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Review article

Tissue plasminogen activator-based clot busting: Controlled delivery approaches

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ABSTRACT

Cardiovascular diseases are the leading cause of death worldwide. Thrombosis, the formation of blood clot (thrombus) in the circulatory system obstructing the blood flow, is one of the main causes behind various ischemic arterial syndromes such as ischemic stroke and myocardial infarction, as well as vein syndromes such as deep vein thrombosis, and consequently, pulmonary emboli. Several thrombolytic agents have been developed for treating thrombosis, the most common being tissue plasminogen activator (tPA), administrated systemically or locally via IV infusion directly proximal to the thrombus, with the aim of restoring and improving the blood flow. TPA triggers the dissolution of thrombi by inducing the conversion of plasminogen to protease plasmin followed by fibrin digestion that eventually leads to clot lysis. Although tPA provides powerful thrombolytic activity, it has many shortcomings, including poor pharmacokinetic profiles, impairment of the reestablishment of normal coronary flow, and impairment of hemostasis, leading to life-threatening bleeding consequences. The bleeding consequence is ascribed to the ability of tPA to circulate throughout the body and therefore can lysis all blood clots in the circulation system, even the good ones that prevent the bleeding and promote injury repair. This review provides an overview of the different delivery approaches for tPA including: liposomes, ultrasound-triggered thrombolysis, anti-fibrin antibody-targeted tPA, camouflaged-tPA, tpA-loaded microcarriers, and nano-modulated delivery approaches.

Keywords: thrombus, tissue plasminogen activator, controlled delivery, clot busting

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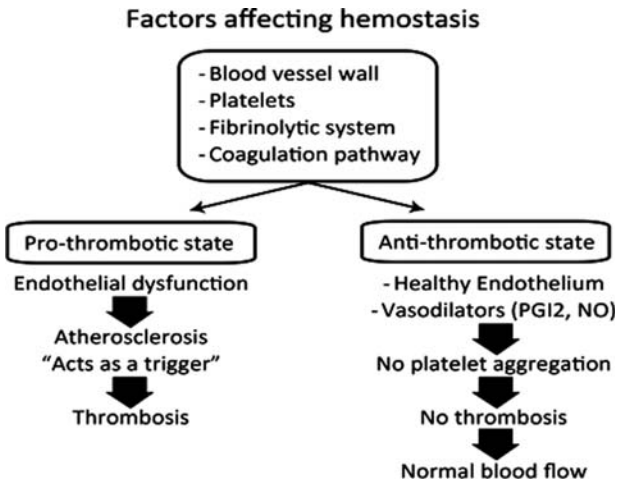

Figure 1. Factors affecting hemostasis.

1. INTRODUCTION

Hemostasis is a multifactorial state that ensures efficient blood flow through peripheral vascular districts. It is affected by the characteristics of blood vessel walls, platelets, the fibrinolytic system, and the coagulation pathway, which are all intimately related (Figure 1). All these factors function normally to produce an equilibrium between antithrombotic and prothrombotic factors.[1,2] Any misbalance in this equilibrium will lead to thrombosis: the formation of a blood clot inside a vessel, causing its occlusion or stenosis, and leading to various clinical presentations, depending on the occluded vessel. For instance, cerebrovascular thrombosis will lead to stroke and coronary artery thrombosis will lead to ischemic heart diseases (angina or myocardial infarction).

Thrombosis takes place in two stages, primary hemostasis, and secondary hemostasis (Figure 2).[3] Primary hemostasis is initiated by platelets' adherence to the damaged vascular endothelium, in a complex involving multiple platelet membrane receptors, to form a platelet plug.[4] Secondary hemostasis includes the activation of the coagulation system, which finally leads to conversion of fibrinogen into fibrin to form a hemostatic clot.[5] Platelet adhesion to the endothelium via the GP Ib

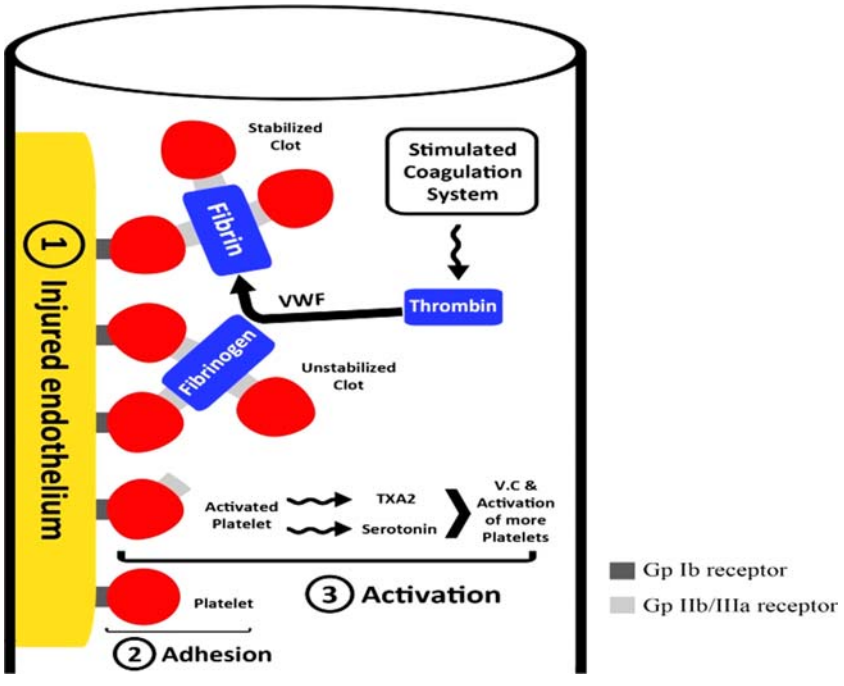

Figure 2. An illustration of the stages of thrombosis.

receptor and von Willebrand factor (VWF) is followed by platelet activation (in the form of shape changes in the platelet, release of thromboxane A_2, serotonin and other agents, and expression of GP IIb/ IIIa receptors on platelets' surface). The final step is binding of fibrinogen and VWF to the activated GP IIb/ IIIa receptors of two platelets, creating a growing aggregate.[3,6]

As the primary and secondary hemostasis phases are dynamically interactive, the plasma coagulation system is activated at the time of formation of the platelet plug. Coagulation is often been represented as two independent pathways (extrinsic and intrinsic) that converge into a single interrelated system or pathway, with thrombin generation as the end point of the cascade.[3,7] Thrombin converts fibrinogen into fibrin, and it also activates factor XIII, leading to stabilization of the fibrin clot. In addition, it is a potent stimulant of platelet aggregation.[8] As a result, thrombin generation is a key goal in the management of thrombosis.

In spite of the relatively large number of available drugs, tissue plasminogen activator (tPA) is still the main and the primary thrombolytic agent used in the treatment of established thrombus in myocardial infarction (MI) and pulmonary embolism.[9–11] Tissue plasminogen factor is one of the physiological plasminogen activators which can be used clinically. This family also includes streptokinase (SK) and urokinase (UK). These agents catalyze the hydrolysis of plasminogen at the Arg561–Val562 bond, which results in the formation of active plasmin. The plasmin then acts by degrading the insoluble fibrin clot, which forms the nucleus of the thrombus.[12–14] However, tPA has a short half-life (< 5 min), which necessitates its administration in a large dose (1 mg/kg) to have the desired effect. This can lead to the degradation of clotting factors and hemorrhage.[15,16] Although a lower bolus dose can be used, followed by prolonged infusion, it is not the ideal manner in which to optimize the benefit of, and minimize side effects from, tPA.

In order to minimize the risk of bleeding, many strategies have been developed to offer local thrombolytic action of tPA, or in other words, targeting of tPA. In this review, we are going to discuss the most common delivery approaches of tPA such as liposomes, ultrasound-triggered thrombolysis, anti-fibrin antibody-targeted tPA, camouflaged-tPA, tpA-loaded microcarriers, and nano-modulated delivery approaches.

2. CONTROLLED DELIVERY APPROACHES FOR TPA

2.1. Liposomes

Liposomes are vesicular drug delivery systems that consist of lipid bilayer arrays.[17,18] Each lipid layer consists of two parts, hydrophilic and hydrophobic. The hydrophilic parts are directed towards the aqueous phase, while the hydrophobic segments are directed towards each other.[19] This structure gives liposomes the opportunity to encapsulate both water-soluble and water-insoluble bioactive materials.[20] Liposomes are highly biocompatible with low immunogenicity,[21] and they are also characterized by their efficient encapsulation of drugs. This separation from external conditions leads to a decrease in side effects resulting from nonspecific actions, such as bleeding in the case of plasminogen activators.[22]

Liposomes have been investigated as a drug delivery system for tPA (Figure 3).[23–25] Heeremans and his team have proved that tPA-loaded liposomes have higher anti-thrombolytic effect as compared to the free tPA.[24] The main drawback of liposomes, however, is their relatively rapid rate of clearance from the circulatory system, due to phagocytosis by the reticuloendothelial system (RES), resulting in their short half-life.[26]

This shortcoming has been overcome via chemical modifications of the surface of the liposome with substances such as polyethylene glycol (PEG) in a process called PEGylation, leading to a significant decrease in the uptake of liposomes by RES.[27,28]

PEG has several advantages, including high water solubility and low cytotoxicity. In addition, the PEGylated-liposomes or vesicles demonstrate high stability due to steric repulsion, which prevents the fusion and disruption of the vesicles.[29] Moreover, liposomal modification with PEG widens the applicability of liposomes by enhancing their circulation time, in addition to improving their targeting capabilities.[30]

Ji-Young Kim et al. for instance, used PEGylated liposomes to prolong circulation of tPA.[25] In their study, encapsulation of tPA into conventional liposomes (EPCL) and PEGylated-liposomes (EPC-PEGL) prolonged the half-life of tPA by 16- and 21-fold, respectively, compared with free tPA. The half-life of free tPA was prolonged from about 5.87 minutes in the terminal phase, to 50.03 min and 132.62 min for EPCL and EPC-PEGL, respectively.

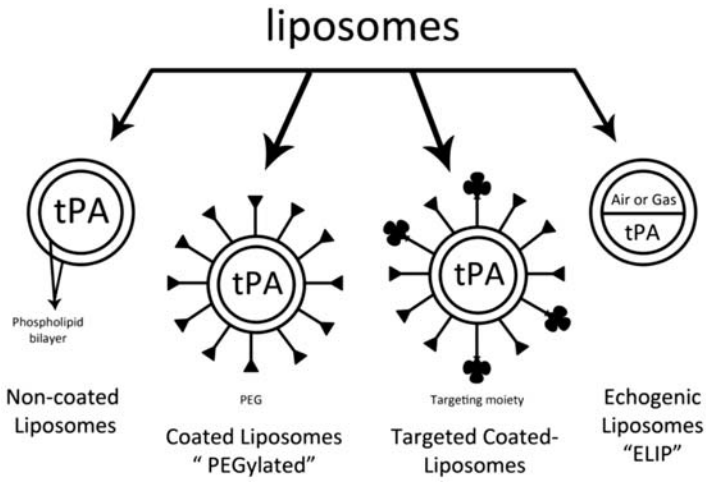

Figure 3. Different investigated types of liposomes-loaded tPA.

In another study,[31] Absar and his co-workers encapsulated tPA into PEGylated and non-PEGylated liposomes decorated with the peptide sequence (CQQHHLGGAKQAGDV) of fibrinogen gamma-chain that has the affinity to bind with the GPIIb/IIIa receptors expressed on activated platelets. This decoration enhanced the liposomal affinity to bind with activated platelets. Consequently, the half-life of tPA has extended from 7 minutes (for free tPA) to 103 and 141 minutes in the case of non-PEGylated and PEGylated liposomes, respectively.

Another advantage of liposomes is the ability to encapsulate gas and fluid to form an echogenic liposome (ELIP). These echogenic liposomes have been utilized as ultrasound contrast agents to assist in ultrasound-enhanced thrombolysis.[32]

2.2. Ultrasound-enhanced thrombolysis

Ultrasound has been found beneficial in thrombolysis since the 1970s,[33,34] and can be used either alone,[35–37] or as the trigger component of a drug delivery system for tPA.[38–40]

Francis[41] has proposed that ultrasound can enhance thrombolysis via two different approaches, mechanical fragmentation of the clot or by enhancing enzymatic fibrinolysis by increasing the enzyme transport to thrombus via static[42] and perfusion[43] systems.

Recently, Everbach and Francis attributed the effect of ultrasound to the growth and/ or collapse of micro-bubbles within the clot, followed by the occurrence of two pathways. In the first pathway, the formed bubbles are enlarged and their diameters exceed that of the pores of the fibrin lattice surrounding them, leading to stretching of the clot fibers. In the other pathway, the bubbles may collapse in a violent way, producing acoustic emissions and inertial cavitations, and consequently altering the structure of clot fibers. Both pathways lead to same result; producing new binding sites for the fibrinolytic enzyme, in addition to a stirring force produced by micro-streaming around the bubbles, which increases the chance of the fibrinolytic enzyme coming in contact with the fibrin strands.[44]

This assumption was in agreement the suggestion of Nyborg and Ziskin in 1985, where they attributed the action of ultrasound to three main mechanisms: acoustic streaming, cavitation and a thermal effect.[45] To explain the process more clearly, Alexandrov stated that *"if you put sugar in a water cup, the sugar promptly goes down to the bottom, and it will take some time for it to dissolve completely due to absence of water motion. Upon stirring water with a spoon, sugar dissolves much faster. In a similar fashion, the enzyme spends a long time to reach the goal because of the stagnant flow near occlusion"*.[46]

In spite of the various advantages of ultrasound, using it alone has some drawbacks. For instance, it causes vessel wall damage[41] and high intensity ultrasound can break the clot into smaller particles, causing embolization.[41,47]

In addition, it was discovered, based on both *in-vitro*[48] and *in-vivo*[49] studies, that ultrasound-induced thrombolysis may cause activation of platelets, leading to re-occlusion. As a result, ultrasound use has been restricted as a trigger for releasing tPA.

Kudo and his group were the first to report using ultrasound as a non-invasive approach to increase the efficiency of systemic tPA.[50–52] With the aid of a canine model, they found that continuous application of transcutaneous ultrasound at frequency of 200 kHz enhanced tPA-induced thrombolysis.[50–52] Many subsequent experimental studies, either using ultrasound with tPA alone[53,54] or, more recently, in the presence of various contrast agents,[39,40,55,56] have shown that ultrasound increased the efficiency of tPA.

Based on the promising results of these experimental studies,[53–56] a number of clinical studies have been carried out which demonstrated the enhanced efficacy of ultrasound-based thrombolysis in stroke patients.[57–60]

Alexandrov and his team used diagnostic ultrasound to improve thrombolysis by tPA.[57,58] They used a 2-MHz transcranial Doppler (TCD) in patients with acute ischemic stroke due to occlusion of middle cerebral artery (MCA), and the TCD was applied in a continuous manner. They observed that patients monitored with TCD during clinical-course treatment with systemic tPA revealed early recanalization and dramatic recovery.[57] Afterwards, this observation was tested through a multicenter clinical trial, CLOTBUST (Combined Lysis of Thrombus in Brain ischemia using transcranial Ultrasound and Systemic tPA).[58] The study involved two groups of patients, each group consisting of 63 patients, where the target group received continuous ultrasound (tPA + TCD) and the control group received placebo (tPA alone). The results showed that 83% of the target group showed recanalization (46% complete and 27% partial) versus 50% (17% complete and 33% partial) for the control group. In addition, 3.8 % of both groups demonstrated symptomatic intracerebral hemorrhage.[58] As a result, it was postulated, by Alexandrov et al. that continuous monitoring by TCD might have augmented the thrombolytic effect of tPA by exposing more clot surface to tPA. Exposing more surface of the clot surface was attributed to pressure gradients formed at the clot site by the ultrasonic energy emitted from TCD, where this pressure leads to tPA molecules being forced and lodged into the clot.[57]

In another clinical trial, Eggers et al.[60] used diagnostic ultrasound to improve the thrombolytic effect of tPA. A total of 25 patients were used in the study; a target group of 11 patients received duplex monitoring and tPA, while the control group, consisting of 14 patients, received tPA alone. The results showed better recanalization and neurological outcome after 3 months in the target group. However, the intracerebral hemorrhage rate was higher in the target group. These results were not considered sufficient to judge the effect of transcranial duplex due to the small number of patients investigated in the study.[60]

In another clinical trial using therapeutic low-frequency ultrasound, Daffertshofer et al.[61] found that low-frequency (300 KHz) ultrasound led to a considerable increase in the rate of symptomatic intracerebral hemorrhage, up to 36%, and consequently, the TRUMBI trial (TRanscranial low-frequency Ultrasound-Mediated thrombolysis in Brain Ischemia) was terminated. As a conclusion, and surprisingly, it has been found that lower ultrasound frequencies (in kilohertz) are causing higher rates of intracerebral hemorrhage,[61] while, the diagnostic frequencies (in megahertz) did not and are safe enough to be used in humans.[58,60]

For further improvement of ultrasound enhanced-thrombolysis, the concept of using contrast agents for better imaging and delivery of thrombolytic agents was tested. Micro-bubbles and echogenic liposomes were two principle examples of these utilized contrast agents.

2.3. Micro-bubbles

Micro-bubbles (MBs) are tiny gas- or air-filled microspheres and were first used as contrast agents for imaging due to their acoustic characteristics.[62–64] In diagnostic ultrasonograpghy, MBs create acoustic impedance that is higher than that of red blood cells,[65] giving them the ability to send stronger echoes and leading to better reflection. The mechanism by which the MBs enhanced ultrasound-accelerated thrombolysis was attributed to stable and inertial cavitation as these MBs act as nuclei for cavitation decreasing the amount of energy required for the cavitation.[66,67] Stable cavitation leads to oscillations of MBs, resulting in micro-streaming, and therefore, erosion of clot surface which enhances the penetration of the clot by fibrinolytic enzymes.[44] Inertial cavitation is induced by increasing the acoustic power on MBs, leading to an explosion which emits the absorbed energy.[68,69] The effect of MBs on thrombolysis depends on many factors, such as bubble size, concentration of the MBs in the clot area, and the stability of the bubbles in blood stream.[67] Being air-filled and encapsulated by a weak shell, this leads the first generation of MBs to be cleared rapidly from systemic circulation due to their low stability. In addition, their relatively large size reduced their ability to cross into lung

circulation and get into the thrombus.[70,71] As a result, a second generation of MBs was introduced by filling the MBs with a high molecular weight gas in addition to encapsulation of the MBs by phospholipids[72] – galactose in the case of levovist, or thin shell albumin in the case of Albunex.[67,68]

Before being introduced to the clinical trials, the effect of MBs on enhancing ultrasound-based thrombolysis was tested by many experimental studies.[73–75] In one of these clinical trials, Molina et al.[67] evaluated 111 patients with acute stroke caused by MCA occlusion. Patients were divided into 3 groups. The first group included 38 patients who received tPA plus continuous two-hour TCD monitoring plus galactose-based MBs (tPA + US + MBs). The second group included 37 patients who received tPA with continuous two-hour TCD monitoring (tPA + US). Finally, the third group consisted of 36 patients receiving tPA with placebo monitoring (tPA alone). It was found that complete recanalization was significantly higher in the first group (54.5%) than in the second (40.8%) and third (23.9%) groups. Furthermore, 2.6 % of the first group, 2.7% of the second, and 5.5% of the third group showed symptomatic intracranial hemorrhage. These results were in agreement with the results obtained by Viguier and his group.[73]

In another pilot study, Alexandrov et al. tested perflutren-lipid microspheres in patients with acute ischemic stroke.[74] Fifteen patients were divided into two groups; target group (12 patients), and a control group (3 patients). The target group received tPA with perflutren-lipid microspheres in addition to two-hour continuous TCD monitoring (tPA + MBs + TCD), while the control group received tPA with monitoring by TCD only (tPA + TCD). The results demonstrated that within 2 hours after tPA bolus, 50% of the target group showed complete recanalization (6/12 patients), and 33% showed partial recanalization (4/12) while 2 patients showed no recanalization (17%). No patients in the control group showed complete recanalization.

Both studies by Alexandrov et al.[74] and Molina et al.[67] are considered as an extension for the CLOTBUST study,[58] introducing the concept of testing contrast agents in the form of MBs. Table 1 summarizes and compares the results of CLOTBUST[58] with Molina's study,[67] and Alexandrov's study.[74]

The second example of contrast agents is echogenic liposomes (ELIP). Echogenic liposomes are multifunctional liposomes, phospholipid-bilayer encapsulated vesicles, which can be used as contrast agents for sonography, and at the same time, as a drug delivery system.[19,75]

On encapsulating a gas in a liposome, the gas acts as a hydrophobic drug and stays trapped between the two monolayers of the lipid-bilayer of the liposome.[76] The overall entrapment efficiency tPA into the liposomes was about 50%. Of that 50%, around 35% of the loaded tPA were associated with the lipid bilayer and only 15% were encapsulated within the liposome.[77] Therefore, the term 'tPA-loaded echogenic liposome' refers to the total of the tPA associated with the lipid shell, in addition to the part encapsulated in the aqueous phase.[23]

The exposure of the liposomes to ultrasound induces the disruption of the lipid shell and hence the release of the drug.[19] As a result, ELIP loaded with tPA (t-ELIP) acts as a targeted drug delivery system by increasing the concentration of tPA in the area of the thrombus, leading to a decrease in the required high systemic dose of tPA, and consequently lowers the possibilities of hemorrhage. The gas encapsulated into t-ELIP will exert a cavitation-related mechanism, as explained earlier, leading to more lytic effect against the thrombus.[78–80] In a study aimed at evaluation of the effect of exposing tPA-loaded ELIP to ultrasound in thrombolysis, Laing et al. found that ELIP-induced thrombolysis improved by 49.5% when ultrasound was added to the treatment protocol.[77] This was in agreement with what Shaw and his team found when they compared the thrombolytic efficacy of tPA alone with that of tPA with ultrasound (120 kHz), and t-ELIP with t-ELIP exposed to ultrasound.[81] It was found that ultrasound enhanced the efficacy of both tPA (from 31% alone to 71% with ultrasound) and t-ELIP (from 48% alone to 89% with ultrasound) and the study confirmed that t-ELIP can be used as an effective delivery system for tPA.[81]

In conclusion, contrast agents can enhance ultrasound-induced thrombolysis. Further modifications of MBs and echogenic liposomes, as well as the trials of targeting MBs[82,83] and echogenic liposomes,[84] may lead to the introduction of new efficient thrombolytic strategies that could be applied at the clinical level.

2.4. Anti-fibrin antibody targeting method

One of the main strategies of targeting tPA, or enhancing the local effect of tPA on thrombus, is the use of anti-fibrin antibody targeting.[85,86] Yang and his co-workers designed an approach[87–90] and called it "ATTEMPTS" (Antibody Targeted Triggered Electrically Modified Prodrug Type Strategy). It was designed

Table 1. A summary of the progress of ultrasound-enhanced thrombolysis through using micro-bubbles (MBs), and comparing the studies outcome including both recanalization and symptomatic intracerebral hemorrhage (SICH).

	CLOTBUST[58]		Molina's study[67]			Alexandrov's study[74]	
Number of subjects	Target group 63	Control group 63	Target group 38	Control group 73		Target group 12	Control group 3
Treatment	tPA + TCD	tPA alone	tPA + TCD + MBs	tPA + TCD	tPA	tPA + TCD + MBs	tPA + TCD
Complete recanalization after 2 hrs from tPA administration	29/63 (46%)	11/63(18%)	54.5%	40.8%	23.9%	6/12 (50%)	0%
SICH; In the active treatment group (target)	3.8%		2.6%			0%	

to deliver tPA to the clot site in an inactive form, followed by triggering its activation locally, and consequently reducing the risk of bleeding.

This system depends on tight reversible electrostatic interaction between the targeting moiety in the form of an anionic heparin molecule (Hep) conjugated to an anti-fibrin antibody (Ab), and the drug moiety in the form of a tPA molecule decorated by a cationic peptide.

The antibody plays a vital role in targeting the system to the site of thrombus. The heparin-antibody conjugate blocks the active site of tPA, inhibiting its anti-fibrinolytic effect. Then, to activate this system, protamine sulfate is administered as an antagonist for heparin and triggers the dissociation of the Hep-antibody complex, resulting in the release of tPA from the inhibitory action of this complex.

This approach was tested experimentally *in-vitro* and *in-vivo*, and did not elicit any considerable degradation of the coagulation factors when compared to free tPA.[87,91]

In 2005, Yang and his group presented a new strategy to overcome the problem of random results of modification tPA with cationic peptide.[90] They used a genetic engineering approach of site-directed mutagenesis to develop an inherent region with surface expressing positive charges to trigger binding with heparin.[90] This led to a safe strategy for enhancing thrombolysis.

2.5. Camouflaged-tPA delivery approach

In a number of studies,[92–94] Absar et al. tried to introduce an efficient delivery system for tPA. Camouflaged-tPA was produced by conjugating tPA with low molecular weight heparin (LMWH) followed by complexion of this conjugate with albumin-protamine.[92] The administration of heparin with tPA led to 71% clot lysis, which is more effective than tPA alone (52% clot lysis). However, using heparin with tPA increased the bleeding risk, which led to a two-fold increase in activated partial thromboplastin time (aPTT). In a contrary situation, albumin-camouflaged heparin triggered strategy achieved a similar clot lysis activity (70%) compared to tPA plus heparin administration, but with no prolongation of aPTT after 1 hour of treatment. This indicates that the camouflaged-tPA can be applied for targeted thrombolysis with a reduced risk of hemorrhage.[92] Based on the fact that LMWH is a therapeutically active molecule, Absar et al. in another study[93], have tested a relatively inert negatively-charged compound to synthesize oligoanion-modified tPA to avoid any potential side effects. The tPA was conjugated to polyglutamate, and separately the human serum albumin (HSA) was conjugated to protamine. The conjugation of tPA with polyglutamate forms a reversible electrostatic complex that could be disrupted by negatively charged heparin via competitive binding. The electrostatic complex formation between polyglutamate and protamine can be reversed by heparin also. It was found that camouflaged-tPA heparin triggered delivery system demonstrated higher activity than the un-camouflaged tPA, where this higher activity may be attributed to the protection of camouflaged tPA from its macromolecular inhibitors. In another modification for the camouflaged-tPA delivery system, Absar and his colleagues[94] have conjugated tPA with HSA via a thrombin-cleavable peptide or linker (GFPRGFPAGGC). In addition, the surface of albumin was decorated by the peptide sequence (CQQHHLGGAKQAGDV) of fibrinogen gamma-chain that has the affinity to bind with GPIIb/IIIa receptors, which are expressed on activated platelets. This conjugate showed an activity of 25%, which increased to about 86% of that of native tPA when the conjugate was incubated with thrombin. This approach can introduce an efficient delivery system for tPA working in an on/off triggered manner (Figure 4).

2.6. Microcarriers delivery system

Microcarriers (MC) have many advantages, such as offering a large volume for encapsulating drugs, and the possibility of co-encapsulation of magnetic nanomaterials to facilitate the control of microspheres against vascular flow.[95] Torno and Kaminski compared the lysis effect of free tPA with that of tPA combined with magnetic microcarriers (MMC), and with that of tPA combined with MMC upon exposure to external magnetic field (MF). They also examined the lysis effect of tPA combined with MMC, exposed to both MF and ultrasound (US). They found that under static and no-flow conditions, thrombolysis efficiency improved by 1.7 and 2.7 fold for red and white clots, respectively in the case of (tPA + MMC + MF). Whereas under flow conditions, they reported a two-fold increase in lysis with a significant reduction in recanalization time by 7-fold. Furthermore, in the case of (tPA + MMC + MF + US), a maximal lysis efficiency of almost 98% was achieved for both red and white clots.[96] Later, Kaminski et al.[95] developed a new magnetic-targeting delivery system via encapsulating tPA in magnetic poly (lactic acid)-poly(ethylene glycol) (PLA-PEG) microcarriers.

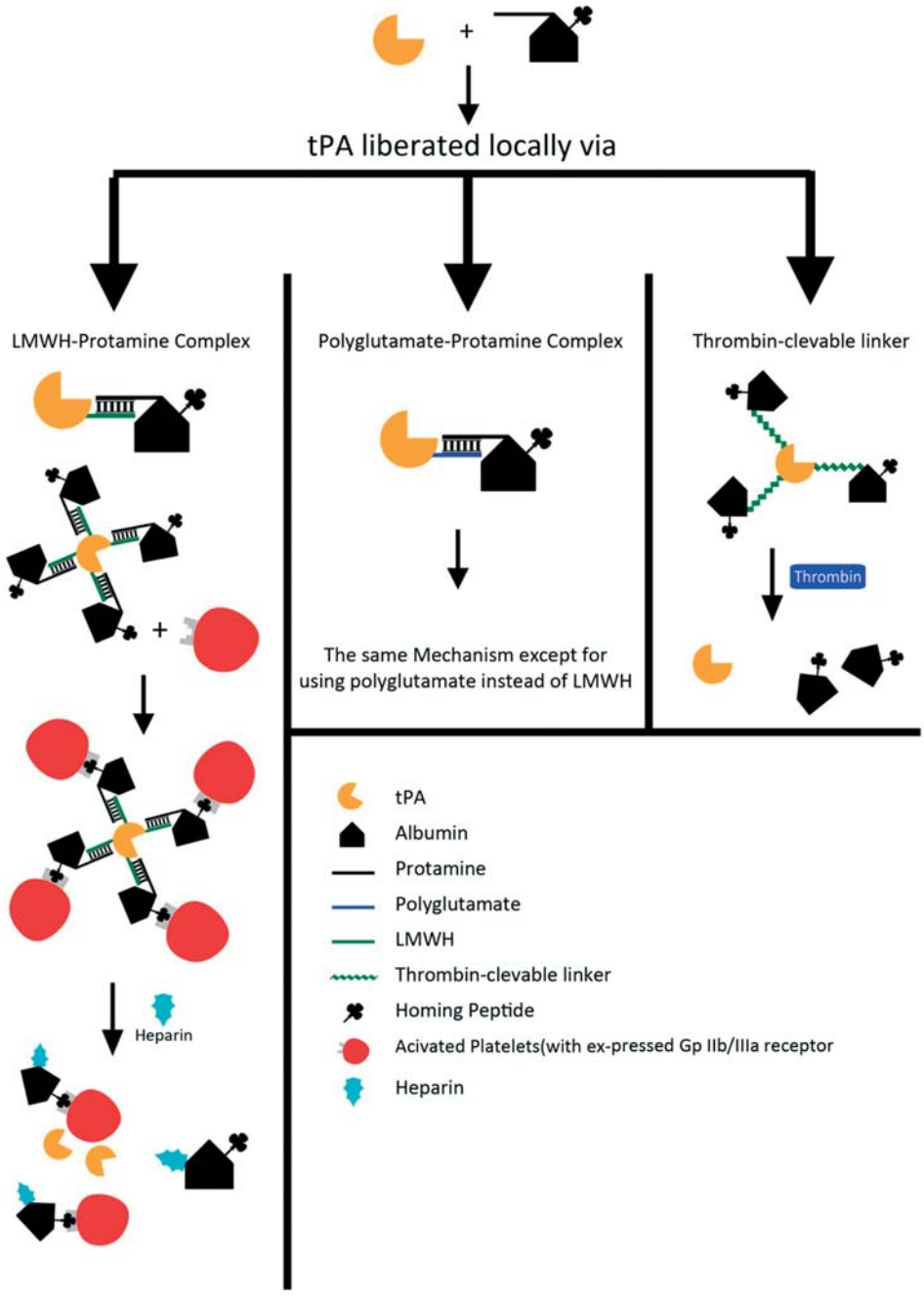

Figure 4. An illustration of the various camouflaged-tPA delivery approaches. Adopted with modification from Absar et al.[92–94]

They reported that this delivery system has increased the overall activity of tPA by preserving its concentration up to 74 ug/ml, which is significantly higher than the concentration needed for efficient clot lysis (1–4 ug/ml).[95]

2.7. Nano-modulated delivery approaches

One of the first nano-modulated approaches is the use of tPA-loaded magnetic nanoparticles (MNPs). MNPs offer a good drug delivery system because of their composition and tailorability. MNPs are usually composed of a core made of iron oxide, Fe_3O_4, and a polymer coating. The core is responsible for the supermagnetic characteristics, which can be controlled by applying an external magnetic field, while the polymer coating is beneficial in increasing the stability and inhibiting the particles

aggregation.[97,98] In addition, being composed of iron oxide, the MNPs demonstrate very limited toxicity and high biocompatibility making their use safe and popular.[99,100]

In this context, Maet et al. studied the efficacy of magnetic targeted delivery of recombinant tPA (r-tPA) in a rat embolic model.[101] A blood clot was produced *in vitro* and then injected into the iliac artery. Then, a permanent magnet was placed above the left iliac artery in order to manipulate the MNPs. It was found from the study that the intra-arterial infusion of the r-tPA significantly reversed the iliac flow within 15 minutes. Moreover, retention of MNPs against the hemodynamic dragging force in the iliac artery of the rat occurred. On the other hand, the MNPs retention was very limited in the absence of the magnetic field.

In another study using the same embolic rat model, Ma and his team have developed polyacrylic acid-coated MNPs to target r-tPA.[102,103] Polyacrylic acid (PAA) was used in this study to stabilize MNPs because it produces electrostatic and steric repulsion preventing the particles aggregation. The study showed that static magnetic field produced by the external magnet with PAA-MNP-r-tPA didn't produce reversal in hemodynamics. On the other hand, applying the magnetic field periodically in an on/off way did the job, indicating that thrombolysis is achieved by mechanical dragging force created by movement of MNPs under the effect of external magnetic field. In conclusion, moving the external magnetic field led to suspension of MNPs and enhancing the penetration of r-tPA into the thrombus.[102]

In accordance with Ma's results,[101–103] Chen et al.[99] used chitosan coated-MNPs to deliver tPA. Chitosan is a hydrophilic polysaccharide obtained from chitin which is naturally extracted from the shells of shrimps and crabs. Chitosan has many beneficial characteristics, such as excellent biodegradability and low toxicity.[104] Chen et al.[99] found that clot lysis time was reduced when using chitosan-MNP-tPA in the presence of an applied external magnetic field by 58% compared to other runs without magnetic targeting, or by 53% compared to free tPA at the same dose of tPA (0.1 mg/ml).

The concept of on/off triggering of thrombolysis has been utilized in several approaches,[92–94,102] and a novel ultrasound-responsive nano-delivery system for tPA has been developed.[38,105,106] This system is composed of tPA complexed with cationic gelatin and PEG-gelatin. This composition depends on the electrostatic interaction between tPA, with its negative zeta potential, and a positively charged cationic gelatin leading to inactivation of tPA. Upon exposure of this composite to ultrasound, the tPA regains its activity. Upon administration of the delivery system without ultrasound, none of the animals showed recanalization. In the case of free tPA, however, half of the animals showed recanalization.[38]

Uesugi et al. modified this system by adding zinc ions to the complex of tPA and gelatin.[105] It was found that zinc ions modified the resulting complex through coordinate and ionic bonds leading to greater suppression of tPA activity. Exposing this modified system to ultrasound at 1 MHz and 0.75 W/cm^2 for 5 minutes, the system showed a 2-fold increase in tPA activity compared to the original level.[105]

Kawata and Uesgui et al. evaluated this modified system *in vivo*,[106] where they found that the plasma tPA activity, determined immediately after injection of tPA-loaded nanoparticles, was almost 71.4% lower than after administration of the same dose of free tPA. On the other hand, the activity of nanoparticles recovered to a similar level to free tPA after being exposed to transcutaneous ultrasound for 5 minutes.

After 40 minutes the plasma tPA activity produced by nanoparticles after being exposed to ultrasound reached a level higher than that produced by free tPA.[106] This approach gives hope for a safe drug delivery system for tPA, leading to reducing the risk of bleeding resulting from using high doses of free tPA.

FUTURE PROSPECTIVE

Nanomedicine potentially opens up novel delivery approaches and new methods of targeting tPA to decrease the risk of bleeding. Shear-activated nanotherapeutics (SA-NTs) represent one of these novel approaches in controlled delivery and targeting of tPA.[107] The mechanism of SA-NT relies on the fact that there is difference between normal blood vessels and stenosed or thrombosed one. As a result SA-NTs can be targeted to the site of thrombus in a size similar to that of platelets. Upon exposure to high local shear stress, they break up into nanosized particles. In addition, they are safe to be used as they are rapidly cleared (80% clearance in 5 minutes).[107]

Nanomedicine also allows for the development of "stealth" technology in which tPA is encapsulated in gelatin nanoparticles complexed with zinc ions.[38,105,106] This opens up the possibility of developing protein therapeutics in vascular diseases, another promising field.

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