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Engineered Micro- and Nanoparticles for Fibrinolysis

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Summary

Fibrinolytic agents including plasmin and plasminogen activators improve outcomes in acute ischemic stroke and thrombosis by recanalizing occluded vessels. In the decades since their introduction into clinical practice, several limitations of have been identified both in terms of efficacy and bleeding risk associated with these agents. Engineered nano- and microparticles address some of these limitations by improving circulation time, reducing inhibition and degradation in circulation, accelerating recanalization, improving targeting to thrombotic occlusions, and reducing off-target effects; however, many particle-based approaches have only been used in pre-clinical studies to date. This review covers four advances in coupling fibrinolytic agents with engineered particles; (1) modifications of plasminogen activators with macromolecules, (2) encapsulation of plasminogen activators and plasmin in polymer and liposomal particles, (3) triggered release of encapsulated fibrinolytic agents and mechanical disruption of clots with ultrasound, and (4) enhancing targeting with magnetic particles and magnetic fields. Technical challenges for the translation of these approaches to the clinic are discussed.

Keywords

Drug delivery systems; fibrinolysis; magnetic fields; plasminogen activators; ultrasonic waves

Introduction

Plasminogen activators (PA) were introduced into clinical practice in the 1980s for indications of myocardial infarction and in the 1990's for ischemic stroke [1]. Large clinical trials show the benefits of recombinant tissue plasminogen activator (tPA) in acute ischemic stroke [2,3]. During the last 30 years of clinical practice several limitations have arisen including a limited time window (4.5–6 hrs from onset of symptoms), neurotoxicity, and bleeding [4,5]. Over this same time, mechanical thrombectomy devices have emerged as an

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effective treatment for many types of occlusions in large arteries and veins [6–8]. In some cases, the combination of fibrinolytics and mechanical thrombectomy have been used successfully [9,10]. However, their use is limited to stroke centers that are not always available to individuals outside of major urban areas. Furthermore, mechanical thrombectomy devices cannot reach all types of clots. For example, in the brain, these devices cannot reach the M3/M4 branches where many strokes occur. Taken together, these considerations motivate the need for alternative drug delivery strategies for fibrinolytic agents.

The principle of fibrinolytic therapy is to deliver plasmin or PA to an occlusion to lyse fibrin, thus robbing the thrombus of its mechanical stability, and restoring blood flow in the vessel [11]. Plasmin binds directly to and lyses fibrin, however its efficacy is limited by rapid binding to its endogenous inhibitor α-antiplasmin. PA such as tPA and urokinase plasminogen activator (uPA) act indirectly by first cleaving plasminogen to plasmin, and SK forms a proactivator complex with plasmin(ogen) that converts other plasminogen molecules in the presence of fibrin [12]. Intravenous (IV) delivery provides an elevated systemic concentration of these fibrinolytic agents. PA are more effective in IV delivery than plasmin because their endogenous inhibitors, plasminogen activator inhibitor 1 (PAI-1) and 2 (PAI-2), are found at lower plasma concentrations compared to α-antiplasmin. Alternatively, local delivery to occluded vessels accessible to catheters can overcome some of the effects of these inhibitors with a high local concentration of plasmin or PA.

The most cited complication of fibrinolytic therapy is the risk for iatrogenic bleeding since these agents degrade hemostatic as well as thrombotic clots [13,14]. PA cause bleeding in a small fraction (1–6%) of patients when used to treat ischemic stroke [13], pulmonary embolism [15], or myocardial infarction [16]. Furthermore, it has been shown that tPA can increase the permeability of the blood brain barrier (BBB) while high concentrations of plasmin can damage the BBB, promoting bleeding [17]. Side effects are not the only limitation of PA; IV delivery of PA has a high incidence of failure for certain types of clots because it relies on blood flow, which is restricted or non-existent, to deliver them to the thrombus location [18]. Fibrinolytic therapy can also fail if a patient has high levels of PAI-1 [19] or SK antibody [20], or if the rate of deactivation exceeds the rate of delivery to the clot [21,22]. These limitations have prompted researchers to improve thrombolysis by developing artificial PA that are more enzymatically active [23], using adjuvants that assist PA [24], or inhibiting PAI-1 [25] or FXIIIa [26]. Another approach is to modify plasminogen activators (PA) with macromolecules to protect them from clearance and degradation in blood, thereby improving circulation half-life [27,28]. Further modifications of PA facilitate thrombus targeting via functionalization with moieties that bind to activated platelets or fibrin [29]. However, these approaches offer only marginal improvements over tPA, rely on blood flow for delivery, and still exhibit high risk of bleeding.

A more promising solution is through the use of nano-and microparticles. These can contain immobilized or encapsulated PA, preventing systemic interaction with the BBB, and can be actuated to improve at-site drug delivery. Nano- and microparticle carriers for PA have been developed to improve circulation time, reduce inhibition and degradation, and target occlusive thrombi [30,31]. Many of these are polymeric or liposomal spheres that

encapsulate or embed PA. In addition to improving half-life and preventing loss of enzymatic activity in the circulation, these particles can be engineered to release PA at a controlled and prolonged rate [30]. Encapsulation allows delivery not only of PA, but also of plasmin itself by protecting it from inhibition by α2-antiplasmin [32]. Other novel fibrinolytic carriers include PA-functionalized red blood cells with prophylactic effects on thrombosis [33], nanoclusters that release PA in response to increased shear stress in stenosed vessels [34], and activated platelet-stimulated liposomes that release their PA cargo [35].

Though such approaches offer improvements, designing PA carriers that selectively target occlusive thrombi without causing bleeding or reaching small vessels remains a challenge and motivates the need for carriers that can be actuated using an external control mechanism. For example, echogenic liposomes only release a PA payload when exposed to ultrasound [36]. Similarly, magnetically responsive liposomes, microbubbles, and micro- and nanoparticles can be driven to accumulate at the thrombus site using external magnetic fields. This topic has been reviewed in the past with focus on targeting [37] and stage of development [38]. In this review, we highlight engineered micro- and nanoparticle PA carriers with special attention to particles externally actuated by ultrasound or magnetics fields.

Macromolecule modification of plasminogen activators

PA coupling to macromolecules can improve circulation time and protect against endogenous inhibitors (Fig. 1). One of the first examples of this approach was coupling streptokinase (SK) to polyethylene glycol (PEG), a process called PEGylation, which reduces α_2-macroglobulin mediated catabolism of SK-plasmin complexes by 50% and extends its half-life from 5 min to 30 min [39]. Additionally, PEGylated SK binding to SK antibodies is reduced by 95%, indicating that this modification reduces the potential immunogenic response to SK [19]. Sakuragawa et al. PEGylated urokinase plasminogen activator (uPA) and showed extended activity over a 6 hr period, compared to a 1 hr period for native uPA, and 6-fold higher antithrombotic capabilities for PEGylated uPA compared to native uPA [40]. Berger and Pizzo showed that PEGylated tPA circulates for ten times longer in mice, rats, and beagles than tPA, though the active half-life is only increased threefold because of inhibition by PAI-1 and PAI-2 [41]. In all of these reports, the fibrinolytic rate of the PEGylated PA is slightly accelerated compared to the non-PEGylated control, likely due to reduced inhibition.

Molecules other than PEG have been used to increase the circulation time of PA. For instance, tPA has been coupled to albumin via a thrombin cleavable peptide [42]. The activity of the albumin coupled tPA is reduced by 25% but, upon exposure to thrombin, regains activity to 90% of the uncoupled tPA control. Additionally, circulation time is increased to 24 hr with a concentrated burst lasting 30 minutes after exposure to thrombin, and fibrinogenolysis is reduced by half. Another method of improving circulation time is embedding PA into branched synthetic polymers called dendrimers. Wang et al. embedded SK within a poly(amido amine) dendrimer and showed that it retained 80% of its activity and was stable in buffer for three-fold longer than free SK [43]. Fibrinolysis rates were

nearly identical for the PA dendrimer and free SK. Other studies using SK in dendrimers report slightly enhanced lysis rates [44] and reduced fibrinogen degradation [45].

Other macromolecule modifications focus on coupling PA to macromolecules that target activated platelets, activated endothelial cells, or fibrin [28]. Bode et al. conjugated uPA to a monoclonal antibody to the integrin $\alpha_{IIb}\beta_3$ on platelets [46]. They found that anti-$\alpha_{IIb}\beta_3$ antibody modified uPA was drastically better at lysing platelet-rich clots in platelet density dependent manner. Similarly, targeting $\alpha_{IIb}\beta_3$ with a peptide attached to staphylokinase (SAK) improved fibrinolysis as well as decreased platelet aggregation relative to SAK without the peptide [47]. For a more detailed review of approaches for targeting platelets, endothelial cells, fibrin and erythrocytes, we refer the reader to the review by Absar et al. [29].

Encapsulation of plasminogen activators in liposomes and polymer particles

While PA modification increases circulation half-life, a primary disadvantage is that it offers moderate (if any) enhancements to fibrinolysis rates. An alternative way to prevent PA degradation and inhibition in circulation while increasing delivery and therefore lysis rates is to encapsulate them within lipid vesicles, liposomes, or polymer matrices (Fig. 1). SK encapsulated in unilamellar phosphatidylcholine liposomes retains 100% of its activity after 30 min incubation in plasma while unencapsulated SK loses more than 50% of its activity [48]. Stability tests indicate that liposomes do not leak SK over a period of 24 hours at body temperature. They release SK through membrane pores during interaction with a thrombus, resulting in similar lysis rates to unencapsulated SK. In another study, tPA encapsulated in poly-(lactide-*co*-glycolide) (PLGA) nanoparticles coated with the polysaccharide chitosan reduces tPA degradation while reducing lysis time of in vitro whole blood clots by 40% [49]. Both liposome and PEG encapsulated SK outperform free SK in thrombi formed in the carotid artery of rabbits; reperfusion was achieved in 75 min for free SK, 19 min for liposomal SK, and 7 min for PEG encapsulated SK [50]. Similarly, SK encapsulation in distearolphosphatidylcholine with PEG in the lipid bilayer increases half-life by 16-fold and activity by 6-fold in rats [51]. Such encapsulated PA are more effective, in part, because they display reduced adsorption to fibrin relative to non-encapsulated PA, and instead support penetration of PA into the thrombus [50].

To mitigate off-target fibrinolysis, micro- and nanoparticles carrying PA have also been decorated with targeting molecules. Huang et al. modified tPA carrying liposomes with PEG and cyclic arginine-glycine-aspartic acid (cRGD), which is found on the γ chains of fibrin and binds to activated $\alpha_{IIb}\beta_3$ [35]. The cRGD supports fusion between the liposomes and activated platelets, causing the liposomes to destabilize and release 90% of their tPA payload within 1 hr of interaction. Liposomes without cRGD motifs or those not exposed to activated platelets only release 10% of their tPA after 6 hr, indicating that tPA release is platelet-sensitive and targeted. Thrombolytic activity of the cRGD coated tPA-liposomes is equivalent to free tPA and three-fold faster than liposomes not triggered by platelets using cRGD. While there is no enhancement to lysis rate over the soluble drug, there are three

advantages of cRGD PEGylated liposomes. First, liposomal encapsulation of tPA protects the drug from degradation in plasma. Second, PEGylation of tPA-liposomes make them less susceptible to unwanted destabilization. Finally, the cRGD motifs ensure that large payloads of tPA are only released in the presence of activated platelets, preventing systemic action of tPA. Nonetheless, liposomal delivery of PA has some disadvantages. Like free PA, liposomes rely on blood flow, which may be reduced or absent in occluded vessels, for delivery to a thrombus. However, because liposomes have larger size, their diffusion in occluded channels will be much slower, increasing the delay between injection and the initiation of fibrinolysis.

Another approach is to target the biophysical environment of thrombosis with pre-circulating nanoconstructs. While not useful for treating fully occluded vessels, this approach can be used prophylactically to prevent thrombus formation or to treat stenosed vessels. Korin et al. fabricated shear-activated nanotherapeutics (SA-NT) using nanoparticle aggregates formed from concentrated solutions of PLGA nanoparticles carrying immobilized tPA [34]. These 1–5 μm aggregates are stable up to shear stresses of ~100 dyn/cm^2 and disassemble into their constituent nanoparticles at higher shear stresses, such as those experienced in stenotic or obstructed vessels. In a microfluidic model of stenosis with peak shear stress of 450 dyn/cm^2, sixteen-fold more nanoparticles released from the SA-NT than in the straight part of the channel with shear stress of 30 dyn/cm^2. Thrombi were lysed within 5 min of SA-NT administration in the ferric chloride thrombosis model in murine mesenteric arteries. This approach also shows promise as a prophylactic antithrombotic as pre-circulating SA-NT delayed time to full occlusion three-fold relative to control mice. In a murine pulmonary embolism model, the SA-NT conferred a survival advantage; 100% of control mice died while 80% of mice receiving SA-NT survived the embolism with >60% reperfusion after 45 min. Another advantage of SA-NT is that tPA-nanoparticles selectively concentrate near occlusions in regions of high shear stress, potentially reducing off-target side effects. However, as SA-NT are primarily useful as prophylactics, they would need to exist in the circulation prior to thrombus formation.

Coupling plasminogen activators to blood cells and blood cell mimics

Another targeting and triggered-release strategy relies on imitating or hijacking platelets or red blood cells (RBC) (Fig. 1). Pawlowski et al. designed platelet microparticle-inspired nanovesicles (PMIN) carrying SK that interact with platelets via ligands for $\alpha_{IIb}\beta_3$ and P-selectin [52]. Once the PMIN interacted with platelets, phospholipase-A_2, an enzyme that is upregulated in sclerotic arteries [53], destabilizes the PMIN and triggers release of SK. In the ferric chloride thrombosis model in the murine carotid artery the SK-PMIN had antithrombotic properties indistinguishable from an equal dose of free SK. In a murine tail bleeding model the bleeding times for mice treated with SK-PMIN is equivalent to those for untreated controls, while bleeding times for mice receiving free SK was three-fold higher [52]. Unlike free SK, these SK-PMIN do not initiate systemic fibrinogenolysis.

PA can be bound directly to blood cells as a targeted therapeutic strategy. For example, tPA has been coupled to biotinylated red blood cells (RBC) to form functionalized RBC (tPA-RBC) [54]. In this case, tPA-RBC are an example of antithrombotic particles that are

entrained in thrombi during their formation and either initiate or enhance fibrinolysis. Their mechanism is prophylactic rather than after a thrombus has formed. In the ferric chloride thrombosis model of the murine carotid artery, tPA-RBC restored blood flow in 20–30 min, where unbound tPA failed to reperfuse the vessel. tPA-RBC were also combined with traditional PA delivery to accelerate fibrinolysis by creating large (>20 μm) pores within the thrombus to promote tPA penetration [55]. Prophylaxis however requires particles to be present in the blood prior to an unpredictable event and therefore are likely most useful in individuals at high risk for thrombosis. See Greineder et al. for further discussion of antithrombotic drug delivery approaches [28].

Rather than using blood cells, Colasuonno et al. synthesized discoidal polymeric nanoconstructs (DPN) mimetics shaped like RBC and functionalized with tPA (tPA-DPN) [56]. DPN are 1 μm in diameter, 0.4 μm in height, biconcave, and can be synthesized from either PEG or PLGA. tPA-DPN have a fibrinolytic activity roughly 50% higher than free tPA at 10% the free tPA concentration. In thrombi formed in the the ferric chloride thrombosis model in murine mesentery venules, 0.1 mg/kg of tPA-DPN recanalizes 70% of thrombi in under 60 min, whereas 1 mg/kg of free tPA only recanalizes 40% thrombi after 90 min. Importantly, tPA associated to DPN is also protected from degradation by PAI-1 for longer than 3 hr in plasma, where free tPA is degraded to 30% activity.

Synthetic platelets have been developed as hemostats [57–60], but also for thrombolysis [61]. In this, polystyrene (PS) spheres are stretched into discoids and used as templates for the growth of cross-linked actin layers. The PS is solvated by a tetrahydrofuran and isopropanol mixture, and the remaining protein structure retains the size, shape and flexibility of platelets. Finally, the protein scaffolding is functionalized with the A1 domain of von Willebrand factor or the amino terminal domain of GPIbα to bind to platelet-rich thrombi. Thrombi formed in ex vivo whole blood perfusion over collagen at concentrations of $1.5–4\times10^5$ particles/μL comprised between 40–80% of the thrombus, while spheres functionalized with the same moieties only comprised between 5% and 10% of the volume. These data indicate that both physical and biochemical properties can be exploited to enhance thrombus targeting, and SP functionalized with PA could be a potent targeted thrombolytic.

Ultrasound mediated lysis and release

Echogenic Liposomes

Instead of relying on systemic circulation, biochemical targeting, or blood flow induced mechanical forces to achieve local thrombolysis, external forces can trigger the release of encapsulated tPA from liposomes (Fig. 1, Fig. 2A). Echogenic liposomes (ELIP) release payloads on demand with application of sonic forces [62]. Shaw et al. used thrombi formed from whole blood ex vivo to test ELIP containing tPA (tPA-ELIP) with 120 kHz ultrasound. tPA-ELIP in the presence of ultrasound lysed thrombi four-fold faster than tPA alone [63]. The efficacy of tPA-ELIP was also shown in a murine model of thrombosis that uses denudation of the aorta followed by injection of 5% sodium ricinoleate and thrombin [64]. Blood flow was restored twice as fast by tPA-ELIP than empty ELIP in the presence of tPA and ultrasound at 5.7 MHz, and tPA-ELIP in the presence of ultrasound was twice as fast

compared to no ultrasound. ELIP has also been used to deliver plasmin [32]. Kandadai et al. used plasmin-loaded ELIP to lyse blood clots formed from human whole blood in vitro, and reported that plasmin-ELIP exposed to 120 kHz ultrasound with 1.7 MHz pulses lysed clots at 15% faster rates than free tPA.

Sonothrombolysis

Sonothrombolysis refers to methods using sonic energy to directly or indirectly degrade thrombi. For the interested reader, Bader et al. reviews the mechanisms of sonothrombolysis [36]. Here we focus on micro- and nanoparticles that enhance the these mechanisms: acoustic streaming, cavitation, and ultrasound-induced temperature rise. Acoustic streaming describes the use of acoustic radiation forces to create flow, which at the site of a thrombus helps overcome the slow, diffusion-mediated transport of PA in occluded vessels (Fig. 2B) [65]. Cavitation directly degrades thrombi with mechanical energy from bubble formation, oscillation, and collapse (Fig. 2C) [66]. Ultrasound can cause local temperature to increase by up to 5 °C and thereby accelerate fibrinolysis; however, when the contributions of each mechanism are decoupled, acoustic streaming and cavitation contribute more significantly to fibrinolytic enhancements than heating directly [67].

Sonothrombolysis is enhanced in the presence of microbubbles (Fig. 2D), gas-filled vesicles typically less than 8 μm in diameter [68]. Microbubbles are used as an ultrasound contrast agent because they scatter sonic waves more than blood, but they have recently garnered attention as agents for drug delivery and gene therapy [68]. Microbubbles can be stabilized with silanes, surfactants, protein shells, polymer coatings, or lipids. Bader et al. investigated the mechanism for microbubble-assisted sonothrombolysis using 50 μm octofluoropropane bubbles stabilized with a lipid monolayer [69]. They concluded that the oscillations and coalescence of microbubbles with a resonant frequency close to the frequency of ultrasound exposure were the primary contributors to lysis during exposure to sonic forces. They also observed more sustained cavitation over 50 s periods in the presence of microbubbles which further contributes to enhanced thrombolysis.

A series of clinical trials show the potential for sonothrombolysis in treatment of ischemic stroke of major cerebral arteries (Table 1). One trial found that ultrasound therapy in the presence microbubbles reduced reperfusion times to less than 20 min, while achieving full recanalization in 71% of patients within 2 hr [70]. By comparison, full recanalization was achieved in only 39% of patients receiving just tPA. There was no increased risk of intracranial hemorrhage due to the microbubbles compared to patients receiving only tPA. In another trial, sonothrombolysis accelerated reperfusion but increased risk of hemorrhage [71]. Out of 35 patients, risk of intracerebral hemorrhage was directly related to the dosage of microbubble infusion. Patients receiving a 1.4 mL dose of microspheres over a 90 min period experienced recanalization in half the time of control patients and did not experience an increase in hemorrhagic events; however, 27% of patients who received a double dose of microspheres experienced hemorrhage without a decrease in reperfusion time. A similar study validated these results [72]. The data from these studies indicate that the effects of microbubble size, composition, viscoelasticity and ultrasound frequency on microbubble-assisted sonothrombolysis merit further investigation.

Magnetic particles and magnetic field control

Ultrasound waves attenuate in tissues, and thrombolytic methods that use ultrasound may be ineffective in vessels away from the body's surface or not easily accessible by surgical intervention. Moreover, both ultrasound mediated drug release and microbubble enhanced sonothrombolysis rely on local catheter delivery or circulation to bring particles near a thrombus—a disadvantage shared by all approaches discussed heretofore. This could be challenging for occlusions in small vessels where catheters cannot reach. Magnetic fields overcome these limitations as they do not attenuate in tissue for frequencies <30 Mhz [73]. They can also be used to concentrate PA-laden magnetic particles at the thrombus periphery [74]. Magnetic nanoparticles (MNP) used for fibrinolysis are made from <30 nm superparamagnetic iron oxide crystals of magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3), FDA approved materials [75,76] with low toxicity in humans [77], embedded in a polymer matrix [75]. The small, randomly distributed domains allow the MNP to orient themselves in the direction of a magnetic field at all times with an induced dipole. Though magnetically guided particles are a promising direction for thrombolytic research, these approaches have only been used in preclinical studies to date.

Synthesis and functionalization of magnetic nanoparticles carriers

As in other fibrinolytic particles discussed above, encapsulating or immobilizing PA on MNP increases enzyme stability during storage [78] and improves half-life [79]. MNP carrying tPA, uPA, or SK have been decorated with coatings that further improve half-life and reduce immunogenicity including dextran [80,81], chitosan [82], silica [79,83], hydrosol [84], PLGA and PLA-PEG [85], polyacrylic acid (PAA) [86], polyethylene glycol (PEG) [87,88], poly[aniline-co-N-(1-one-butyric acid) aniline] [78], and heparin [89]. PA functionalization of MNP can rely on either physical adsorption to the MNP matrix or covalent attachment [90]. Adsorption yields high concentrations of tPA (> 20 μg/mL) near a thrombus [85]; however, PA desorbs from PLGA, PAA, and uncoated magnetite rods within 30 min of injection [85,90,91]. Premature release of PA before particles reach their target is problematic and compounded by the short half-life (5 min) of recombinant tPA in blood [92].

Limitations related to timing of PA release motivate the need for magnetic carriers with triggered release and alternative functionalization methods. Drozdov et al. developed a magnetite composite material that incorporates uPA that does not leach, enabling magnetically responsive drug carriers to have prolonged activity for several hours [84]. In this, negatively charged plasminogen interacts with a positively charged particle matrix containing uPA. Once converted, positively charged plasmin is repelled out of the MNP. A covalent bonding strategy is functionalization of PAA particle surfaces using N-hydroxysuccinimide followed by coupling with tPA [90]. Alternatively, streptavidin-functionalized particles can be conjugated to biotinylated tPA [74]. Such immobilization of PA protects the enzyme from inhibition by PAI-1 and degradation in the liver.

Magnetic field control of magnetic particles

The simplest form of magnetic control is to use a permanent magnet to establish a magnetic field gradient to move MNP towards a target (Fig. 3A, Table 2). This is the most widely reported method for manipulating PA carriers [79,81,87,88,91]. Ma et al. used PAA particles conjugated with tPA to show recanalization of the iliac artery in rats and demonstrated reperfusion using only 20% of the therapeutic tPA concentration (1 mg/kg) [86]. Experiments in rat and mouse models also use a moving permanent magnet to drag MPN from the drug injection site to the thrombus [12,14,19]. Magnetic field gradients are difficult to scale to humans as the strength of a magnetic gradient force decays with the inverse square of the distance ($1/r^2$) from the magnet. As an example, a 1.5 T magnet is capable of creating a 40 mT/m gradient in the carotid artery of pigs, which is sufficient to propel a 1.5 mm sphere at a velocity of 13 mm/s, but a 5 μm sphere only 1 μm/s [46]. The tortuosity of the human vasculature also renders a unidirectional field gradient potentially ineffective as MPN must navigate through vessels in varying orientations.

Magnetic field induced mixing can enhance fibrinolysis by free PA (Fig. 3B, Table 2). Torno et al. used a permanent magnet to move magnetic microspheres back and forth, inducing a flow field near a bolus of tPA [93]. Mixing doubled the thrombolytic efficiency by reducing concentration gradients and, when combined with 20 kHz ultrasound, accelerated lysis three-fold over no mixing or ultrasound. A similar mixing strategy was reported by Huang et al. where rotating permanent magnets made rod-shaped particles spin [94]. In an embolic rat model, they reported that mixing doubled tPA-mediated lysis. Khalil showed thrombi removal using a ~1 mm helical robot made to abrade a thrombus via external magnetic control [95]. This purely mechanical mechanism removed a clot at three times the rate of the clinical dose of SK and may provide a less invasive alternative to catheters. MNP can also be heated with applied magnetic fields. For example, Voros et al. immobilized tPA on nanocubes, particles with cubic rather than spherical geometry, and subjected them to 500 kHz radio frequency (RF) fields to locally raise the temperature to 42 °C (Fig. 3C) [96]. Here, fibrin dissolution occurred an order-of-magnitude faster at 42 °C compared to 37 °C in vitro. In a ferric chloride thrombosis model in murine mesentery vessels, tPA nanocubes lysed clots in <1 min compared to 5–10 minutes in the absence of heating. This enhancement is greater than predicted based on the temperature-dependence of lysis kinetics and is also attributed to faster tPA release from the nanocubes [96].

Some of the most novel MNP strategies initiate thrombolysis through both chemical and mechanical mechanisms (Table 2). De Saint Victor et al. made 0.2–15 μm microbubbles containing air and 10 nm magnetite particles stabilized by phospholipids [97]. The mechanical energy from ultrasound cavitation combined with the biochemical action of free tPA degraded whole blood thrombi 2.5-fold faster than free tPA alone. Magnetic targeting provided further enhancement; retention of a high local concentration of magnetic microbubbles using a permanent magnet resulted in a continuous supply of high cavitation energy. The cavitation energy was itself enhanced by a large concentration of microbubbles resulting from magnetic focusing. This magnetically mediated sonothrombolysis degraded clots roughly twenty-fold faster than soluble tPA alone, ten-fold faster than ultrasound alone,

and four-fold faster than tPA and ultrasound in the presence of microbubbles but in the absence of magnetic focusing.

Tasci et al. demonstrated enhanced fibrinolysis using a combination of biochemical and mechanical action using magnetically powered microwheels assembled in situ [74]. Biotinylated tPA was immobilized on 1 μm streptavidin-coated MNP. The microwheels rolled to the interface of a plasma clot (Fig. 3D) and accumulated tPA at this interface at a concentration 50 times higher than the injected concentration. Once at the interface, a corkscrew-like motion was used to drive the microwheels into the clot yielding a lysis rate six-fold faster than tPA alone. This approach relies on low strength (~10 mT), rotating magnetic fields rather than field gradients which is easier to scale-up to humans. The microwheels assemble to structures greater than 10 μm in diameter, which is within the detection limits of MRI [98], but disassemble into 1 μm spheres upon removal of the magnetic field, making them passable through capillaries.

Outlook

Table 3 provides a summary of the advantages and disadvantages of the different approaches to engineered fibrinolytic particles reviewed here. The external actuation of PA particles using ultrasound and magnetic fields is a promising direction, but several challenges must be overcome to translate these approaches into clinical practice. Ultrasound actuation has received positive results in small clinical trials but further characterization is needed on microbubble dosage [71], size, and composition, especially at lower frequencies [99]. Most studies of magnetic field actuation to date have been in preclinical models. A promising feature of magnetic approaches is the ability to both localize and concentrate fibrinolytic particles near a thrombus, reducing the effective circulating concentration of PA, helping to minimize side effects, and potentially broadening the indications for PA therapy. The scale-up of the hardware required for magnetic control in humans will have to overcome several technical obstacles: (i) the generation of sufficient magnetic forces in deep tissues, (ii) particle navigation in the complex, three-dimensional vasculature, (iii) combining actuation with imaging, and (iv) achieving translation across or against flowing blood. Nonetheless, the development of fibrinolytic particles has produced encouraging results. Some of the most recent work has made it clear that combining PA encapsulation with magnetic localization, ultrasound, and hyperthermia may produce lysis rates up to an order-of-magnitude faster than those achievable using free PA. Engineered particles also allow for the delivery of two or more agents, which could be exploited to couple PA with other emerging thrombolytics targeted to von Willebrand factor [100–102], neutrophil extracellular traps [103], and thrombin-activatable fibrinolysis inhibitor (TAFI) and PAI-1 [104]; alternatively, PA could be delivered alongside adjuvants that enhance PA activity [24], PAI-1 inhibitors [25], or FXIIIa inhibitors to accelerate thrombolysis [26]

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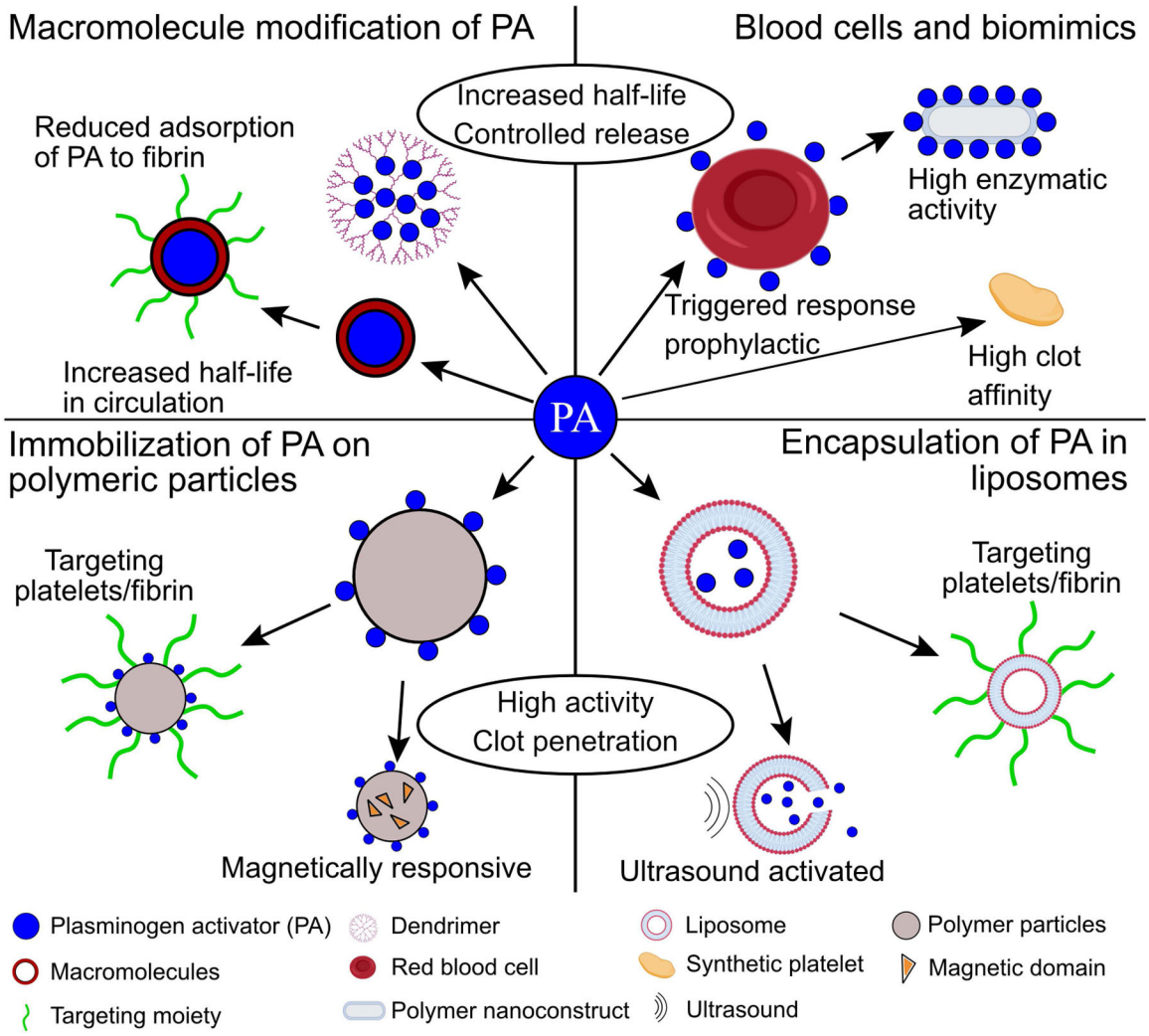

Figure 1.

Overview of engineered particles for the delivery of plasminogen activators (PA) covered in this review with features of different materials, targeting strategies, and actuation.

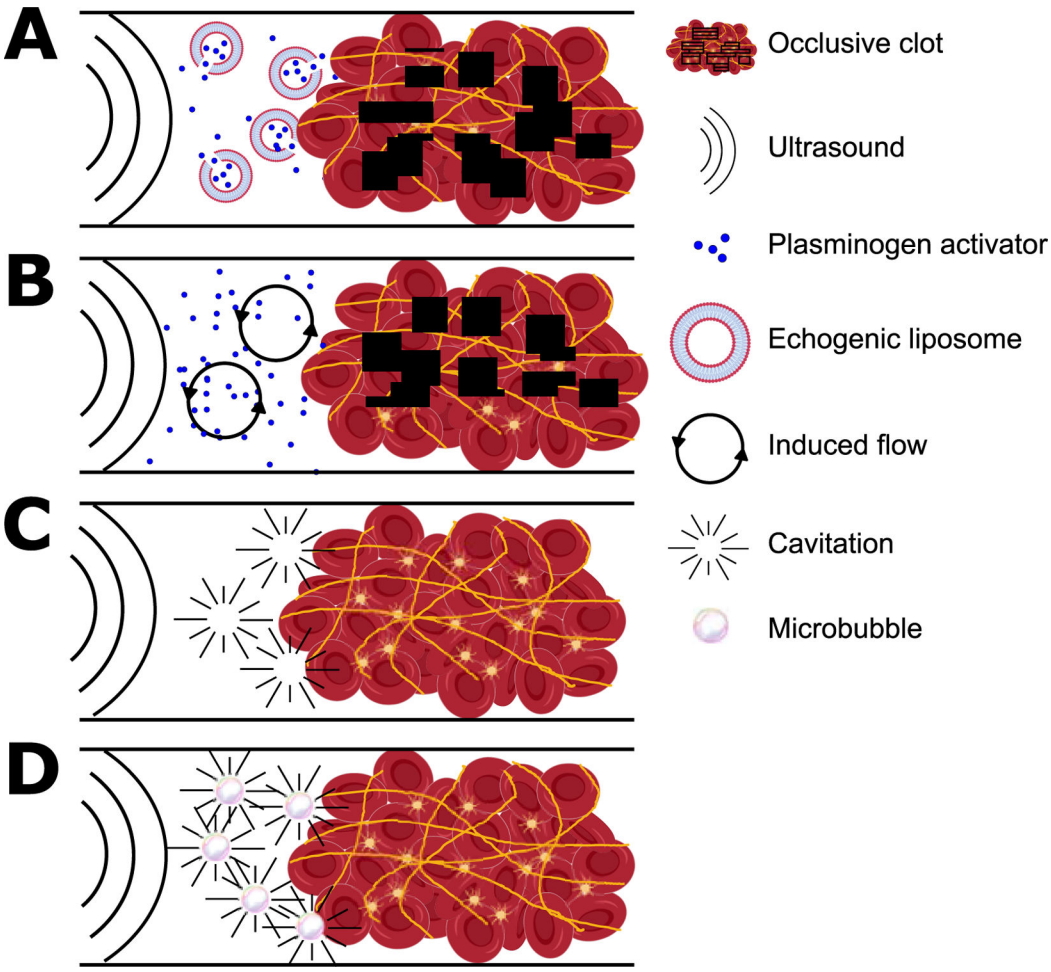

Figure 2.

Mechanisms of assisting thrombolysis using ultrasound. A) Ultrasound forces cause ELIP to deliver a payload of PA near a thrombus. B) Sonic forces cause acoustic streaming that induces flow and improves transport of PA to a thrombus. C) Sonic forces cause cavitation, which imparts mechanical energy into the thrombus and initiates degradation even in the absence of PA. D) Microbubbles increase the frequency of cavitation and accelerate lysis. See Table 1 for details and references.

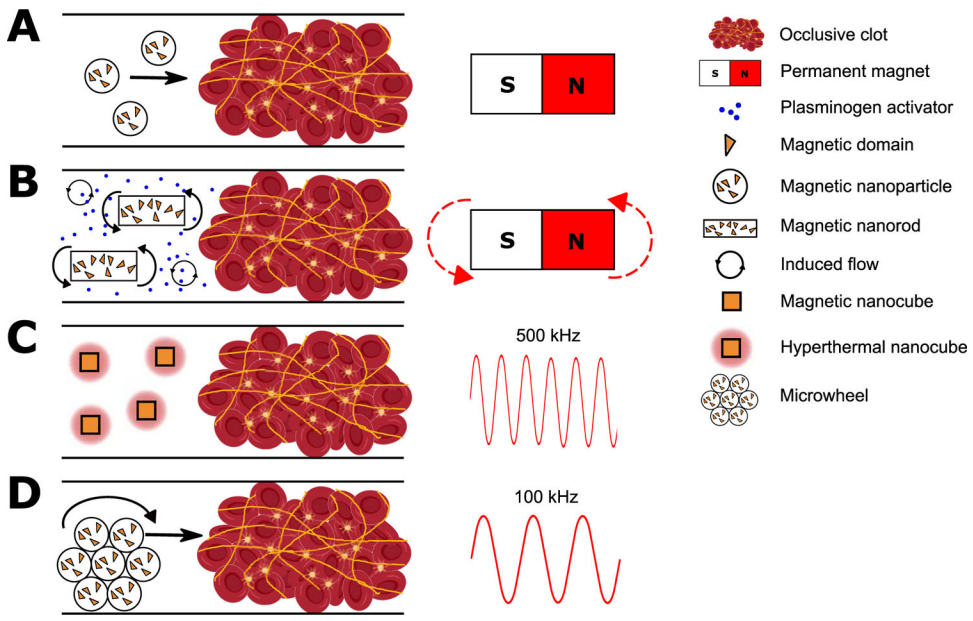

Figure 3.

Methods of magnetic control of magnetic nanoparticles (MNP). A) MNP move down a magnetic field gradient toward a clot using a permanent magnet. B) A rotating magnet is used to rotate rod-like MNP, inducing convection and improving transport of PA to a clot. C) A high frequency magnetic field induces local heating of cube-like MNP. D) A low frequency rotating magnetic field causes MNP to assemble into microwheels and roll into a thrombus. See Table 2 for details and references.

Table 1.

Clinical studies using microbubble-assisted sonothrombolysis.

Vessel	Number of patients	Initial NIHSS score (Range)	3-month favorable outcome	Ultrasound frequency	Gas	Microbubble size	Microbubble stabilizing agent	Ref.
MCA	15	17 (6–28)	40%	2 MHz	C_3F_8	1–2 μm	Lipids	[72]
MCA	111	18 (15–19)	56%	2 MHz	Air	2–8 μm	Galactose	[70]
MCA, ACA or PCA	35	18 (9–21)	72%	2 MHz	C_3F_8	1–2 μm	Lipids	[71]
MCA	138	17 (12–20)	46%	300 kHz	Air or SF_6	2–8 μm (air) or 1.5–4.5 μm	Galactose (air) or Lipids	[99]

National Institute of Health Stroke Scale (NIHSS) rates the severity of an occlusion. A higher score indicates a more severe clot with NIHSS > 4 requiring treatment. A 3-month favorable outcome is characterized by a decrease in the NIHSS score to 0–3. MCA, middle cerebral artery; ACA, anterior cerebral artery; PCA, posterior cerebral artery.

Table 2.

Summary of preclinical studies for MNP targeting and actuation.

PA	Particle matrix	Magnetism	Iron oxide loading	Magnetic guidance	Type of experiment	Lysis rate relative to soluble PA	Ref.
tPA	PLGA	SP	5 wt%	None	None	Not Compared	[85]
uPA	PEG	P	0.54 wt%	PM	In vitro	1.8	[87]
uPA	PEG	P	2 wt%	PM	In vitro	3.7	[88]
tPA	PLA-PEG	SP	Unmedicated	Translating PM	In vitro	2.7	[93]
tPA	Silica	SP	9.4 wt%	PM	In vitro	1.7	[83]
tPA-SK	Silica	SP	0.2 mg/mL	PM	In vitro	2.2	[79]
uPA	Hydrosol	SP	1 wt%	PM	In vitro	3.0	[84]
tPA	None	SP	6 wt%	Rotating PM	In vitro	1.4	[91]
tPA	Dextran	SP	0.25 mg/mL	PM	In vitro	Not Compared	[81]
tPA	PAA	SP	8 wt%	Translating PM	Rat, iliac artery embolism	>1.6	[86]
uPA	Dextran	SP	20000 IU/mL	PM	Rat, arteriovenous shunt	5.0	[80]
tPA	Chitosan	SP	9.5 wt %	Translating PM	Rat, iliac artery embolism	2.1	[82]
tPA	Poly aniline	SP	27.6 wt%	PM	Rat, iliac artery embolism	6.5	[78]
uPA	Heparin	SP	8 wt%	PM	Rat and rabbit, carotid	20.8	[89]
tPA	Ferrolipids	SP	Unmedicated	PM	In vitro	~20	[97]
tPA	Aluminum	M	Unmedicated	Rotating PM	In vitro	3.3	[95]
tPA	Nickel	P	Unmedicated	Rotating PM	In vitro	1.8	[94]
tPA	BSA	SP	~0.5 mg/mL	AC Field	Mouse, mesentery vasculature	~1000	[96]
tPA	Polystyrene	SP	3.6 ug/ mL	Helmholtz coils	In vitro	3.3	[74]

PA, plasminogen activator; PLGA, poly-(lactide-co-glycolic) acid; PEG, polyethylene glycol; PLA-PEG, poly(D,L-lactide)-co-poly(ethylene glycol); PAA, polyacrylic acid; BSA, bovine serum albumin; SP, superparamagnetic; P, paramagnetic; M, permanently magnetic; PM, permanent magnet; AC, alternating current.

Table 3.

Summary of approaches to engineered fibrinolytic macromolecules and particles

Method	Advantages	Disadvantages
	Macromolecules	
Polymer coatings	Increased half-life	Reduced activity
	FDA approved materials available	Low specificity
		Low mobility in occluded vessels
Dendrimers	Increased half-life	Low specificity
	Reduced adsorption	Low mobility in occluded vessels
	Variable loading	
	Modified blood cells and cell mimics	
Targeting	Targets components of thrombi	Low mobility in occluded vessels
	Reduced bleeding risk	
Prophylaxis	Preventive Triggered release	Requires presence in blood before vessel occlusion
	Degrades clot from inside out	Complicated preparation/synthesis
Shear-activated release	Reduced bleeding risk	No shear in fully occluded vessel
	Triggered release	Requires presence in blood before vessel occlusion
	Liposomes and polymer particles	
PA Encapsulation	Increased half-life	Low specificity
	Controlled release	Difficult to control stability
	Reduces adsorption to fibrin	Low mobility in occluded vessels
PA Immobilization	Increased half-life	Reduced activity
	Decreased PA inhibition	Low mobility in occluded vessels
	Increased clot penetration	
	Used in other clinical applications	
	Actuation	
Sonic actuation	Enhanced lysis	Limited mobility in occluded vessels
	Locally induced flows	Limited targeting/specificity
	Successful phase II clinical trials	
Magnetic actuation	Compatible with most engineered particles	Early stages of development (preclinical)
	High mobility, targeting	Challenging scale-up
	Local hyperthermia	
	High local concentration	