reactive and biocompatible, moreover soluble polymers can be used as soluble carriers, whereas a family of insoluble carriers that include microparticles, nanoparticles, liposomes and micelles can also be used.

3.2.1. Targeted delivery by direct attachment of ligand to the active moiety

Murine monoclonal antibodies conjugated to plasminogen activators may be utilized as targeting vectors for the delivery of thrombolytic agents to a thrombus. A thrombus contains fibrin-like and platelet-rich material. Thus, targeting of plasminogen activators may be achieved using either antifibrin or antiplatelet monoclonal antibodies. So many studies have been performed to explore the potential of antibody conjugated plasminogen activators. Some of these studies are discussed in the following paragraphs.

3.2.1.1. Anti-fibrin antibody. Fibrin, the main component of the thrombus, has been considered as a major molecular target for delivery of thrombolytic agents selectively to the thrombus; it is because of the absence of fibrin in blood circulation and in the normal tissue. The fibrin-specificity of thrombolytic agents may be improved by conjugating these with monoclonal antibodies which are fibrin-specific and do not cross-react with fibrinogen (Lijnen and Collen, 1993). Targeting of thrombolytic agents to a thrombus by combining the antigen-binding properties of a fibrin-specific antibody with the catalytic activity of a plasminogen

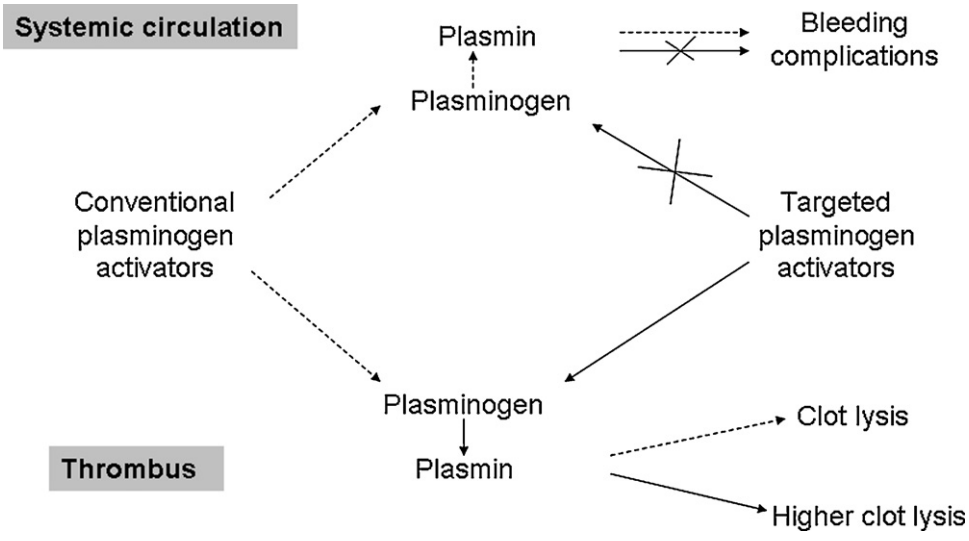

Fig. 1. Schematic diagram showing effects of conventional (dashed lines) and targeted (solid lines) plasminogen activators.

activator in a single molecule has been resulted in significant increase of both their *in vitro* fibrinolytic and *in vivo* thrombolytic potencies. Monoclonal antibodies that have been studied for the purpose include antibodies against the Bβ-chain of fibrin and against cross-linked fibrin fragment D-dimer (Dewerchin and Collen, 1991). The availability of an anti-fibrin monoclonal antibody with an affinity for human fibrin 1000-fold higher than that of tPA might be helpful to improve the thrombolytic efficiency of drug. Bode et al. (1991) developed a complex of fibrin specific antibody (64C5) with either UK or tPA that was found 100 times more efficient than UK and 10 times more efficient than tPA in an *in vitro* fibrinolysis study. Later, they also explored the use of tPA and anti-fibrin monoclonal antibody (59D8) conjugate in an *in vivo* thrombolytic model (Runge et al., 1987). Disulfide-linked tPA-antibody conjugate was synthesized by reacting an N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) derivative of tPA with 2-iminothiolane substituted anti-fibrin monoclonal antibody 59D8. Antibody-tPA conjugate was found 3–10 times more potent than free tPA. Further, a tPA-antibody conjugate was developed using recombinant technology. It was found that the conjugate possessed not only higher thrombolytic activity but also it demonstrated reduced bleeding complications as seen in the case of free tPA (Runge et al., 1996).

3.2.1.2. Anti-platelets antibody. Platelets play a major role in various thromboembolic diseases and have been used as target for the thrombolytic therapy. Haemostatic platelets plug formation is a key event which results from a series of biochemical and cellular process. These processes can be divided in to four categories: adhesion, activation, secretion and aggregation. Membrane glycoprotein receptors are essential for functions of platelets (Langer and Gawaz, 2008; De Meyer et al., 2008). Activation of GP IIb/IIIa receptors is a major and main pathway involved in platelet aggregation. GP IIb/IIIa is a heterodimeric platelet membrane receptor, the major integrin expressed 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 stimulation of platelets by physiologic ligands such as thrombin or collagen (Vyas and Vaidya, 2009). Thus, monoclonal antibodies that recognize epitopes present on the surface of activated platelets, but not on resting platelets, might prove to be effective targeting vector for thrombolytic agents towards platelet-rich clots. GP IIb/IIIa receptors have been explored in the targeted delivery of thrombolytic agents by conjugating monoclonal antibody selective to GP IIb/IIIa with UK (Bode et al., 1991). It was found that platelets directed UK not only accumulated in the thrombus but also have other advantages. Arterial thrombus (platelet rich thrombus) which is resistant to the action of plasminogen activators was dissolved effectively by the use of antibody conjugated UK. Further, it was also observed that the conjugate prevents an early platelet-mediated re-occlusion owing to the presence of anti-GP IIb/IIIa antibody which is clinically used as an antithrombotic agent. Antibodies against other platelet glycoproteins, such as GP IIIa and GP IIb, have also been used for the targeted delivery of plasminogen activators and improved thrombolysis (Dewerchin and Collen, 1991).

3.2.2. Targeted delivery by attaching ligand to the surface of carriers

Site-targeted drug delivery could be of therapeutic importance in the treatment of vascular injury-associated thrombotic and occlusive episodes caused by cardiovascular diseases (*e.g.* atherosclerosis) or interventional procedure (*e.g.* angioplasty and stenting). 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 for the treatment of thromboembolic diseases.

3.2.2.1. Liposomes. Among various drug delivery systems, liposomes have been explored extensively by various research groups for the encapsulations of thrombolytic drugs (Nguyen et al., 1990; Heeremans et al., 1995a; Elbayoumi and Torchilin, 2008; Holt and Gupta, 2010). Liposomes prepared using natural phospholipids are biocompatible. They can entrap practically hydrophilic or hydrophobic drugs into their internal water compartment or into the membrane. The liposomes-incorporated drug remains isolated from the inactivating effect of external conditions, and at the same time it does not cause undesirable side-reactions (Torchilin, 1995). These properties of liposomes make them versatile carriers for the encapsulation of plasminogen activators.

In an *in vitro* experiment, Nguyen et al. (1989) demonstrated ability of liposomes to protect SK in plasma without significant loss of enzyme activity. Plasminogen activators entrapped in liposomes have shown substantial reductions in the time required to restore reperfusion with an efficient digestion of thrombus as compared to freely infused plasminogen activators. One of the various theories proposed to increase the effectiveness of the liposomal plasminogen activators is the plugging of channels by the liposomes which results in the pressure at the leading edge of the thrombus. To prove this theory, Heeremans et al. (1995b) compared the thrombolytic activity of free tPA to free tPA + empty liposomes in a jugular vein model in rabbit. It was found that clot lysis activity of free tPA was nearly equal to that of tPA + empty liposomes with an equivalent dose. The same results were observed by other investigators also. Thus, on the basis of these studies it was concluded that increased thrombolytic activity of liposomes encapsulated plasminogen activator was not due to pressure created by the blocking of the channels in the thrombus. Moreover, it was suggested that liposomal encapsulation reduces the premature inactivation and prevents systemic degradation of enzymes especially in the plasma *vis a vis* releases drug at the site of thrombus by shear stress.

Erdogan et al. (2006) developed three types of vesicular systems, *i.e.*, liposomes, niosomes and sphingosomes and evaluated their relative biodistribution using radiolabelled SK and compared with IV injection of free streptokinase. Results revealed detectable amounts of SK from the thrombus in all three types of vesicles, with higher amount at 4 h than 1 h. Uptake of Tc-99m-labelled SK by the thrombus can be explained by the mechanism of thrombolysis produced by SK. This mechanism involves a series of reactions where SK adsorbs to and penetrates into and around the thrombus; activating plasminogen within the thrombus. This yields sufficient plasmin for fibrin dissolution and thrombolysis.

Recently, Baek et al. (2009) developed liposomes for subconjuctival delivery of SK for the targeting to the thrombus while decreasing the systemic absorption and/or side effect. Results of the study demonstrated lower ocular absorption of SK from liposomal formulation as compared to non-liposomal SK. Additionally, no detectable systemic and ocular side effects were recorded. Liposomal formulations also enhanced the rate of subconjunctival haemorrhage (SH) absorption.

These studies suggested that vesicular carriers, *i.e.*, liposomes could be therapeutically useful carrier for the effective delivery of plasminogen activators targeted to the site of occlusion. However, these carriers accumulated in other non-target sites also. For better therapeutic effects, drug should be localized at the target site subsequently should avoids its non-specific effects to normal non-target tissues. Therefore, a well designed drug delivery system appropriately surface modified with a targeting moiety for delivery of thrombolytic agents to the site of thrombus may provide an effective alternative (Fig. 2). On the basis of these principles, a

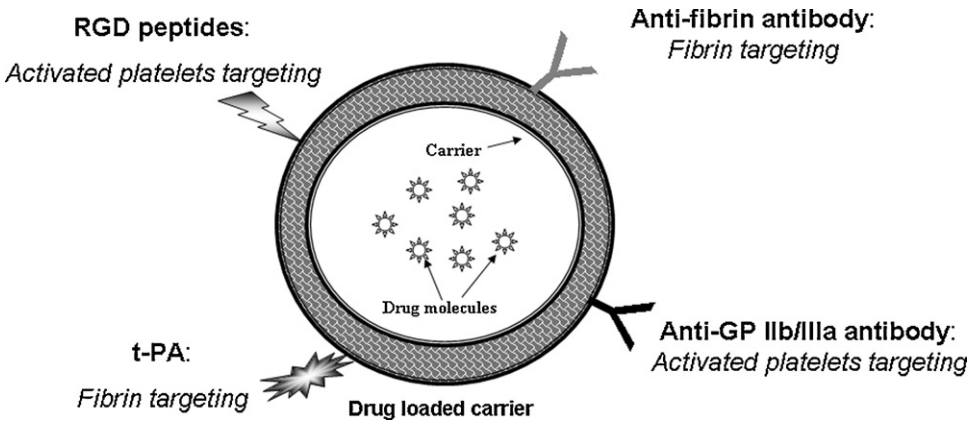

Fig. 2. Schematic thrombus targeted drug delivery system.

specific ligand-receptor based approach has been used by various research groups.

In a recent review (Vyas and Vaidya, 2009), we have discussed the role of integrin receptors in the targeted delivery of thrombolytic agents. To target GP IIb/IIIa integrin receptors expressed on the activated platelets, Arg-Gly-Asp (RGD) peptides have been explored by researchers as a homing device (Gupta et al., 2005). Lestini et al. (2002) developed RGD-modified liposomes for the targeting activated platelets. It was found that RGD-liposomes interact more selectively with the activated platelets as compared to the resting platelets. Thus, it was concluded that RGD-liposomes may be useful for the delivery of thrombolytic agents to the platelets embedded in the thrombus. In other study, Wang et al. (2003) developed RGD peptide conjugated liposomes for the targeted delivery of UK to the site of thrombus and observed an increased thrombolytic efficacy. RGDS conjugated DSPE-PEG_{3500}-COOH was incorporated in to the liposomal bilayer. Thus, hydrophilic PEG imparts long circulation whereas RGD provides selectivity to the carrier system towards activated platelets. It was observed that developed systems could significantly improve thrombolytic efficacy as compared to plain UK liposomes. Suggested mechanism for the improved thrombolysis is the site specific release of UK at the thrombus acting thereby converting plasminogen in to plasmin and simultaneously reducing the bleeding complications. Later, conformationally constrained cyclic RGD peptide conjugated liposomes were developed for yet improved selectivity towards GP IIb/IIIa integrin receptors expressed on the activated platelets (Huang et al., 2008). Recently, we have developed target sensitive (TS) liposomes for the delivery of SK (Vaidya et al., 2011). TS liposomes were prepared using dioleylphosphatidyl ethanolamine (DOPE) and acylated cyclic RGD peptide. It was found that these liposomes not only specifically targeted the blood clot but also released drug instantly after interaction with activated platelets in the clot. *In vitro* clot dissolving study revealed that RGD conjugated TS liposomes could not only reduce the clot lysis time but also increase the total clot dissolution.

3.2.2.2. Polymeric carriers. Although liposomal encapsulation of thrombolytic agents has proven to be an effective method, however such delivery systems suffer a stability problem. To improve the stability, polymeric carriers have now been used for the delivery of various therapeutic proteins. Encapsulation in polymeric carriers avoids protein deactivation during systemic circulation and improves systemic circulation to an acceptable half-life. Leach et al. (2003) developed both types of formulations [liposomal (LESK) and polymeric microcapsules (MESK)] and evaluated and compared for various thrombolytic parameters. It was found that both the formulations could reduce reperfusion times, residual clot mass and improve return of flow compared to identical dosages of free streptokinase in a thrombosed rabbit carotid. The MESK showed comparatively better results. In another experiment, the mechanism for increased thrombolysis by MESK was suggested; MESK resists adsorption to the leading edge of the thrombus, a common limitation for the permeation of free plasminogen activators. Thus, higher thrombolytic activity is due to improved penetration of the MESK to the interior of clot (Leach et al., 2004).

Polymeric carriers for the delivery of thrombolytic agents should possess following properties: (i) they should be small enough to circulate without the risk of vascular occlusion; (ii) they should possess anti-opsonizing properties; (iii) they should selectively accumulate at the site of thrombus; and (iv) they must release thrombolytic agents in sufficient concentration for clot lysis. To confer these properties, Chiellini et al. (2008) synthesized a polymeric material for the development of nanoparticles with targeting as well as long circulatory property. Developed carriers could release fibrinolytic agents in a controlled manner in to the fibrin clots (Piras et al., 2008). Polymer was synthesized by covalently binding poly(ethylene glycol) moieties and monoclonal antibody anti-fibrin Fab fragment to the polymer chain, Poly[(maleic anhydride)-alt-(butylvinyl ether)]s. In another study, microspheres of a FDA approved polymers (polylactide-co-glycolide; PLGA and polylactic acid-polyethylene glycol; PLA-PEG) were developed for the delivery of tPA and found that these carriers could retain 5% (w/w) tPA and released the drug at the site of thrombus resulting in to localized concentration, exceeding 4 μg/ml that is required for the effective clot lysis (Xie et al., 2007).

Moreover, size of the particles is a major concern in systemic drug delivery. Thus, polymeric nanoparticles have been developed for effective delivery of thrombolytic agents to the site of occlusion and subsequently to the interior of the clot. In the case of microparticles it was hypothesized that the permeation of particles in to the clot was due to pressure created at the clot. However, in the absence of hydrodynamic pressure, the pores of fibrin clots are highly resistant to the penetration and permeation of carriers with a size of 1 μm or larger. Therefore, systems should be developed that permeate through the fibrin network into the interior of the clot for intra-clot lysis even in the absence of pressure drop. Furthermore, surface of the particles should be modified to promote the interaction of particles with the components of the clot and to increase the permeation of the particles in to the interior of the clot. Chung et al. (2008) developed tPA loaded PLGA nanoparticles coated with chitosan (CS) and CS-GRGD for electrostatic interaction between positive charge of chitosan and negative charge of fibrin as well as for ligand-receptor interaction between GRGD (Gly-Arg-Gly-Asp) peptide and GP IIb/IIIa receptors expressed on the activated platelets. Thrombolysis study in a blood clot occluded

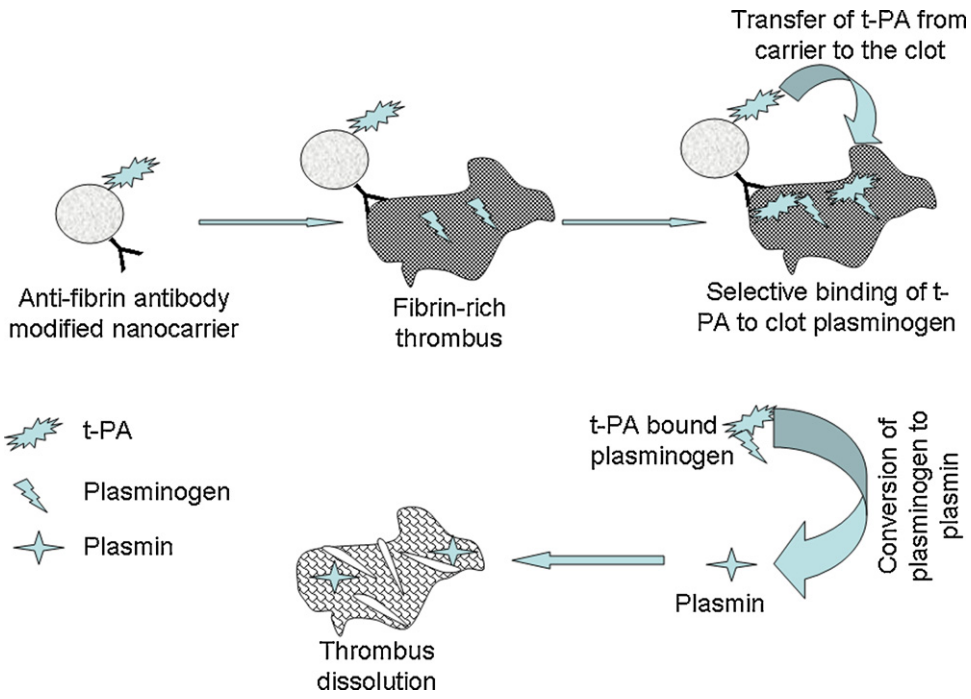

Fig. 3. Schematic mechanism of action of anti-fibrin antibody modified nanocarriers.

tube model revealed that PLGA/CS nanoparticles showed minimum clot lysis time whereas PLGA/CS-GRGD nanoparticles showed the highest weight percentage of digested clots. The permeation study further revealed that PLGA/CS-GRGD nanoparticles were adhered more to the clot front and aggregated in the interior of the clots compared to PLGA/CS and plain PLGA nanoparticles.

As discussed above in the text, anti-fibrin/anti-platelets antibody conjugated plasminogen activators exhibited higher thrombolytic efficacy as compared to their unconjugated versions. However, these approaches were found to be unacceptable for clinical applications because of low stability and heterogeneity of the cross-linked proteins and inhibition of fibrinolytic activity by the conjugated antibody. In recent times, these targeting moieties have also been explored for the delivery of drug loaded nanocarriers at the site of blood clot. Recently, Yurko et al. (2009) developed a nanodevice in which antifibrin antibody and tPA were chemically anchored to the surface of 40 nm polystyrene latex nanoparticles. Earlier reports depicted that enzymes conjugated nanoparticles possess enhanced stability as well as high activity. It was hypothesized that antifibrin antibody transfers the carriers directly to the surface of clot with simultaneous reduction in systemic toxicity of tPA (Fig. 3). Results of the study showed that *in vitro* fibrinolytic activity of antifibrin antibody conjugated nanoparticles was comparable to that of free tPA.

3.2.2.3. Magnetically modulated carriers. An alternative to the above systems, a mono-responsive nanocarrier based system was developed. The carriers could be retained at or guided to the target site by the application of an external magnetic field of appropriate strength (Widder et al., 1983; Widder and Senyei, 1983). Retention of magnetic carrier at target site delays the reticulo-endothelial clearance, facilitates extravasation and thus prolongs the systemic action of drug (Vyas and Khar, 2002). After reaching to the target site, drug can be released from the carrier in an active form or may remain in an immobilized state yet therapeutically active. Up to 60% of an injected dose can be deposited at the site which may release in a controlled manner to the selected non-reticuloendothelial organs (Ranney and Huffaker, 1987). Magnetic targeting has several advantages:

- It achieves therapeutic responses in target organs at only one tenth of the conventional drug dose.
- It offers controlled drug release within target tissues from 30 min to 30 h.
- It avoids acute drug toxicity to endothelium and normal parenchymal cells.
- Adaptable to any part of the body.

Magnetically modulated carrier systems have also been evaluated for the delivery of thrombolytic agents. Various carriers, *i.e.*, resealed erythrocytes, liposomes and microspherse have been used for the loading of magnetite for magnet guided delivery of drug at the target site with the help of an external field. It was evaluated in a dog and rabbits models that IV administration of aspirin loaded magnetite autologous RBCs showed better local prevention of thrombosis when these systems were localized to the clot area using strong SmCO magnet placed externally to the artery where the thrombus was formed (Orekhova et al., 1990). Torchilin et al. (1988) also developed a system in which SK was loaded in dextran coated iron oxide microparticles and evaluated for the targeted thrombolysis in carotid arteries of dogs. Rat embolic model was also used to study the effects of targeted thrombolysis using magnetic nanoparticles (Ma et al., 2007). Recently, Bi et al. (2009) have developed UK conjugated magnetic nanoparticles. In the study, UK was covalently conjugated to the dextran coated iron oxide nanoparticles and thrombolysis study was performed in rat arteriovenous shunt thrombosis model. Results showed that magnetic nanoparticles exhibited 5-fold higher thrombolytic activity as compared to free UK. Moreover, unchanged plasma residual fibrinogen level and little change in bleeding time revealed low systemic toxicity of UK loaded magnetic nanoparticles.

During last decade, biodegradable polymeric carriers have also been used for the loading of magnetic particles and drug molecules and subsequently to trigger the release of active molecules by

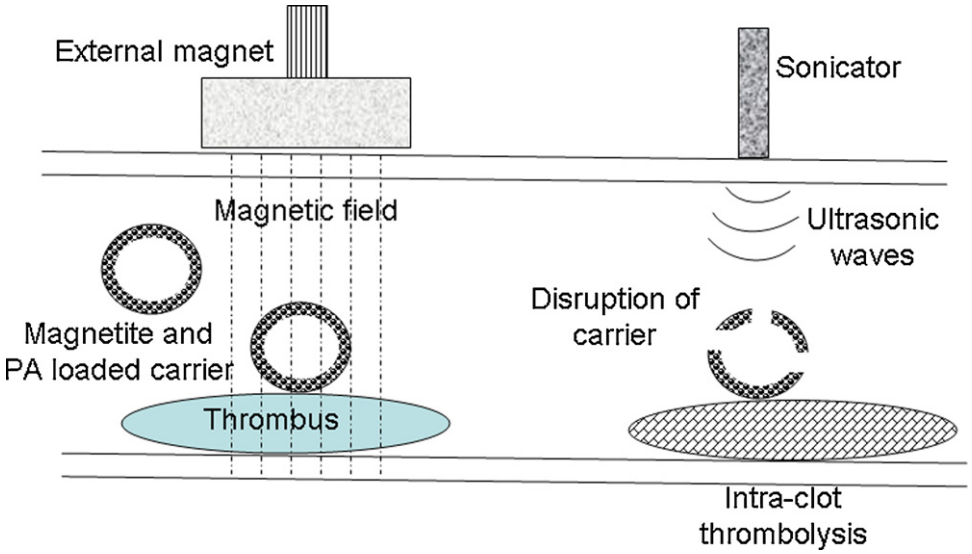

Fig. 4. Combined effects of magnetically guided carrier and ultrasound based drug delivery. By using external magnetic field carriers may be located to the thrombus where by application of sonication waves drug will be released from the carrier for better therapeutic activity.

using external ultrasound (Kaminski et al., 2008). External magnet localizes the carrier(s) to the site of clot where by using external ultrasound burst release of drug may be affected (Fig. 4). In addition to burst release, these systems also utilize thrombolytic potency of sonication. Sonication increased the porosity of the thrombus thus enhanced the penetration of particles in to the interior of the clot whereby drug releases through insonation that enhances intra-clot lysis. Using this technology, Torno et al. (2008) developed a carrier system which was consisted of non-toxic diblock polymer PLA-PEG. tPA and magnetite loaded microspheres of the co-polymer were evaluated for thrombolytic activity. Results of the study revealed that tPA loaded magnetically guided microspheres exhibited 2-fold clot lysis activity and 7-fold reduction in the recanalization time as compared to plain tPA. Further, immunohistochemical studies confirmed the deeper penetration of the tPA in the clot.

3.2.2.4. Ultrasound triggered drug delivery systems: microbubbles. Microbubbles, small gas-filled microspheres, have specific acoustic properties that make them useful as a contrast agent in ultrasound imaging. First-generation microbubbles are room air microspheres; however they disappear rapidly from the systemic circulation (Alter et al., 2009). To improve the stability of microbubbles several attempts have been made, *e.g.* stabilizing microbubbles with a thin shell of albumin (Albunex) or galactose palmitic acid (Levovist) (Dijkmans et al., 2004). However, the microbubbles suffer stability problem as they can not resist arterial pressure gradient. Further improvement in the stability of microbubbles was made using heavy-molecular weight gas (*e.g.* sulphur hexafluoride) in place of room air. Phospholipids have also been used for stabilizing the microbubbles. Sonoveu is an example of phospholipid stabilized microbubbles, which have been used in diagnostic imaging (Sidhu et al., 2006).

During last decade, it has been investigated that ultrasound can be used for the improved thrombolysis. Several studies confirmed the increased clot lysis *in vitro* as well as *in vivo* (Francis, 2001; Culp and McCowan, 2005). It has also been investigated that concomitant use of thrombolytic agents with ultrasound augments the fibrinolytic activity of the thrombolytic agents (Everbach and Francis, 2000; Tsivgoulis et al., 2008). It was found that thrombolytic activity increases from 30% to 80%. The increased fibrinolytic activity might be due to better penetration of drug in to the interior of clot assisted by a combination of various mechanisms such as pumping effect, improved diffusion, cleavage of fibrin polymers resulting in to an increased the surface area for thrombolytic action, and increased binding of tPA to fibrin (Culp and McCowan, 2005).

Ultrasound can also generate cavitation, which can cause large molecules and particles to penetrate cells (sonoporation); this property is actively being explored for drug and gene delivery (Tachibana and Tachibana, 2001). Addition of a contrast agent as capitation nuclei can lower the threshold for these ultrasound bioeffects (Tiukinhoy-Laing et al., 2007). Microbubbles reportedly increase the permeability of the cell membrane on simultaneous use of ultrasound (Fig. 5). Various mechanisms for the increased permeability have been proposed and discussed in detail in a recent review by Dijkmans et al. (2004).

Microbubbles have now been proposed as a new vehicle for the delivery of drugs and genes (Li et al., 2003; Lu et al., 2003; Mayer and Bekeredjian, 2008). In several studies, these have also been evaluated for the delivery of thrombolytic agents. The proposed mechanism was that after insonation, microbubbles destroy and increase the permeability of the clot resulting in an increased penetration of drug in to the interior of the clot. Thus, improved clot lysis was obtained. Drug loading in the microbubbles can be affected by using different ways: (i) drug incorporation in the core, (ii) drug incorporation or intercalation in the wall of the microbubbles, (iii) covalent attachment of the drug at the surface of membrane, and (iv) attachment of the drug to a ligand appended on the bubble surface (Hernot and Klibanov, 2008).

To further improve the fibrinolytic activity of microbubbles and ultrasound, microbubbles have been modified using clot selective targeting moieties. As discussed earlier in the text, carriers can be modified to target either fibrin network or platelets glycoprotein receptors. Hence, antibody/antibody fragments and any ligand which has affinity to the clot can be attached at the surface of microbubbles. In one study Fab fragments of humanized antibody against glycoprotein IIb/IIIa receptor (Abciximab) was attached to the surface of microbubbles and evaluated for their ability to target the clot (Martina et al., 2008). Results revealed that Abciximab modified microbubbles accumulated in higher concentration in to the clot as compared to non-targeted microbubbles. The study confirmed the targeting ability of modified microbubbles and suggested microbubbles as promising carriers for the site directed delivery of thrombolytic agents (Alonso et al., 2009). In other study, RGDS peptide has been conjugated on to the surface

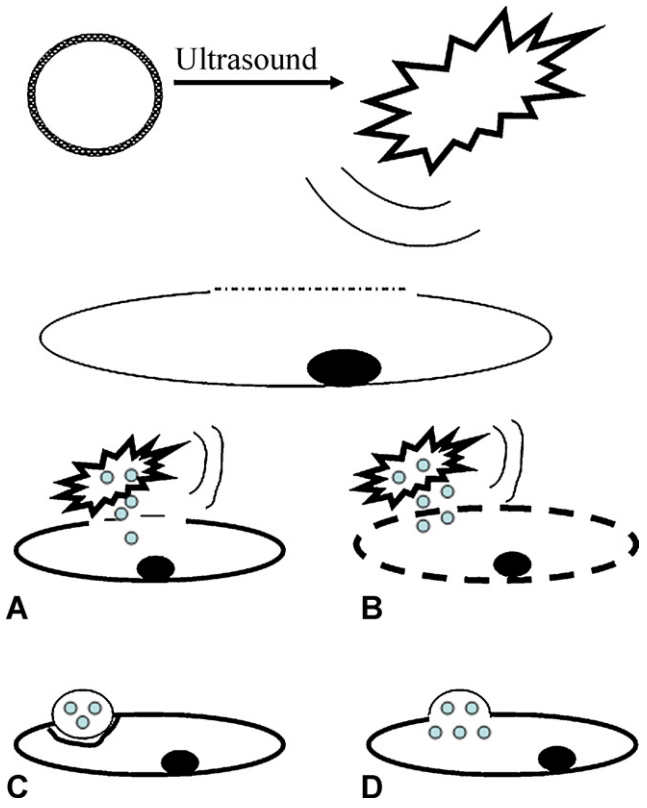

Fig. 5. Destruction of microbubbles by ultrasound resulting in increased membrane permeability by shear stress, temperature rise and activation of reactive oxygen species. Drug delivery by microbubbles by: (A) transient holes induced by shear stress; (B) increase in membrane fluidity; (C) endocytosis of microbubbles; (D) fusion of the microbubble membrane with the cell membrane.
From Dijkmans et al. (2004).

of microbubbles to impart clot selectivity (Mu et al., 2009). UK was loaded as a thrombolytic agent. It was found that these carriers have thrombolytic ability *in vitro*. Further, *in vivo* study demonstrated that after IV administration these carriers aggregated on the surface of arterial thrombus.

Another ultrasound based carrier developed known to as Echogenic liposomes (ELIP) has been reported. They are phospholipid vesicles filled with gas and fluids. These vesicles have the potential to be used for ultrasound-enhanced thrombolysis in the treatment of various cardiovascular pathologies including acute ischaemic stroke, myocardial infarction, deep vein thrombosis or pulmonary embolism (Smith et al., 2010). tPA loaded ELIP revealed higher thrombolytic activity as compared to plain tPA solution with or without application of ultrasound (Tiukinhoy-Laing et al., 2007; Shaw et al., 2009). Various modifications have also been incorporated and evaluated for improved thrombolytic activity of these vesicles. It has already been reported that tPA possesses higher affinity to the fibrin thus tPA was conjugated to the surface of ELIP in order to target the fibrin rich thrombus for better activity of liposomes (Tiukinhoy-Laing, 2007; Klegerman et al., 2008). Fibrin binding of ELIP-associated tPA was found 2 times higher than that of free tPA. This strong affinity for fibrin was confirmed using echogenicity analysis of porcine clots *in vitro*.

3.2.3. Targeted delivery of thrombolytic agents for thromboprophylaxis

Plasminogen activators are most commonly used for the dissolution of nascent thrombi and minimizing ischaemia reperfusion injury in patients at high risk of imminent thrombosis. However, these agents are not suitable for thromboprophylaxis due to unfavourable pharmacokinetic and toxicity. These limitations of PAs might be circumvented through the use of more targeted approaches.

Targeting fibrin and activated platelets promotes the delivery of antithrombotic agents to the existing blood clots. However, such clot-targeting strategies are unlikely to be useful for thromboprophylaxis. Targeted delivery of antithrombotic drugs to the vascular lumen prior to the clot formation may permit thromboprophylaxis in patients with a high risk for thrombosis (Murciano et al., 2003b).

Targeted delivery of antithrombotic enzymes to the endothelium has been achieved in various animal models. It may provide local thromboprophylaxis in patients with high risk of thrombosis. Thus, clot extension may be prevented. Recent studies showed that angiotensin-converting enzyme (ACE) and especially intercellular adhesion molecule (ICAM) and platelet endothelial cell adhesion molecule (PECAM) are potential site directing anchors for targeting antithrombotic drugs to endothelium because endothelial cells expressing these receptors are actively involved in the process of thrombosis (Carnemolla et al., 2010). Further, endothelium as such does not internalize anti-PECAM and anti-ICAM; allowing antithrombotic activity to retain and maintain on the luminal surface. Different studies showed that drugs coupled to PECAM-1 and ICAM-1 antibody selectively bind to the endothelium and exert therapeutic effects *in vivo* (Ding et al., 2005, 2008). For example, tPA chemically conjugated with anti-ACE retained fibrinolytic and antigen-binding activities and also exhibited sustained and uniquely preferential accumulation in rat pulmonary vasculature. After IV injection in rats, pulmonary uptake of anti-ICAM/tPA conjugates was estimated two orders of magnitude higher than control IgG/tPA, with an enhanced fibrinolysis of subsequent pulmonary emboli. Further, these conjugates could also deliver drugs to the endothelium of other organs, such as cardiac or cerebral vasculature, through infusion to their respective afferent arteries (Scherpereel et al., 2002).

Collagen receptors which are exposed after vessel injuries and help in the activation of coagulation cascade may also be used as a molecular target. It has been reported that anti collagen antibody can serve as vectors for drug targeting to the site of vessels injuries. Muzykantov et al. (1986) developed a complex, anti collagen antibody-erythrocytes-streptokinase and evaluated for its activity. Results showed that targeting of such erythrocytes led to local lysis of fibrin clot located in the target zone. Thus, the study open ups the possibility of using such strategy for the prevention of re-occlusion especially during surgical manipulations on vessels.

Other approach for altering pharmacokinetics and therapeutic activity of plasminogen activators is based on the coupling of these agents to a large biocompatible carrier, such as red blood cells (RBCs). This approach provides a safe and effective thrombolytic prophylaxis (Murciano et al., 2003a). RBCs have been potentially used as a carrier for intravascular drug delivery particularly of the drugs which have their action in the blood owing to their longer circulation half-life (Murciano et al., 2009). Recent animal studies demonstrated that coupling of tPA to RBCs makes it a therapeutically effective and safe thromboprophylactic agent (Danielyan et al., 2008). It has been demonstrated that RBC-bound tPA (RBC/tPA) does not permeate or dissolve post-surgical clots, even those formed just 10 min prior to administration (Murciano et al., 2003a). However, due to longer circulation time, it gets incorporated in to intravascular clots if formed hours after RBC/tPA administration, thus prevents vascular occlusion (Ganguly et al., 2005).

Ex vivo conjugation of tPA to isolated RBCs followed by re-injection of the RBC/tPA complexes may be applicable to the conditions in which the risk of thrombosis is anticipated. However, phlebotomy, *ex vivo* loading, and re-infusion of the modified RBCs are impractical in most settings where the use of such

therapy could be envisioned (Zaitsev et al., 2006). Thus, it has been hypothesized that targeting plasminogen activators directly to the circulating RBCs would significantly improve the efficacy and utility of this approach without compromising the survival of RBCs. It has been reported earlier that complement receptor type 1 (CR1) receptors are expressed on the surface of RBCs thus might be used as a target site for the delivery of PAs. In an experiment Zaitsev et al. (2006) tested the hypothesis and found that anti-CR1/tPA conjugate effectively target CR1 positive RBCs and load fibrinolytic agents to the RBCs. The study was performed in transgenic mice expressing human CR1 on their RBCs (TgN-hCR1). Results of the study suggested that by using this strategy rapid dissolution of subsequently formed pulmonary emboli may be affected. Thus, development of occlusive arterial thrombi may be prevented.

4. Concluding remarks and future prospects

Thrombolytic drugs (plasminogen activators) play a critical role in the treatment of various cardiovascular diseases including acute myocardial infarction, pulmonary embolism, deep vein thrombosis, arterial thrombosis and peripheral vascular thromboembolism. The problems with plasminogen activators are short half-life and immunogenicity because of their foreign nature. By encapsulating proteins within the novel carrier systems, an increased half-life and decreased immunogenicity might be obtained. Further, targeted delivery of thrombolytic agents may reduce the risks of haemorrhage and toxicity associated with systemic administration. Thus, it may offer a promising, minimally invasive approach that could control and treat the thrombosis.

Among various carriers discussed above, liposomes may be considered as a versatile carrier for the use. These carriers may be constructed by using simple and easy processes and can be easily modified for better encapsulation efficiency and targeted delivery to the specific site. It has been demonstrated by various studies that liposomes could convincingly improve the serum stability of plasminogen activators which in turn increases the half-life. At the same time liposomes retained the activity of encapsulated drug. However, vesicular carriers suffer from stability problem. Therefore, selection of lipid and other components of the structures are critical parameters considered for the stability of carriers in the blood circulation as well as on storage for longer period of time. Other carriers discussed above are also equally effective. Magnet guided carriers have the advantages of easy localization to the site of occlusion with the help of external magnet. Thus, without surface modification of the carriers they may be accumulated at the site of action.

For better and instant action drug should be released in the vicinity of thrombus at a desirable therapeutic concentration. Therefore, in the designing of novel carriers or in the encapsulation of plasminogen activators, release behaviour of the carriers should be optimized for better therapeutic action.

Nowadays, uses of strategies which have dual property of imaging and therapeutic action (theranostics) are considered to be better option for the treatment of various diseases. This approach may also be used in the case of vascular diseases. As discussed above, microbubbles based contrast agents have already been evaluated by various researchers for the imaging of thrombus area by decorating the surface of microbubbles with molecules having affinity towards the components of thrombus, *i.e.*, fibrin and GP IIb/IIIa receptors expressed on activated platelets. These carriers might also be useful for the encapsulation and release of thrombolytic agents specifically in the vicinity of thrombus. Thus, carriers help drug-carrier localization in the vicinity of thrombus where drug may continue to release in response to the external stimuli, such as ultrasound. Furthermore, by targeting of the thrombus using ligand–receptor mediated targeting, thrombolytic activity might be increased with reduced chances of haemorrhage. Thus, it can be concluded that novel carrier based targeted delivery of thrombolytic agents might be a pragmatic approach for the treatment of life-threatening thromboembolism.

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