2.1. Molecular targets available for antithrombotic nanomedicine

Numerous promising targets have been reported and explained in the literature, according to their specificity and presence on thrombi which are depicted in **Figure 2.1A**. Antithrombotic drugs are categorized into three major classes anti-coagulants, anti-platelets, and thrombolytic agents. Each category of these drugs has its own individual targets in the coagulation cascade to control or manage the proper hemostasis. Anti-coagulants are the drugs mainly used in the prevention of thrombotic events as they have no therapeutic effect on already formed thrombi. These drugs reduce the catalytic activity of serine proteases (clotting factors) involved in the coagulation cascade and stop the growth of the thrombus by keeping clotting factors inactivated. Anticoagulant drugs target mostly the central part of the cascade, which involves clotting factors such as factor VII, factor IX, factor Xa, and thrombin [8, 48-50]. Antiplatelet drugs mainly interfere with platelet function and are used for both prophylaxis and acute treatment of thrombosis. These drugs target the upstream part of the coagulation cascade and prevent platelet aggregation/adhesion. The molecular targets exposed during this phase of the cascade are the thromboxane A2(TXA2) receptor, ADP receptor (mainly P2Y12), GPIIa/IIIb receptor, and PAR1 [9, 51-53]. GPIIa/IIIb integrin receptors are well-explored targets in the field of thrombosis. The antagonists of these receptors halt the platelet aggregation process by inhibiting the binding of fibrinogen with the activated platelets [54]. Additionally, PAR1 and PAR4 were identified as newer targets for antiplatelet therapy and their antagonists represent the current generation of antiplatelet drugs [48, 55, 56]. Thrombolytic agents (fibrinolytic) are mainly plasminogen activators that cleave the bond between the Arg-Val of plasminogen and induce the activity of the proteolytic enzyme plasmin. Plasmins further degrade the fibrin and thus, these are useful in thrombolytic applications [8]. Schematically, different targets of antithrombotic drugs have been presented in **Figure 2.1 B**. These

molecular targets were reported based on their role in the hemostatic mechanism and the categories of antithrombotic drugs and interestingly, they all can serve as targets for antithrombotic nanomedicines. The prerequisite criterion for nanoparticulate antithrombotic targets is that the thrombus site ought to be accessible by the nanoparticle that is administered peripherally into the bloodstream. At the same time, the optimal antithrombotic nanomedicine should have a considerable level of specificity toward the desired area, thus minimizing the overall systemic effect. The molecular targets currently being investigated for the prophylaxis and treatment of thrombosis are presented in **Table 2.1**.

Antiplatelet drugs mainly interfere with platelet function and are used for both prophylaxis and acute treatment of thrombosis. These drugs target the upstream part of the coagulation cascade and prevent platelet aggregation/ adhesion. The molecular target exposed during this phase of the cascade is the thromboxane A2 (TXA2) receptor, adenosine 5-phosphate (ADP) receptor, GPIIa/IIIb receptor, and PAR1 [57]. Platelet activation is the initial step in the coagulation cascade, comprises of shape change and activation of different catalytic proteins and enzymes that amplify further steps. Aspirin is the most commonly prescribed antiplatelet drug in day-to-day practice. It inhibits the activity of the cyclooxygenase enzyme that is needed for the synthesis of TXA2 (potent platelet activator). Aspirin is used as a preventive drug in cardiovascular disease to reduce the risk of myocardial infarction (primary prevention) as well as also in patients who already have myocardial infarction to avoid further severity due to the growth of thrombus (secondary prevention) [58]. Another category of antiplatelet drugs targets the platelet adhesion process by acting predominantly on the ADP receptor (P2Y12) and inhibits them, followed by the reduction in platelet activation. Clopidogrel is the most common drug in this category, mainly prescribed to patients suffering from acute coronary syndromes like unstable angina (which involves

chest pain and subsequently reduced blood supply) [52, 53]. GPIIa/IIIb integrin receptors are a well-explored target in the field of thrombosis. The antagonist of these receptors ceases the platelet aggregation process by inhibiting the binding of fibrinogen with the activated platelets. They serve as a substitute for fibrinogen and bind themselves with the GPIIa/IIIb receptors and stop the further amplification step of the coagulation cascade. Examples of GPIIa/IIIb receptor antagonists are abciximab, eptifibatide, tirofiban, etc. Generally, these drugs are given as short-term therapy for patients with percutaneous coronary intervention [54]. The protease-activated receptor 1(PAR 1) antagonist is the current generation of antiplatelet drugs. PAR 1 and PAR4 were identified as newer targets for antiplatelet therapy. Vorapaxar is a reversible PAR 1 inhibitor given orally. In an earlier study, it was reported, the addition of vorapaxar in the antiplatelet therapy of coronary artery disease decreases the risk of mortality but increases the risk of intracranial hemorrhage [48, 55].

Thrombolytic agents (fibrinolytic) are mainly the plasminogen activators that cleave the bond between the Arg-Val of plasminogen and induce proteolytic enzyme plasmin. These plasmins further degrade the fibrin and are used in thrombolytic applications. They are categorized into three-class streptokinase, urokinase, and tissue plasminogen activators (tPA)such as alteplase, reteplase, anistreplase, etc. Streptokinase and anistreplase mechanism of action is slightly different from other thrombolytics as it acts by complex formation with plasminogen in plasma and creates an activator complex. The formed complex transforms residual plasminogen into fibrinolysin (proteolytic enzyme) [8].

Molecular targets at the thrombus site ought to be attainable by the nanoparticle that administered peripherally into the bloodstream. At the same time, the optimal nanoparticle should have a quite elevated level of specificity at the desired area, thus minimizing the overall systemic effect. In designing the targeted nanoparticulate systems a major challenge

comes across, that the expression of receptors varies according to individual vascular entities. In other words, each of the cellular structures has its own expression of receptors that regulates their functions inside the body (**Table 2.1**). The type of antithrombotic drug loaded into the nanocarrier decides their use in different stages of a thrombotic event. Mainly anticoagulant is used for both acute and chronic condition before and after surgical intervention. Anti-platelet drugs are used mostly during and after surgical intervention (stenting) and thrombolytics are mostly used for the initial treatment of acute and chronic thrombosis.

Table 2.1. Molecular targets currently being studied for nanotherapeutics in the prophylaxis and treatment of thrombosis

Target	Drugs available for target	Available Targeting peptides	Nanocarriers used with targeted peptides for antithrombotic drug delivery	Ref.
P-selectin	------	Fucoidan,	Polymeric nanoparticles	[59]
		EWVDV		[60]
Thrombin	Heparin, Lepirudin, Desirudin, Bivalirudin, Argatroban	Thrombin cleavable sequence(rkrk(LVPR G-rkrk)3,	Nanocomplexes, PFH based nanoparticles	[12]
		PPACK peptide		[13]
Fibrin	Streptokinase, urokinase, reteplase, alteplase, tenecteplase	CREKA peptide,	Polymeric nanoparticles	[61]
		GPRPP peptide sequence		[62]
GPIIa/IIIb	Abciximab, eptifibatide,tirofiban	CQQHHLGGAKQA GDV Peptide sequence	Liposomes, polymeric nanoparticles, mesoporous silica nanoparticles	[28]
		RGD peptide		[63-65]
		cRGD peptide		[66]
		KGDS peptide		[67]
GP Ia	----------	----------	-----	
PAR	Vorapaxar	SFFLRN peptide sequence	------	[68]

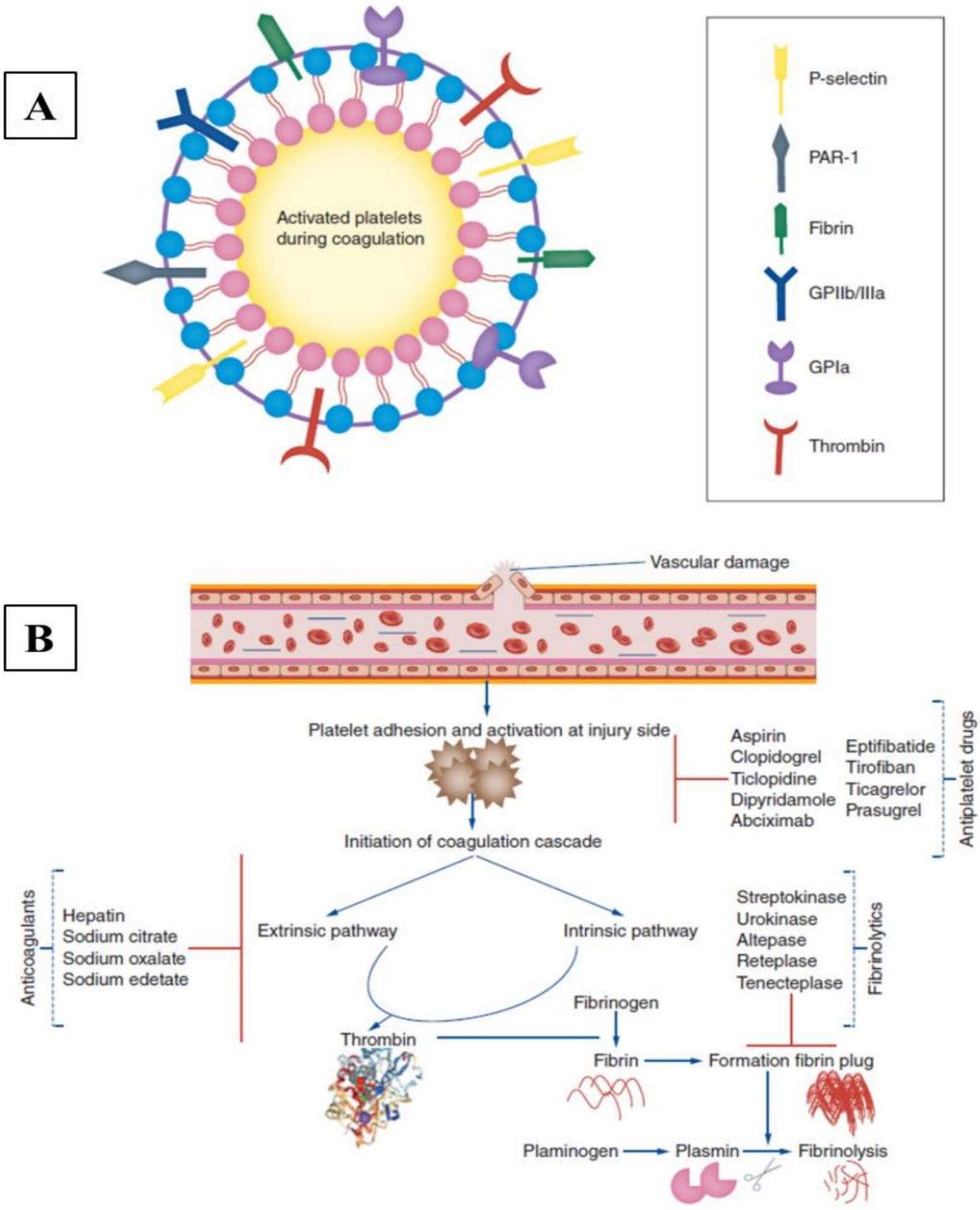


Figure 2.1. Molecular targets of thrombus and the available drugs for targeting. (A) Molecular targets available at the thrombus site during hemostasis and (B) drugs used to control thrombosis: targets of different antithrombotic drugs [18]

2.2. Nanoplatforms developed for anti-thrombotic therapy

Nanomedicines allow better API partitioning in the bloodstream when administered parenterally due to albumin and other binding processes. Because many small-molecule drug candidates can be potentially harmful due to high serum concentrations (Cmax) after injection and during systemic circulation, encapsulating them in nanocarriers allows their release to be

adjusted through controlled release. Additionally, rapid clearance of an API after injection can be avoided by encapsulating the nanoparticle and decorating the surface with appropriate functional groups for prolonged release and enhanced circulation. By directing a medicine to a specific site or countering the drug's release, nanoparticle-based therapies have the potential to reduce systemic toxicity and improve efficacy.

A plethora of delivery methods are available for stabilizing proteinaceous drugs with due consideration to their bioavailability and pharmacokinetics. Among these, the encapsulation of drug molecules into different nanocarriers is a widely used method [69]. Under the protection of these nanocarriers, the protein (anti-thrombotic drugs) is shielded from the external environment and does not affect normal tissues or cells. They are also kept protected against degradation by different hydrolytic enzymes when administered parenterally. Another advantage of these carriers is that they are capable of delivering multiple drugs at the same time. In addition, because of their small size, they can also evade the renal filtration process and are defended against RES uptake, mainly in the liver and spleen [8].

2.2.1. Liposomes

Generally, liposomes are bilayered lipid vesicles that are composed of phospholipid and cholesterol and have an aqueous inner compartment. They are biocompatible as well as non-toxic in nature. Usually, they are self-assembled during preparation by the lipid components and form a spherical sac-like structure enclosing the aqueous phase covered with the lipoidal membrane [70, 71]. To incorporate proteinous drugs like streptokinase, tissue plasminogen activator (t-PA), etc., liposomes can be utilized as a carrier to stabilize and modulate the pharmacokinetics of these molecules [72]. These drugs can be added to liposomal vesicles via different techniques based on their physicochemical properties. Hydrophilic proteins are mainly encapsulated in the aqueous core of liposomes. In contrast, lipophilic proteins arrange

themselves in the phospholipid bilayer and a portion of remaining proteins can be attached to the surface by electrostatic interaction.

Additionally, liposomes permit surface modification by the attachment of different peptides/proteins (streptokinase, t-PA, urokinase, etc) and provide a system for site-specific targeting [73]. Encapsulation of plasminogen activators (PA) into liposomes allows steric separation from local media and offers protection during preparation, storage, and circulation after administration. This can enhance PA stability and bioavailability with the potentiation of bioactivity as well. Moreover, the administration of these PA-tagged liposomal nanocarriers by parenteral route allows targeted access to the thrombotic sites (blood cells, clots, and endothelial cells) [74, 75]. There are many types of liposomes that are used to deliver anti-thrombotic drugs, like conventional liposomes, PEGyalated liposomes, targeted liposomes, and echogenic liposomes (especially for t-PA). A number of studies performed on animal models have suggested that streptokinase encapsulated in liposomes reduces reperfusion time with a reduction of dose, significantly increases clot lysis or thrombolytic activity, decreases further clot formation and also enhances the accumulation of streptokinase near to thrombus [72, 76]. Various liposomes have been fabricated to encapsulate t-PA in prior studies and it was noted that the presence of lipid content reduced the extent of plasminogen activation due to negatively charged compounds (such as phosphatidylserine, and sulphatide) [77]. Later, egg phosphatidyl choline-based liposomes, prepared by the film hydration method, showed up to 92% encapsulation. However, the addition of detergent (0.05% tween) to avoid non-specific interaction during preparation led to the loss of most of the encapsulated t-PA (only 25% left) because of damaged integrity of liposomal vesicles during ultracentrifugation [78]. PEGylated liposomes, loaded with anti-thrombotic drugs (PA, streptokinase, urokinase), have been proven to have prolonged circulatory effects with increased half-life and also exhibit higher thrombolytic activity than free drug [45, 79]. In

addition to these examples, thrombus-targeted liposomes have been progressively investigated by numerous groups of scientists [37, 73], which are summarized in the section 'ligand-based thrombus targeted nanomedicines'.

2.2.2. Micelles

These are self-assembled colloidal spherical nanostructures composed of a lipophilic core and hydrophilic shell. Micelles generally consist of several (usually more than 100) block co-polymer chains and possess a diameter of 20–50 nm. They have two spherical concentric zones; one is closely packed hydrophobic blocks and the other is a dense hydrophilic brush-like shell. Their application in drug delivery has emerged recently in the treatment of various diseases, especially cancers. These micelles have multiple advantages over other nanocarriers such as higher drug loading efficiency, prolonged circulatory half-life, and the ability to be conjugated with many ligands at a time for targeted delivery of drugs [80, 81]. In regard to anti-thrombotic therapy, very few studies have been reported that utilize micelles as a drug carrier. Dabigatran is an anticoagulant drug that suffers bioavailability-related problems due to its limited absorption. D-alpha tocopherol polyethylene glycol 1000 succinate (TPGS) is a water-soluble vitamin E derivative and was used for fabricating dabigatran-loaded micelles. The cellular uptake of these micelles was enhanced significantly (2- to 2.6 times) in the Caco-2 cell monolayer and the oral bioavailability of these micelles was also improved by 3.37-fold in rats [82]. In another study, heparin micelles loaded with indomethacin were explored. They were shown to synergistically enhance antithrombotic activity and caused less coagulation or thrombus formation as compared with free heparin [83]. A site-specific thrombus targeting approach was also explored by using annexin V conjugated micelles. These micelles consisted of a triblock copolymer loaded with lumbrokinase and linked with annexin V by using carbodiimide chemistry. *In-vitro* and *in-vivo* studies suggested a higher

affinity of these tagged micelles toward thrombi, as well as increased thrombolysis by targeted micelles as compared with nontargeted micelles or free lumbrokinase [84].

2.2.3. Polymeric nanoparticles

These are nanocarriers consisting of different polymers obtained from synthetic (poly-d,l-lactide-co-glycolide [PLGA], poly-ε-caprolactone [PCL], polyethylene glycol [PEG], Poly-alkyl-cyano-acrylates [PAC]) and natural (polylactic acid [PLA], chitosan [CT], gelatin [GT]) sources. These polymers are mainly biodegradable, biocompatible, and less toxic, and hence have been widely used in the preparation of polymeric nanoparticles (PNPs) [85]. PNPs can be fabricated as a solid assembly or as a bilayer of amphiphilic atom-like liposomes. As per published reports, PEG functionalization of nanoparticles defends against RES recognition and enhances their circulation time [86]. A study was carried out to encapsulate t-PA into chitosan-coated PLGA nanoparticles conjugated with arginyl-glycyl-aspartic acid (RGD) peptide sequence. It was demonstrated that compared with free t-PA, PLGA-chitosan-nanoparticles, and RGD-PLGA-chitosan nanoparticles dissolved clots in shorter times and also considerably enhanced the weight of clots digested [87]. A few other studies have also been reported that utilize polymeric nanoparticles as a carrier system for antithrombotic drugs, shown to enhance the drug efficiency and half-life with a significant increase in clot lysis [30, 61].

2.2.4. Material-based nanoparticles (inorganic nanoparticles)

These nanoparticles mainly consist of compounds that contain silica, iron, gold, silver, and copper salts. They tend to agglomerate and to avoid this, polymeric coatings (organic molecules) have been extensively utilized to form a protective outer shell. In the delivery of antithrombotic drugs, iron-based nanoparticles are widely used due to their MRI contrast property. Generally, they comprise of a core (made of paramagnetic iron oxide) and a shell (made of lipids and polymers) to improve their colloidal stability in a medium. The oxides

used in core fabrication are either magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) or a combination of both. According to size, they are categorized into two types; ultra-small superparamagnetic iron oxide (USPIO) nanoparticles which have a particle size less than 50 nm, and superparamagnetic iron oxide (SPIO) nanoparticles with a particle size ranging from 50 to a few 100 nm. These magnetic nanoparticles are biocompatible and biodegradable, as they take part in the homeostasis process [88]. Another crucial aspect of magnetic nanoparticle's application in drug delivery includes their extraordinary magnetic capabilities. When a strong magnetic field is applied externally, the magnetic nanoparticles can accumulate at a particular site. This property can be utilized for target-based therapy in cardiovascular diseases [32]. For example, a urokinase conjugated magnetic nanoparticle was designed for targeted thrombolysis. The application of an external magnetic field to guide these nanoparticles enhanced the thrombolytic effect by five-times with little prolongation in bleeding times [27]. Further, mesoporous silica nanoparticles have also shown the potential to deliver anti-thrombotic drugs. In one study, heparin assembly with mesoporous silica nanoparticles (MSN) and gold nanoclusters (GNC) was explored. Electrostatic interaction was used for heparin (negative) binding on the surface of positively charged MSN (functionalized with the amino group) and GNC was further added to enhance the fluorescence, providing an analytical tool for heparin analysis [89].

2.2.5. Other biological vectors

These drug delivery carriers include viral vectors and nonviral vectors (like platelets, red blood cells [RBC]). Modified viral particles have been utilized as nanocarriers for drug delivery applications in various diseases [90-92]. They draw considerable attention because of their natural origin, biocompatible nature, and reduced toxicity and thus can be given the title of nature's nanoparticles. Considering their symmetrical and well-established structure, plant-originated viruses have advantages over other synthetically derived nanocarriers [90].

Also, they are more favored viral vectors compared with their mammalian counterparts due to their safety profile. The Cowpea mosaic virus (CMV) is a plant-derived viral nanoparticle with a size of 30 nm and is widely utilized for biomedical applications mainly in the areas of cancers and cardiovascular diseases. These viruses are used commonly because of their ordinary binding ability to vimentin on the cell surface and have been extensively exploited to target vascular lesions [90]. Numerous studies have reported RBCs as a drug carrier as well. PA coupled with RBCs demonstrated *in-vivo* properties useful for thromboprophylaxis. They can prolong circulation times, minimize extravasation, and perform lysis of nascent clots preferentially over preexisting ones [93-95]. Various nanocarriers employed for the delivery of antithrombotic drugs have been illustrated in **Figure 2.2 A**.

2.3 Strategies involved in nanocarrier based thrombus targeting

The mechanisms involved in the delivery and release of drugs from nanovesicles at the target site can be classified as active and passive targeting. Active targeting deals with the stimulation of drug release by manipulating the features of the carrier. It gives narrow control of drug distribution and specifies the area of action. A driving stimulus is needed to guide this process, possibly overexpressed receptors, or some external force like magnetic/electric field, sound waves, temperature, light or pressure, etc. The working principle for targeted thrombolysis is presented in **Figure 2.2 B**.

On the other hand, passive targeting utilizes the regular human physiological mechanisms or immune response as a driving principle to deliver the active molecules to the target area. The drug is released by a simple diffusion process from the nanocarrier at the local site through the metabolic process present in individuals [47]. In comparison with conventional therapy, nano carrier-based formulations demand a much reduced amount of drug considering the higher localization of the drug at the target site. This way, the overall side effects of the drug will be reduced due to minimized doses. Additionally, the outer shell conjugation with

thrombolytic agents or thrombus-specific ligands can potentiate the localization of these nanoparticles that prevent the further formation of thrombus and serves as a part of thrombus targeted therapy [96]. Numerous strategies reported to target thrombotic events via exploiting active and passive targeting are summarized in **Figure 2.3**.

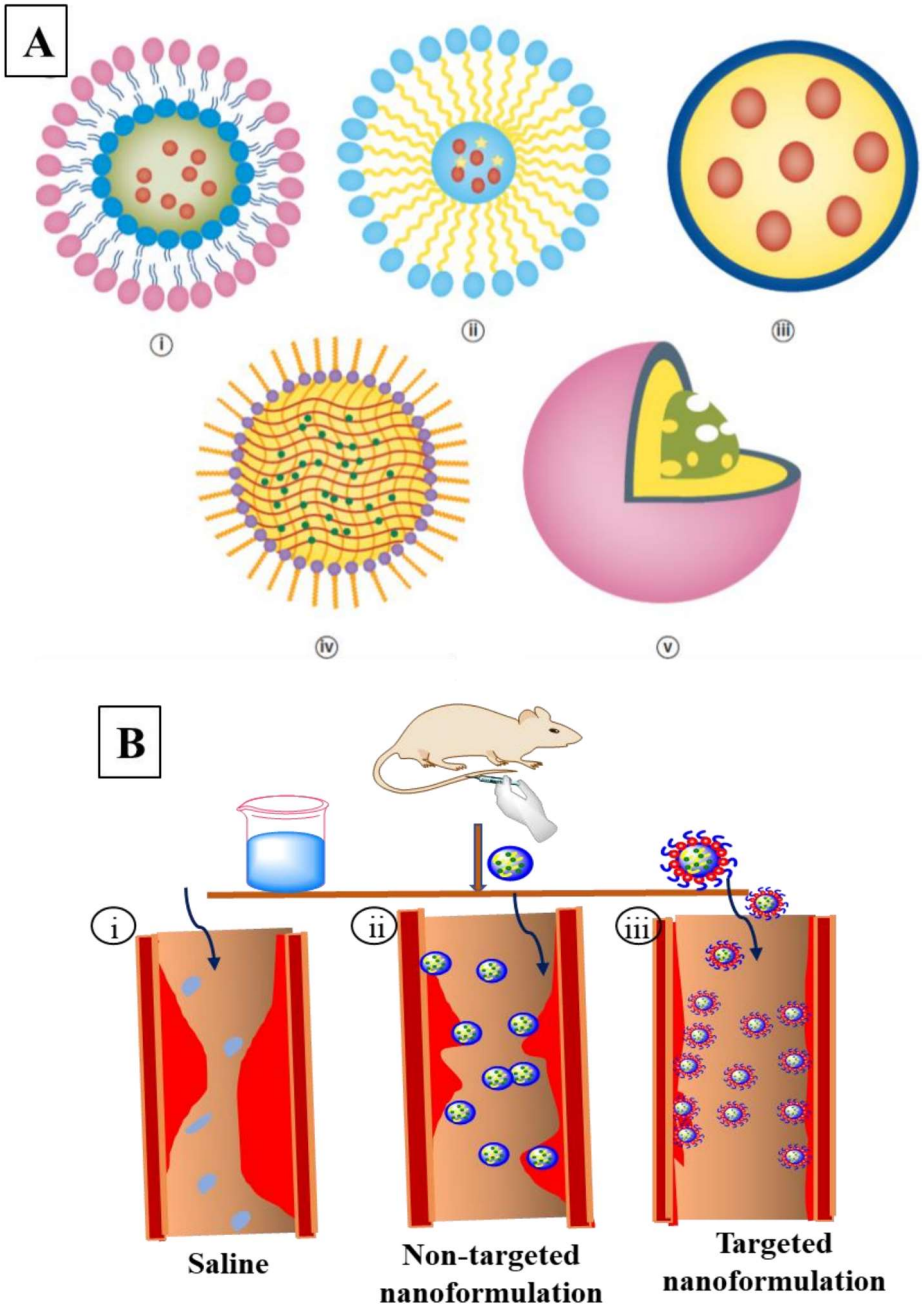


Figure 2.2. Anti-thrombotic nanomedicine and their mechanism of action. (A) Nanocarriers for targeted anti-thrombotic drug delivery (i) liposomes (ii) micelles (iii) biological vectors (iv) polymeric nanoparticle (v) material based (silica) nanoparticle, (B) working principle of targeted thrombolysis. (i) thrombus remains with saline treatment, (ii) nanoformulation binding to local thrombus site helps to reduce clot formation, (iii) targeted nanomedicine works more potently and dissolves the clot [18]

2.3.1. Passive targeted antithrombotic nanotherapeutics

2.3.1.1. Shear-activated nanotherapeutics

Occluded or thrombosed blood vessels contain physical attributes distinct from normal vasculature. Mainly fluid shear stress of blood vessels varies from 70 dyne/cm2 (normal) to >1000 dyne/cm2 (obstructed), depending upon the extent of constriction in arteries. Generally, circulating thrombocytes are activated locally by high shear stress and adhere to the nearby surface of the lumen, which makes blood vessels narrower, and leads to the formation of atherosclerotic plaque. The same natural phenomenon, which involves shear force-stimulated platelet aggregation, can be utilized as a thrombus targeting strategy for the treatment of thrombosis [97]. Generally, thrombosis develops in a large area of blood vessels (e.g., in arterial stenosis, fatty deposition of plaques on the vessel's wall) and causes total cessation of blood flow. In this condition, an increase in hemodynamic shear rate (>5000 s−1) can be observed. This shear force is mainly responsible for shear-induced platelet aggregation which remains the common pathway for thrombosis resulting from hemostasis and arterial pathology [98]. Recently, a novel passive targeting approach has been developed, named 'shear activated nanotherapeutics' (SAN). SANs are approximately similar in size to platelets; however, fabricated as micro aggregates composed of a number of nanoparticles. They usually remain intact in normal physiological bloodstream, but when they come in contact with higher shear stress, they break apart. As a consequence, nanoparticles get separated and adhere efficiently to an adjacent surface (blood vessel lumen) due to lower drag forces exhibited by the small size. The site-specific adhesion can be further improved by the surface coating of these nanoparticles with molecules that bind to fibrin clots. In this way, a significant elevation in therapeutic concentration at the local thrombus site can be achieved. This SAN takes benefit of the distortion of blood flow in vessels that occurred due to the clot itself [97]. Lenticular nanovesicles made from synthetic 1,3-diaminophospholipid were also

explored for shear stress-sensitive delivery in constricted blood vessels. Using a cardiovascular *in-vitro* model consisting of polymeric tubes and a pump to mimic constricted vessels with a high shear stress of the human heart, these nanovesicles were analyzed for their efficiency. The lenticular nanovesicles release the encapsulated drug at high shear stress due to constricted vessels and were proven as a potential alternative for antithrombotic drug delivery [99]. The extent of drug release depends on the number of nanoparticles. Moreover, for using these methods, extremely specialized nanocarriers that can load specific drugs effectively and keep them safe from conformational changes while binding to the fibrin are needed [100]. The passive targeting of thrombus with nanotherapeutics is depicted in **Table 2.2**.

2.3.1.2. Stimulus-sensitive antithrombotic nanomedicines

The emergent role of nanotechnology in drug delivery applications has allowed a plethora of strategies to improve the safety and efficacy profile of therapeutic agents. Many of these approaches have been inspired by different biological mechanisms that exist naturally by the combination of multiple components to trigger a response. In order to develop nanosystems that can precisely symphonize and control biological processes occurring in the human body, these approaches can be utilized. Recently, a novel approach for the application of antithrombotic therapy has been used by Lin and coworkers. They have developed a bio-responsive peptide that is able to form nanocomplexes and exploits negative feedback mechanisms to self-titrate the release of anticoagulants (heparin) with reference to differing coagulation activity. These nanocomplexes are composed of PEGylated cationic peptide, which is mainly thrombin-cleavable substrate and anionic heparin. Release of heparin from these complexes takes place due to thrombin activity. However, released heparin interacts with anti-thrombin and inhibits thrombin activity. Thus, the initial release generates the negative feedback mechanism and helps to regulate the further release of heparin from nano

complexes. By this mechanism, more anticoagulants are deployed at the time of the thrombotic event and do not disturb the healthy coagulation process significantly [12]. Recently, the use of polymeric prodrugs with stimuli-responsive features (such as pH, temperature, light, redox, etc.) has been exploited for drug delivery applications. A novel polymer prodrug as a nanocarrier of antithrombotic drugs (diosgenin, proven to prevent thrombosis) has been fabricated recently. These prodrugs had prolonged circulation time and could self-assemble into micelles in the presence of aqueous media. When it came into contact with the acidic environment adjacent to the thrombus, it was cleaved to release the diosgenin at the target site. Additionally, it was also found that these diosgenin micelles are capable of preventing thrombosis without any bleeding complications by inhibiting platelet activation and apoptosis and were more efficient than clinically used antithrombotic drug aspirin [101].

Table 2.2. Different approaches to thrombus targeting by external mechanical force or by passive targeting

Type of approach	Materials used/drug	Model used	Advantages of nanocarrier	Ref.
Passive targeted nanotherapeutics				
Shear activated therapeutics				
PLGA nanoparticles microaggregates loaded with t-PA	PLGA./ t-PA	Mice model for pulmonary embolism, mesenteric injury model	Biophysical methods follow natural phenomena to target thrombus, shown potential in drug targeting with reduced dose and side effects.	[97]
Lenticular liposomes	DPPC, POPC, Pad-PC-Pad	*In-vitro* model (An extracorporeal heart pump is connected to a plastic model of healthy or constricted arteries)	These vesicles release drugs at high shear stress present in a mimicked constricted blood vessel.	[99]
Stimulus sensitive delivery				
Self-titrating nanocomplexes	PEG, thrombin activatable peptide, /heparin	Pulmonary Embolism Assay in mice, tail bleeding assay in mice	Utilizes a negative feedback mechanism to target thrombus and does not significantly affect healthy coagulation.	[28]
pH-responsive polymeric prodrug	PEG prodrug/ diosgenin	$FeCl_3$-induced arterial thrombosis model in rats, Middle cerebral artery occlusion model in mice	Novel antithrombotic nanosystem with pH-responsive character, has no bleeding complication and side effects.	[101]

Miscellaneous other strategies for nanotherapeutic based thrombus targeting				
By application of ultrasound				
Gelatin-t-PA nanoparticles	Gelatin, zinc oxide /t-PA	swine acute myocardial infraction model	This drug delivery system utilizes controlled stealth activity, shown recanalization in 9 out of 10 swine	[42]
Ultrasound responsive nanoparticles	Gelatin, PEG, ethylenediamine/t-PA	Rabbit thrombosis model.	Prolonged the half-life of t-PA about 3 times by the application of ultrasound.	[29]
u-PA loaded nanogel	Glycol chitosan, aldehyde capped PEG/u-PA	*In-vitro* clot lysis	They enhance the rate of *in-vitro* clot lysis	[102]
Dodecafluoropentane nanoparticle	Dipalmitoyl phosphatidylcholine, distearoyl phosphatidylethanolamine	Artificial ultrasound irradiation model	As compared to microbubbles, these NPs provide a better acoustic cavitation effect and improve thrombolysis efficiency	[103]
By application of an external magnetic field				
Superparamagnetic iron oxide nanoparticles	NH_4OH, $FeCl_3.6H_2O$, $FeCl_2.4H_2O$, Dextran	*In-vitro* models	These nanoparticles were very stable and were well tolerated in cell culture studies	[104]
Urokinase magnetic nanoparticle	Ferric chloride, dextran, ammonium hydroxide/ urokinase	Rat arteriovenous shunt thrombosis model	Urokinase conjugated with magnetic nanoparticles directed by the external magnetic field shows the local thrombolytic effect with reduced hemorrhagic complication	[27]
Surface functionalized agar coatedFe_3O_4 nanoparticle	Agar, sodium hydroxide, iron(III), iron(II), /urokinase	*In-vitro* model (microfluidic channel)	Efficiency to clear clots by lysis has been improved as compared to free urokinase.	[105]
Magnetically powered nanomotor	Fe_3O_4/ t-PA	Mice embolism model, *in-vitro* fluidic channel experiment	These nanomotor based delivery approaches enhance thrombolysis speed by 2-fold.	[100]
Dual targeting				
Platelet targeted dual pathway inhibition	SCE5-TAP	$FeCl_3$-induced arterial thrombosis model in mice	Inhibits two pathways by using anticoagulant and antiplatelet peptide recombinant fusion, shown promise enhancement in antithrombotic therapy	[106]
Mesoporous silica-polyglutamic acid dendrimers	RGD, Boc-L-glutamic acid, hexahydrate ferric chloride /nattokinase	$FeCl_3$-induced arterial thrombosis model in mice	Peptide targeting and applied magnetic field approach combined to deliver these NPs at the thrombus site showed significant thrombolysis.	[64]
Platelet microparticle inspires clot responsive liposomes	Cholesterol, BSA,DSPC/streptokinase	$FeCl_3$-induced arterial thrombosis model in mice	They utilize two targeting ligands to localize the encapsulated drug and found a significant increase in targeting efficiency over other nanosystems	[34]

PLGA- poly(lactic-co-glycolic acid), DPPC- Dipalmitoylphosphatidylcholine, POPC-1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, Pad-PC-Pad- 1,3-dipalmitamidopropan-2-yl 2-(trimethylammonio)ethyl phosphate, u-PA- urokinase-type plasminogen activator, BSA- Bovine serum albumin, DSPC-1,2-distearoyl-sn-glycero-3-phosphocholine, SCE5-single-chain antibody, TAP- tick anticoagulant peptide, $FeCl_3.6H_2O$- Ferric Chloride Hexahydrate, NH_4OH- Ammonium hydroxide

2.3.2. Active targeted antithrombotic nanotherapeutics

2.3.2.1. Ligand-based thrombus targeted nanomedicines

Nanomedicine approaches for targeted antithrombotic drug delivery for the treatment and management of thrombosis are a subject of growing interest [32, 107, 108]. Thrombolytic drugs encapsulated in lipid and PEGylated polymer, reported earlier, showed an increase in drug half-life as well as improvised safety aspects from drug degradation [45, 109]. Recently, these processes are combined with the active targeting approaches by using a short peptide sequence as a substrate of specific enzymes or cells at the target site. These peptide sequences facilitate the accumulation of nanovesicles at the local thrombus site and expedite thrombolysis while at the same time eliminating the hemorrhagic risks [110, 111]. Arginyl-glycyl-aspartic acid (RGD) tripeptide sequence exhibits affinity toward GPIIa/IIIb receptors present on activated platelets that are embedded in thrombus [37, 73]. The RGD peptide decorated nanocarriers, loaded with anti-thrombotic drugs, exhibit target-sensitive attributes and display improved anti-thrombotic therapy *in-vitro* [30, 44, 86, 87]. Similarly, a few others reported that the cyclic-RGD (cRGD) peptide sequence is having the same binding interaction as RGD and is available for the same purpose. However, the binding of cRGD with GPIIa/IIIb receptors is better than linear RGD peptides [7].

In an earlier report, polyglutamic acid-based dendrimers were fabricated, with the incorporation of nattokinase (as a thrombolytic drug) and RGD (as a targeting molecule) at the periphery by chemical bonding. The nattokinase incorporated in the dendrimer is protected by the polyglutamic acid layer, improving its enzymatic activity. *In-vitro* and *in-vivo* studies suggested that the chemical bonding did not degrade or reduce the activity of nattokinase and the dendritic particles can target the thrombi successfully [63]. Additionally, the mesoporous silica nanoparticles were also exploited for nattokinase delivery in combination with polyglutamic acid by the same group of scientists. They prepared dual-

targeted dendritic nanoparticles consisting of magnetic mesoporous silica core, sequentially bonded with polyglutamic acid and RGD. When a magnetic field was applied externally, the magnetic mesoporous silica with nattokinase can be guided to the target area. Simultaneously the RGD present on the surface of these nanoparticles allows specific binding to GPIIb/IIIa receptor and inhibits the platelet aggregation and improvises the effect of thrombolytic drug [64]. Moreover, thrombus-targeted liposomes have also been progressively investigated by numerous groups of scientists as targeted nanocarriers for anti-thrombotic drug delivery. Liposomes loaded with streptokinase and RGD surface decoration were prepared by Vaidya and colleagues for platelet directed targeting-based approach. They found that the targeting ability of these liposomes was relatively higher as they accumulated near to clot and even they were able to increase the thrombolytic effect of streptokinase [65]. In another study, a similar approach was used for thrombus targeting by using cyclic RGD (CNPRGDY(OEt)RC). An analysis through flow cytometry and fluorescence microscopy revealed higher target sensitivity of these liposomes. An *in-vitro* clot lysis assay of these liposomes showed a reduction in clot lysis time and simultaneously increased the extent of clot lysis [112]. In a later work, streptokinase-loaded liposomes, linked with cRGD, were prepared for the same purpose. An *in-vivo* study performed on a human clot-inoculated rat model for detecting thrombolytic efficacy suggested that targeted liposomes lysed more part of thrombi (28.27%) as compared with non-liposomal streptokinase (17.18%) [113]. Additionally, urokinase-encapsulated liposomes decorated with cRGD were investigated for thrombus targeting. *In-vitro* experiments demonstrated the plateau release (60%) within 5 h of iv. administration. A thrombolysis study performed in a mouse mesenteric thrombosis model suggests the reduction of urokinase dose by 75% when given in liposomal form [66]. Similarly, Zhang and coworkers synthesized the urokinase-loaded liposomes functionalized with cRGD for thrombolytic therapy. The presence of cRGD drove the liposomes to the target

area and facilitated the continuous local release of urokinase. *In-vivo* evaluation of thrombus lytic activity was done by the mouse mesenteric thrombosis model which suggested that these liposomes improved the thrombolytic activity by four times than plain urokinase [55]. In another report, the cRGD decorated N,N,N-trimethyl chitosan nanoparticles loaded with a thrombolytic agent (lumbrokinase) have been prepared by the ionic gelation method. The thrombolytic efficacy of these nanoparticles was significantly enhanced in clot occluded tubes (*in-vitro*) as well as in the carotid artery thrombus model in rats (*in-vivo*) [7].

A few other studies have also been reported that target the integrin GPIIb/IIIa receptor with other targeting peptides. In one of those studies, tPA-loaded liposomes were attached with a targeting peptide sequence (CQQHHLGGAKQAGDV) for targeted thrombolytic therapy. Liposomes were made as both PEGylated and non-PEGylated; the attachment of the targeting peptide facilitated the targeted delivery and PEGylation allowed control release for a longer duration and also provided a stealth effect. The formulation showed initial burst release (40–50% in 30 min) accompanied by later sustained release (up to 80–90%) in 24 h. In comparison with free t-PA, liposomal t-PA has shown a 35% increment in clot lysis *in-vitro* [28].

In contrast to GPIIa/IIIb receptors, P-selectin is limitedly explored for targeted thrombolysis. Moreover, it is a glycoprotein absent in resting platelets and expressed only in activated platelets, serving as a promising target to promote targeted anti-thrombotic drug delivery. An approach was demonstrated to make fucoidan functionalized polymeric nanoparticles that target P-selectin and guide the site-specific delivery. The thrombolytic efficiency of these newly fabricated nanoparticles was found to be improved in the *in-vivo* study performed on a mouse model of venous thrombosis [59]. Furthermore, an approach for fibrin-based thrombus targeting was investigated using perfluorocarbon nanoparticles, surface-functionalized with thrombolytic drugs and anti-fibrin monoclonal antibodies. However, this approach did not improve the clot dissolution but increased the enzymatic payload,

subsequently increasing the thrombolytic effect. These nanoparticles (rhodamine tagged), when observed under a fluorescence microscope, displayed a dense accumulation at intraluminal thrombus [114]. Besides, ferritin is a protein available biologically in the human body reported for its super-molecular nanosized assembly and can be functionalized easily without any perturbation of its nanocage-like structure. Ferritin nanocages were designed in conjugation with clot-targeting peptidic sequence (CLT:CNAGESSKNC), which only recognizes the complex of ferritin and microplasmin present in the clot while not affecting the free fibrins. Consequently, it showed higher efficacy toward the thrombus site after the iv. followed by a decrease in bleeding risks associated with current therapies [115]. The nanomedicine surface conjugated with targeting peptides/molecules for targeted thrombolysis is summarized in **Table 2.3**.

Table 2.3. Peptide conjugated targeted nanocarriers for delivery of anti-thrombotic drugs

Types of nanocarriers	Materials/incorporated drug	Targeting agent	Target	Size of particles	Outcomes	Ref.
Liposomes						
PEGylated liposomes	Cholesterol, phosphatidyl choline, DOPE, DSPE-PEG_{2000}-MAL/ t-PA	CQQHHLGGAK QAGDV peptide sequence	GPIIb/IIIa	~187nm	Pegylated and non-pegylated liposomes decorated with peptide sequences were synthesized to test the reduction in hemorrhagic risk by improving clot lysis by 35%.	[28]
cRGD functionalized liposomes	DSPE-PEG2000-NHS, DMF/ urokinase	Cyclic RGD peptide	GPIIb/IIIa	~157nm	cRGD liposomes reduced the dose of urokinase by 75%	[66]
Polyethylene glycol conjugated liposomes	DSPE-mPEG, DSPE-PEG-COOH/ streptokinase	RGD peptide	GPIIb/IIIa	~114 nm	These liposomes are long circulatory and shown clot lysis(26%) higher than free streptokinase(23 %).	[65]
Target sensitive cRGD conjugated liposomes	DOPE, DOPC/ streptokinase	CNPRGDY(OEt) RC peptide	GPIIb/IIIa	100-120nm	The *in-vitro* clot lysis of cRGD targeted liposomes was improved by 50% as compared to free streptokinase(30 %)	[112]

Dual targeting						
Platelet microparticle inspired nanovesicles	DSPE-PEG2000-Mal/ streptokinase	(GSSSGRGDSPA) (DAEWVDVS)	GPIIb/IIIa P-Selectin	-----	Induce targeted fibrinolysis without affecting systemic hemostasis. Provide safe and site targeted technology for thrombosis treatment.	[34]
Micelles						
Targeted mixed micelles for thrombolysis	Caprolactone, PMDETA, Stannous octoate, DMAEMA/ lumbrokinase	Annexin V	Phosphatidylserine on the surface of activated platelet at thrombus site	~167nm	Lumbrokinase loaded targeted micelles stability was improved by incorporating PEG, followed by increased targeting efficiency with reduced bleeding risk.	[84]
Polymeric nanoparticle						
PEG-PCL conjugated rt-PA nanoparticles	PEG-PCL/ rt-PA	Recombinant tissue plasminogen activator	Fibrin	~129nm	rt-PA pharmacokinetics parameters were improved and 73% of enzyme activity remained after conjugation with nanoparticles	[30]
Polysaccharide-poly isobutyl cyanoacrylate nanoparticles	Isobutyl cyanoacrylate, polysaccharide, carboxymethyl-dextran/ rt-PA	Fucoidan	P-selectin	~136nm	Recombinant tissue plasminogen activator loaded nanoparticles improved the thrombolysis efficiency in *in-vitro* and *in-vivo* models.	[59]
Phase transition nanoparticles	Fe_3O_4, India ink, PLGA, PFH	EWVDV short peptide	P-Selectin	~387nm	These particles capable of multimodal imaging by the US, MRI, and PA. and used both in the diagnosis and treatment of thrombosis.	[60]
LIFU phase transitional nanoparticles	Fe_3O_4, PLGA, Perfluorohexane	CREKA Peptide	Fibrin	~311.3± 4.3nm	Low intensity and frequency ultrasound responsive phase transition nanoparticles synthesized that induce thrombus burst	[61]

Aspirin polyconjugate nanoparticles	HEMA, IR780 iodide, Aspirin	GPRPP pentapeptide	Fibrin	300 to 400nm	Aspirin polyconjugate targeted nanoparticles has proved contrast agent based imaging and H2O2 activatable thrombolytic behavior successfully	[62]
Polyglutamic acid peptide dendrimer	Boc-glutamic acid /Nattokinase	RGD peptide	GPIIb/IIIa	~650nm	Polyglutamic acid dendrimer loaded with nattokinase was synthesized with RGD functionalization, tested for thrombolytic therapy with non-toxic degradation process	[63]
Material based nanoparticle(inorganic nanoparticles)						
Magnetic Nanoparticles	Ferric chloride, dextran, ammonium hydroxide/ urokinase	Urokinase Magnetic nanoparticles (iron oxide)targeted towards thrombus site by the magnetic field	Thrombus formation site	~116nm	Urokinase conjugated with magnetic nanoparticle directed by the external magnetic field shows local thrombolytic effect with reduced hemorrhagic complication	[27]
Mesoporous silica nanoparticles	TEOS, CTAB/ Nattokinase	RGD peptide	GPIIb/IIIa	~90nm	Nattokinase delivered to thrombus site by the recognition of RGD peptide, shows efficient thrombus degradation.	[64]
Nanocages	Gadolinium, NHS, EDC, FITC	KGDS Peptide	GPIIb/IIIa	11.59±0.37nm	It provides the benefit of thrombosis detection by MRI as well as thrombolytics can be loaded inside nanocages to use as theranostics purpose	[67]
Biological vectors						
Erythrocyte-tPA nanocarrier	Plasminogen, RBCs/t-PA	t-PA and rt-PA	Fibrin	---	RBC-coupled with PA prolonged circulation time and improve the fibrinolysis	[93]
Ferritin microplasmin nanocages	Ferritin, mictroplasmin/	CNAGESSKNC peptide	Fibrin-fibronectin complex	---	These nanocages shown prolonged circulatory time with efficient lysis of preexisting clot	[115]

					provides high efficacy and safety than existing therapies	
Others						
Perfluorocarbon nanoparticles	Perfluorooctylbromide, glycerine, surfactant	PPACK(phenylalanine-proline-arginine-chloromethyl ketone)	Thrombin	~250nm	Anti-thrombin peptide was used at the surface of nanovesicles to upregulate the deposition on clot site	[13]
Nanoparticles	Perfluoro-octylbromide, glycerin, lecithin	Active Anti-fibrin monoclonal antibodies	Fibrin	~354nm	Urokinase nanoparticles in conjugation with fibrin targeting ligand were prepared and an increased in targeting efficiency was noted	[114]
Nanoscale self-titrating activatable therapeutic (Nano STAT)	Heparin, peptide, Polyethylene glycol	(Thrombin cleavable sequence(rkrk(LV PRG-rkrk)$_3$))	Thrombin	150nm	Thrombin sensing peptide with anionic heparin nanocomplex for targeting thrombosis	[12]

2.3.3. Miscellaneous strategies involved in thrombus-targeted nanomedicine

2.3.3.1. Sonothrombolysis (US-mediated echogenic nanomedicine)

Sonothrombolysis is the process in which US is utilized to perform the thrombolysis alone or in association with a drug to potentiate its action in the nanoparticulate drug delivery system. The combined therapy shows a high degree of recanalization [116]. In sonothrombolysis based anti-thrombotic therapy, low-intensity focused US is applied for the treatment of ischemic stroke and deep vein thrombosis in combination with thrombolytic agents or contrast agents (such as microbubbles), facilitating an early clinical recovery or recanalization as compared with conventional intravenous drug administration. However, the combination of sonothrombolysis with thrombolytic drugs can still bring the associated risk of hemorrhage [117, 118]. High intensity focused ultrasound (HIFU) alone, to lyse the thrombi, has been explored as thrombolytic therapy *in-vivo* and *in-vitro*. The advantages of this approach over others include site-targeted treatment, thrombolysis in lesser time, and

non-requirement of thrombolytic drugs. However, some clinical problems remain unchecked as the production of clot debris during lysis. When this clot debris size is larger, it can obstruct the downstream blood vessels and result in emboli-based occlusion risks. Phase-change nanodroplets were designed to collaborate with HIFU to solve this issue. The clot debris particle size distribution resulting through sonothrombolysis with or without these nanodroplets was checked in the same conditions. The results suggested that these nanodroplets significantly reduced the volume percentage and average diameter of the clot debris and proficiently reduced the risk of occlusion caused by the application of HIFU alone [118]. The application of the US to improvize the action of thrombolytics (such as alteplase or t-PA) has been studied by a number of scientists with positive feedback. Uesugi et al. prepared tissue plasminogen activator (t-PA) loaded PEG-gelatin nanocomplex for targeted thrombolytic therapy. Gelatin was cationized by ethylenediamine and conjugated with t-PA, resulting in the complexing of gelatin to t-PA. Further, it was mixed with PEG to produce nanocomplexes containing PEG chains on their surface. This complexation process reduced the activity of t-PA to 45%, but it was completely recovered by US application [29]. Later, Kawata et al. developed US responsive nanosystem composed of gelatin and zinc acetate in which t-PA was loaded. When US was applied to the thrombus site, these nanoparticles released the t-PA following the degradation of the gelatin complexes [42]. Similarly, in another study, urokinase (u-PA) loaded nanogels were designed for US-mediated thrombolysis. These u-PA loaded nanogels have shown prolonged circulation times than u-PA alone. Additionally, by the US application, the release of u-PA was triggered that enhanced the thrombolysis significantly [103]. In the consecutive study, these nanogels were evaluated for *in-vivo* sonothrombolysis in the rat acute ischemic animal model. The study revealed the excellent controlled release profile of u-PA around the thrombus under US application [119]. In another approach, the dodecafluropentane nanoparticles were fabricated

without any antithrombotic agents. The US irradiation following administration of these nanoparticles showed improved thrombolysis with an increase in the acoustic cavitation effect [103]. In conclusion, thrombolysis can be improvised by concurrent utilization of nanomedicines, loaded with or without thrombolytic drugs, and US. The nanocarrier-based ultrasound-assisted thrombolysis may be a safe and effective process for pulmonary embolism patients with minor side effects.

2.3.3.2. Magneto-thrombolysis (magnetic field mediated magnetic nanomedicine)

Magneto-thrombolysis is a physical targeting technique employing an external magnetic field for site-specific delivery of magnetic nanocarriers for thrombolytic therapeutics. These carriers are usually composed of a magnetic nucleus/core and an outer covering shell (organic/inorganic). Generally, the shell of magnetic nanovesicles shares some extra attributes like keeping the magnetic carriers protected from aggregation, providing surface functionality, and improving biocompatibility. The potent magnetic properties and biologically compatible features of magnetic carriers contribute to various applications in the field of drug targeting (magnetically guided), MRI, and magnetic diagnosis of diseases [6]. In magnetic field-based targeting techniques, the location of the magnetic carrier is controlled by the external magnetic field; therefore eliminates many of the drawbacks that occur during systemic administration and can directly interact with the proteins available at the local thrombus site [47].

Mostly, Fe_3O_4 and γ-Fe_2O_3-based magnetic nanoparticles are used due to their strong magnetic property and biocompatibility [100]. Iron oxide-based magnetic nanoparticles (IONPs) are enormously exploited as magnetic carriers because of their biocompatibility and biodegradability (by the iron metabolism pathway). Thrombolytic agent (plasminogen activator) loaded IONPs have shown potential for thrombolytic therapy guided by an external magnetic field. Several organic polymers (chitosan [39], polyacrylic acid [46], dextran [104],

and inorganic compounds (silica) [120]) are used as a shell to protect and stabilize IONPs loaded with plasminogen activator. t-PA loaded dextran and polyacrylic acid magnetic nanoparticles have proven to be more efficient in targeting than plain t-PA and at the same time, protected t-PA from degradation [6]. Many of the other magnetic nanocarriers were used to encapsulate magnetite as well as t-PA with a shell of biodegradable polymers, for example, polyethylene glycol polylactic acid copolymer. A few others are the core-shell type of nanoparticles, consisting of superparamagnetic (iron oxide, Fe2O3) inner core and polymeric shell functionalized with thrombolytic drugs (t-PA) or targeting peptide or both [121].

The magnetic carrier, consisting of a magnetite core with polyacrylic acid (PAA) shell, has been explored for rt-PA delivery. Application of the external magnetic field guides these magnetic nanoparticles for site-specific delivery and also produces effective thrombolysis with only<20% of rt-PA regular dose [46]. However, this method was restricted by some drawbacks such as poor loading of rt-PA that leads to suboptimal therapeutic concentration at the target site and further extends the risk of occlusion due to the accumulation of a high amount of magnetic nanocarrier. To overcome these disadvantages, poly [aniline-co-N-(1-one-butyric acid) aniline] coated magnetic nanoparticles were constructed with Fe3O4core and rt-PA loading. The size of these particles was 14.8 nm and possessed a superparamagnetic character. An *in-vitro* study using the tubing system demonstrated a significant increase in thrombolysis by these magnetic nanoparticles than free rt-PA that too in considerably less time (39.2 min for rt-PA and 10.8 min for magnetic nanoparticles). The additional assay, done in the rat embolic model by application of the magnetic field, has shown recanalization of the occluded vessel within 15–25 min without any hematological toxicity [111].

In another work, chitosan-coated magnetic nanoparticles were developed by Chen, and coworkers by using the ionic crosslinking method.Water-soluble chitosan was used instead

of acid-soluble, high molecular weight chitosan to prepare formulation at neutral pH and avoid denaturation of thrombolytic enzymes (rt-PA). *In-vitro* analysis has proven the biocompatible and non-cytotoxic nature of the nanoparticles. Also, an *in-vivo* assay in a rat embolic model demonstrated the controlled release profile of rt-PA from magnetic nanoparticles, leading to the restoration of blood flow at only 20% dose of regular rt-PA dose [122]. Dextran-coated magnetic nanoparticles, conjugated with urokinase, were fabricated to achieve site-specific thrombolysis. Under the applied external magnetic field, this formulation has shown a five fold enhancement in thrombolytic efficacy than the free urokinase. The *in-vivo* study carried out in the rat arteriovenous shunt thrombosis model has demonstrated that magnetic nanoparticles exclusively reached the specific target site by taking the benefit of the applied magnetic field and prolonged the half-life of urokinase in the biological system [27].

Later, a magnetically activated nanomotor was designed to increase the effectiveness of t-PA and localized (near to thrombus, reached by targeting) nanocarriers loaded with thrombolytic drugs. The nickel nanorod was fabricated and given along with t-PA by the application of a rotating magnetic field. The availability of a rotating nanorod with t-PA significantly enhanced the speed of thrombolysis. By increasing the concentration of nanorods and keeping other conditions or variables constant, the speed of thrombolysis was enhanced as a function of nanorod concentration in the *in-vitro* analysis. This combination of therapy, tested for clot lysis in a rat embolic model, showed accelerated clot removal [100]. The use of a rotating magnetic field to accelerate thrombolysis by thrombolytic agents (urokinase) was reported by another group of scientists recently. They employed Fe_3O4 nanoparticles in combination with urokinase to induce significant thrombolysis in the *in-vitro* model (microfluidic channel-based thrombolysis model). The application of a rotating magnetic field generated a vortex that manipulated the diffusion of urokinase through the surface of the thrombi resulting in

accelerated thrombolysis. This approach enhanced the thrombolytic efficiency of urokinase twofold and has proven to enhance thrombolytic therapy which makes it a subject to explore further [123].

Despite the fact that magnetic nanoparticles are well suited for use *in-vivo*, their retention in different body parts is restricted due to their small size. However, US-based nanoparticles are extensively practiced because of their cost–effectiveness, imaging capability, and high patient compliance in therapeutic applications. US-based nanoparticles have significant benefits over magnetic nanoparticles as they can bear a higher payload. Further, it is also postulated that the US exposure at optimized intensities elicits temporary permeabilization of biological membranes (or sonoporation) and enhances nanoparticle targeting efficiency [47].

2.3.3.3. Dual targeted antithrombotic nanomedicines

Despite numerous advancements in antithrombotic therapy, the associated complications with the recurrence of cerebro/coronary vascular ischemia and thromboembolism remain huge. Dual targeting approaches offer the promise of a better outcome from a thrombotic event, although widespread use of these techniques can bring major bleeding risks. Dual targeting approaches involve either the use of two strategies or two pathways to target the thrombus. According to a previous report, employing both anti-coagulant and anti-platelet agents together to block dual pathways during thrombosis can be regarded as a promising strategy in anti-thrombotic therapy [60, 106]. Another approach proposed with dual targeting attributes was using two targeting peptides to localize the liposomal nanosystem at the thrombus site. The liposomal vesicles, loaded with an anti-thrombotic drug (streptokinase), have been surface modified with two targeting units of peptide sequences; GSSSGRGDSPA (targets GPIIb/IIIa receptors) and DAEWVDVS (targets P-selectin). These nanosystems mainly target activated platelets, therefore named as ‘platelet microparticle-inspired clot-responsive nanomedicine’ (PMIN). Considerably these PMINs can not only able to protect

encapsulated drugs but also hold into the thrombus via hetero-multivalent ligand-mediated binding at GPIIb/IIIa and P-selectin sites available on activated platelets. *In-vivo* experiments done in mice carotid artery thrombotic models have shown the higher targeting efficiency of these liposomal carriers without disturbing normal hemostasis [34]. Moreover, a number of other researchers reported the combination of two targeting approaches to target thrombus. Nattokinase-loaded mesoporous silica-polyglutamic acid dendrimers were fabricated with magnetite core and surface functionalized with RGD. The application of an external magnetic field improved the localization and RGD binding to clot receptors elicited synergistic outcomes. They have shown significant clot lysis in both *in-vitro* and *in-vivo* models [64].

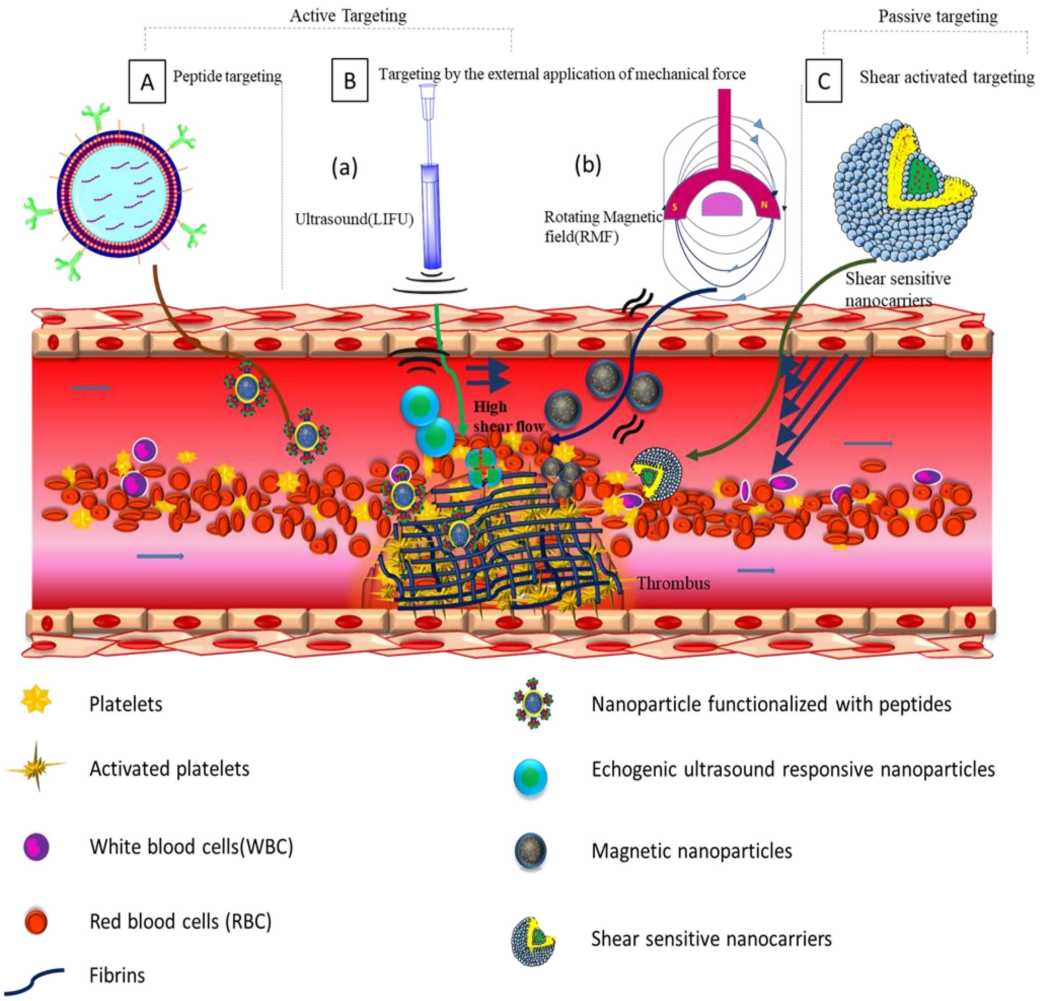


Figure 2.3. Different strategies of nanocarrier based thrombus targeting. (A) ligand based targeting with peptide conjugated nanoparticles, (B) irradiation based targeting approach by applied mechanical force (a) ultrasound based targeting with echogenic nanoparticles (b) magnetic field based thrombus targeting with magnetic nanoparticles (C) targeting by the high shear rate exists at the thrombus site [18]

2.4. Antithrombotic nanomedicine for theranostics applications

The concept of thrombus imaging, which emerged in the mid-1970s, has been growing continuously to date, to the application of modern techniques and equipment. This persistent effort is propelled by unresolved issues in the clinical analysis of thromboembolic diseases [124, 125]. The main objective of thrombus imaging is to diagnose and localize the thrombus site at an early stage [126]. By predicting the necessary information regarding the size and site of the thrombus, the dosage regimen, route of administration, and delivery systems of

drugs can be decided. Therefore, there is an immense need for *in-vivo* imaging techniques to improvise the overall thrombosis treatment [107]. The current techniques available for thrombus imaging include single-photon emission computed tomography (SPECT) [60, 106], positron emission tomography (PET) [127, 128], US imaging [129], magnetic resonance imaging (MRI) [130], photoacoustic imaging (PAI) [131] and fluorescence (near-infrared [NIR]) [62]. Despite the availability of a lot of techniques, a few drawbacks tend to limit their clinical applications. The SPECT and PET imaging techniques make use of ionizing radiation and are thus associated with radiation-induced risks that can further increase mortality in patients [132]. MRI, though harmless and provides a detailed note on body components, lacks a few attributes such as a prolonged examination process, weak or unsteady signals, and inadequate spatial resolution [133]. Additionally, the PET and MRI instruments are expensive and bulkier which makes them immovable. Patients in critical condition, who cannot able to walk, face a lot of inconvenience during examination [131]. As for PAI- and NIR-based imaging, they are still in the development stage and not significantly used in clinical settings. Among all these techniques, the US has been the mostly used in clinical practice (mainly for venous thromboembolism). It has various advantages over other techniques like real-time data generation, high penetration depth in body tissues, lower risk, extensive availability, and portability [134]. Molecular imaging of therapeutic interventions loaded with targeted agents and simultaneously carrying drugs or genes for local delivery is appealing. In the past few years, molecular imaging by the utilization of targeted contrasting agents, delivered by nanovesicles, to diagnose distinct molecular mechanisms of various diseases like thrombosis has generated a lot of attention. The remarkable aid of this technique in the diagnosis and treatment of diseases is the reason that made it prominent, as it presents a least invasive method in addition to elevated sensitivity and specificity. In one report, thrombin activatable MRI nanosensors were constructed which can provide knowledge of the age and progression

of thrombus in blood vessels. These nanosensors give T1 and T2 signals in MRI scanning based on the type of thrombus. According to the age and progression of thrombi, the strength of T1 and T2 signals were varied which help in the precise detection and sensing of thrombi in cardiovascular diseases [130]. In a recent study, KGDS peptide (substrate for GPIIb/IIIa) modified magnetic nanocages, consisting of gadolinium (Gd) as an MRI contrast agent and apoferritin as a cage-forming component, were synthesized. They reported that the prepared contrast agent was able to carry enough Gd inside the apoferritin cage and showed specific binding at the thrombus site guided by KGDS decoration and serves as a promising MRI contrast agent *in-vitro* [67]. PAI technique has been established for its brilliant spatial resolution and high contrast, although their application is limited due to its signal off effect caused by ischemia occurring in thrombosis. To overcome this problem, organic semiconducting NPs were fabricated to enhance PAI contrast in the diagnosis of thrombus. The amphiphilic compounds were used to self-assemble these NPs (perylene-3,4,9,10-tetracarboxylic di-imide) and functionalized with cRGD to provide site-specific targeting. These compounds have shown enhancement in PAI contrast for thrombus diagnosis in living mice with good stability and half-life in serum [131]. As discussed earlier, each of the imaging techniques contributes distinctive advantages as well as drawbacks. To expedite the overall imaging process, a combination of these imaging techniques furnishes potential benefits with respect to sensitivity, specificity, and risks. Molecular imaging by a multimodal approach can provide considerable data related to thrombi. However, not too many findings were recognized so far. Several other studies have reported multimodal imaging by employing targeted nanoparticles which have both diagnostic and therapeutic benefits for thrombotic diseases [60, 61, 127, 128].

Multimodal molecular imaging nanoparticles have been promising as a complementary approach for thrombus detection. However, simultaneous noninvasive detection and lysis of

thrombi, in the case of cardiovascular diseases, remain challenging. Although, multiple techniques are already in clinical use or currently under development, exploring novel imaging techniques and strategies to obtain the most precise information at the early thrombosis stage is extremely essential to improve the therapeutic outcome and subsequently, the quality of life.

2.5. Mesoporous silica nanoparticle (MSN)

In the past few years' research in drug delivery by nanotechnology is widely evolved. Nowadays nanotechnology is widely applied in the pharmaceutical field for the prevention, treatment, and diagnosis of diseases. There is a number of nanosystems that are used to deliver drug including inorganic nanoparticle, polymeric nanoparticle, metallic nanoparticle, mesoporous silica system, nanocapsule, nanosphere, polymeric micelles, liposomes, dendrimers, carbon nanotubes, nanocrystals, and hydrogels, etc [135]. In these, MSN has gained popularity because of their significant advantages i.e., the uniform porous structure of silica, easy functionalization at the surface and pores, availability of pores externally and internally, high drug loading, and gating mechanism for drug release through the pore [126, 136, 137] (**Figure 2.4**). The utilization of MSN as a carrier for cargo loading ranging from small drug molecules to macromolecules like proteins, peptides, nucleic acid, and saccharides is successfully demonstrated by different scientists [136]. Functionalization of the surface and pores of the MSN provides a change in the surface properties that increase the interaction with the therapeutic molecule [138]. Conventional drug products are mainly available for oral and intravenous administration that does not provide optimal delivery for macromolecules i.e. proteins or nucleic acids. Macromolecules, when given by the conventional route of administration, show less efficacy and unwanted degradation by enzymes or gastric acid [139]. MSN are novel drug carrier that has a special mesoporous framework that provides chemical stability and surface functionality. It is also biocompatible and used for targeted and

controlled drug delivery [138]. The different physicochemical parameters of MSN can be controlled by altering the amount and ratio of different components such as size of an MSN can be controlled by amount of TEOS, pH and reaction time. The shape and porosity of MSN can be directed by changing the concentration of surfactant. However, for the surface charge the functionalization of surface can be performed that can provide specific interaction. According to earlier reports MSN are considered as biocompatible, biodegradable and safe. As for long term effects and accumulation, MSN can be modified with biodegradable linkages such as peptide, disulfide, and tetrasulfide inside the framework structure, allowing them to decompose in bodily fluid media for a few days. However, the MSN mentioned in this research work was only fabricated for short term use and have no accumulation issue as it can be easily metabolized in the form of silicic acid. The other advantage of fabricating MSN for parenteral purpose that conventional sterilization methods such as thermal treatment, ethylene oxide, ultraviolet, and gamma irradiation can easily be employed as MSN is stable and has less sensitivity of these physical factors.

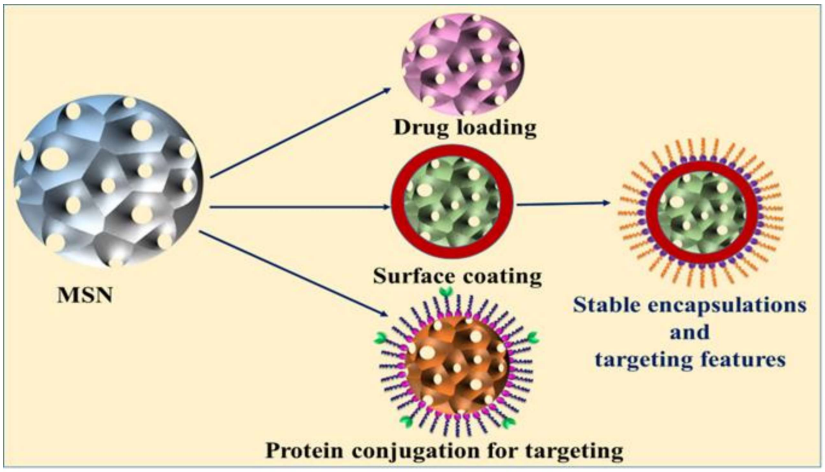


Figure 2.4. Surface functionality of MSNs

2.5.1. Methods of preparation of mesoporous silica nanoparticle

2.5.1.1. Sol-gel method

The wet chemical method known as the sol-gel process is popular in the fields of ceramic engineering and materials science. This procedure is also known as the chemical solution deposition method. The sol-gel method begins with the preparation of a colloidal suspension (also known as sol) for the formation of an inorganic network and continues with the gelation of the sol to create the network in a continuous liquid phase (also known as a gel). The precursors that are utilized to produce these colloids typically include a metal or metalloid element that is encircled by a variety of reactive ligands. When the starting material comes into contact with water or diluted acid, the processed starting material transforms into a sol. The gel is formed by removing the liquid from the sol, and the sol/gel transition determines the particle size and physical form. Oxide is created during the calcination of the gel. The majority of the sol-gel chemistry processes are based on the hydrolysis of metal alkoxides, which is followed by the condensation of corresponding oxides with various stoichiometry [140, 141].

2.5.1.2. Template assisted method

It is a widely recognized and less expensive approach that can be used to create structured mesoporous materials. This method constructs mesoporous materials using a template. It can be categorized into two groups: endotemplate (soft matter templating) and exotemplate (hard matter templating) techniques. A surfactant (a structure-directing agent) is employed as a template in endotemplate method to produce ordered mesoporous materials. However, a porous solid is utilized as the template in the exotemplate method (also known as "nanocasting") in place of the surfactant [142].

2.5.1.3. Chemical ethching method

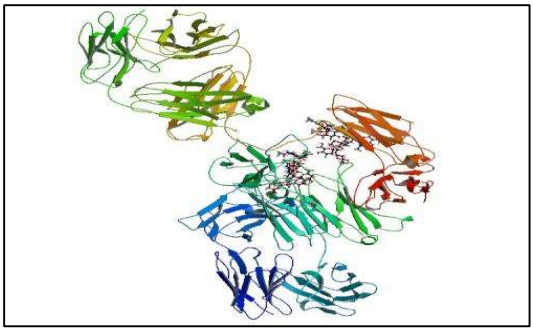
This method uses structural variations between a silica core/mesoporous silica structure's core and shell to create hollow type mesopores, which are then used to construct hollow interiors. This method allows for the synthesis of widely dispersed hollow mesoporous silica with adjustable pore size, which can be employed as a transporter for drugs with a high loading capacity. A homogeneous templating method called "structural difference-based selective etching" is used in the chemical etching technology to construct porous structures and produce distinctive core/shell structures. A hollow structure develops when a suitable etching agent is applied because only the interior is etched, while the exterior is mostly left unaltered [143].

2.5.1.4. Microwave-assisted method

In terms of research technology, the microwave-assisted synthesis process used to create molecular sieves is quite new. This process involves stirring a structure-directing agent in deionized water at 400 ^{o}C for 4-6 hours. Then, while the homogeneous solution is still being stirred, a mixture of, 2-bis(triethoxysilyl)ethane, deionized water, and HCl, is added. The finished liquid was poured into Teflon containers, which were placed into a microwave oven set to 1000^{o}C. Under microwave irradiation, block copolymer and organosilane precursors self-assembled, followed by hydrothermal treatment. The synthesis mixture was agitated using magnetic beads at 400 ^{o}C for 2–24 h in the first stage. Following the initial stage, the temperature was raised to 1000 ^{o}C and maintained there for 8–48 h in a microwave oven without any stirring. The finished product was then filtered, cleaned with deionized water, and dried at 800 ^{o}C in the oven. The use of microwave-assisted technology enabled the effective synthesis of periodic mesoporous organosilica with ethane and disulfide groups, including mesoporous hectorites [144].

2.5.2. Biosimilar's profile (Abciximab)

2.5.2.1.Chemical structure



2.5.2.2. Description: ABX is the first chimeric antibody approved as an antagonist of glycoprotein (GP) IIb/IIIa receptors that mainly appear on the surface of activated platelets. It is a Fab fragment of the chimeric human-murine monoclonal antibody 7E3. Platelet activation starts with a number of agonists and processes such as vascular injury, hypercoagulability, atypical blood flow, etc., allowing the expression of a specific receptor on the surface of platelets termed glycoprotein (GP) IIb/IIIa receptors. The natural agonist of these receptors is mainly fibrinogen which regulates the hemostasis in a person with normal physiology. ABX as an inhibitor of GPIIb/IIIa receptors inhibits the binding of its natural ligand (fibrinogen) and simultaneously ceases the platelet aggregation process and consequently produces a potent antithrombotic effect [145].

2.5.2.2. Molecular weight: 145651.1 Da

2.5.2.3. Molecular formula: $C_{6462}H_{9964}N_{1690}O_{2049}S_{48}$

2.5.2.4. Half-life: 30 min

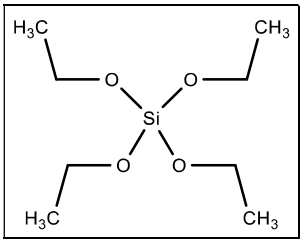
2.5.2.5. Mechanism of Action: Abciximab binds to the glycoprotein (GP) IIb/IIIa receptor of human platelets and inhibits platelet aggregation by preventing the binding of fibrinogen, von Willebrand factor, and other adhesive molecules.

2.5.2.5. Indication: Percutaneous coronary intervention

2.5.2.6. Side effects: chest pain, abdominal pain, backache, hypotension, nausea, minor hemorrhage, vomiting, gross hematuria, and pain at the injection site. Rarely seen side effects like thrombocytopenia, major hemorrhage in pulmonary and cerebrovascular area, anaphylaxis, etc [146].

2.5.3. Excipients profile (Tetraethyl orthosilicate, TEOS)

2.5.3.1. Chemical structure:



2.5.3.2. Description: TEOS is primarily employed in the semiconductor sector as a precursor to silicon dioxide and as a crosslinking agent in silicone polymers. It is also used for the synthesis of various zeolites, as a source of silica. TEOS is easily converted to silicon dioxide when water is added. A sol-gel process is exemplified by this hydrolysis reaction. The TEOS molecule is transformed into a solid that resembles a mineral, by the production of Si-O-Si bonds as the reaction progresses through a succession of condensation reactions. Acids and bases, which both function as catalysts, have an impact on the conversion rate. Monodisperse and mesoporous silica can be synthesized via the Stöber technique [147, 148].

2.5.3.3. IUPAC name: Tetraethoxysilane

2.5.3.4. Molecular formula: Si $(OC_2H_5)_4$

2.5.3.5. Molecular weight: 208.33 g/mol

2.5.3.6. Physical properties

- **Appearance:** Colorless liquid
- **Solubility:** Soluble in ethanol, and 2-propanol Reacts with water
- **Melting point: -**77^{o}C

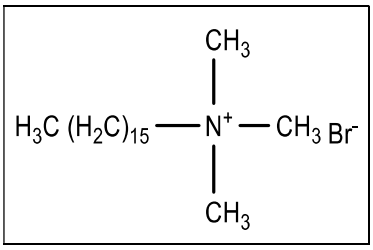
- **Boiling point:** 168°C

2.5.3.7. Metabolism: Naturally undergoes hydrolysis, beginning with the production of hydroxy esters and ethyl alcohol, progressing to the final products of silicic acid or hydrated silicic acid.

2.5.3.8. Uses: Silica precursor

2.5.4. Excipients profile (Cetyltrimethyl ammonium bromide, CTAB)

2.5.4.1. Chemical structure:



2.5.4.2. Description: It is a quaternary ammonium surfactant, widely used in the preparation of mesoporous silica nanoparticles and gold nanoparticles. CTAB is used as a template in the synthesis of ordered mesoporous silica materials. It forms micelles in an aqueous medium as other surfactants. It is mainly used as an antiseptic agent in creams, a component of DNA extraction buffer and in protein electrophoresis [149].

2.5.4.3. IUPAC name: N,N,N,-Trimethylhexadecan-1-aminium bromide

2.5.4.4. Molecular formula: $C_{19}H_{42}BrN$

2.5.4.5. Molecular weight: 364.45g/mol

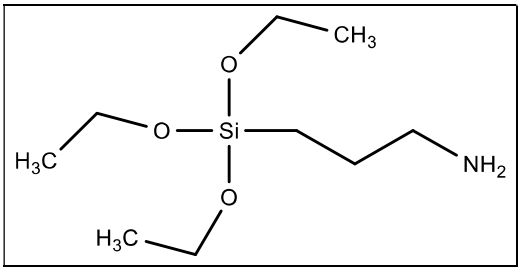
2.5.4.6. Physical properties

- **Appearance:** White solid
- **Solubility:** Soluble in water
- **Melting point:** 237-243 °C

2.5.4.7. Uses: Structure directing agent/template

2.5.5. Excipients profile ((3-Aminopropyl) triethoxysilane, APTES)

2.5.5.1. Chemical structure:



2.5.5.2. Description: An aminosilane called APTES is often used in the silanization process, which involves functionalizing surfaces with alkoxysilane molecules. Additionally, it can be used to covalently attach organic films to metal oxides like titania and silica. In order to facilitate bio-conjugation, APTES provides an amino group to the functional silane [150].

2.5.5.3. IUPAC name: 3-(Triethoxysilyl)propan-1-amine

2.5.5.4. Molecular formula: $C_9H_{23}NO_3Si$

2.5.5.5. Molecular weight: 221.372 g/mol

2.5.5.6. Physical properties

- **Appearance:** Pale liquid
- **Melting point: -**70^oC
- **Boiling point:** 217^oC

2.5.5.7. Uses: Surface functionalization

2.6. Liposomes

Liposomal vesicles are proven potential nanocarriers for targeted drug delivery due to their composition (phospholipid bilayer) which is biocompatible, non-toxic, and non-immunogenic. Along with these qualities it can also use as a nanocarrier for both hydrophilic and hydrophobic drugs [151]. Moreover, these liposomal carriers can be surface modified for stealth activity and to enhance the targeting efficiency to localize the drug delivery [152]. Many of the liposomal formulations have been approved by regulatory

authorities for parenteral use to deliver a few anti-cancer drugs[153]. These features of liposomes make them suitable carriers for drug delivery applications. Many groups of scientists have already worked on thrombus targeted liposomal formulation and has been found to be one of the suitable nanocarriers for anti-thrombotic drug delivery [154]. However, liposome sterilization remains a concern in liposome manufacturing due to its sensitivity and proclivity for physicochemical changes. Conventional sterilization methods such as thermal treatment, ethylene oxide, ultraviolet, and gamma irradiation are deemed unsuitable for liposome sterilization, therefore, filtration and aseptic manufacturing are recommended techniques for achieving liposome sterility in parenteral formulations.

2.6.1. Methods of preparation of liposomes

2.6.1.1. Thin film hydration method

The oldest, most widely used, and easiest technique for developing MLVs is thin-film hydration. The primary phospholipid components are dissolved in an organic solvent to ensure a homogenous mixture. The organic solvent can be eliminated gradually through evaporation under a vacuum pump at a temperature of 45 to 60 °C. Small volumes (less than 1 mL) of the organic solvent may be evaporated in a fume hood using a dry nitrogen or argon stream until all traces of the organic solvent are eliminated, whereas rotary evaporation is typically used for larger volumes. After the organic solvent is eliminated, a uniform, dry, thin lipid film (of stacked bilayers) is generated. The lipid film is then hydrated using a suitable aqueous media (buffer) or a phosphate saline buffer with a pH of 7.4. The hydration procedure (which lasts between one and two hours) is often carried out between 60 and 70 °C, which is always greater than the phase transition temperature of a lipid. The agitation (stirring) at this point could be beneficial in separating the lamellae of the (swelling) lipids from the surface of the internal surface. The resulting liposome suspension is then kept overnight at 4 °C to complete lipid hydration. The hydration stage causes the lipid to swell and hydrate, which

causes the creation of a highly heterogeneous MLV suspension in terms of size and lamellarity [155].

2.6.1.2. Detergent removal method

The lipids are hydrated (and solubilized) using a detergent solution in the detergent removal technique. A mixture of detergent/lipid micelles develops when the detergent binds to the phospholipids during mixing (protecting the hydrophobic sections from direct contact with the aqueous phase). The mixed micelles increase in lipid content as the detergent is gradually (successively) removed, leading to the development of unilamellar vesicles. High CMC detergents such as sodium deoxycholate, Triton X-100, sodium cholate , and alkyl glycoside are frequently used detergents. Different methods can be used to remove detergent such as the dilution method, dialysis method, and column chromatography method [156].

2.6.1.3. Solvent injection method

The lipid is dissolved in an organic solvent using the solvent injection method, and the solution is then injected into an aqueous phase. For the production of liposomal nanoformulation, ethanol, and ether have been used as the two primary solvents.

2.6.1.3.1 Ethanol injection method

The phospholipids are promptly injected into a (pre-heated) aqueous phase in the ethanol injection method. The dispersed lipids in the aqueous phase are more likely to self-assemble when the concentration of ethanol in the aqueous phase is reduced below a critical level. The precipitation of lipid molecules and the subsequent production of bilayer planar components (stacks), which enclose the aqueous phase, are both facilitated by the rapid ethanol dilution (in the aqueous phase). At last, the ethanol depletion (evaporation), facilitates the association of the lipid components and allows the subsequent production of closed unilamellar vesicles [157].

2.6.1.3.2. Ether injection method

The ether injection method involves the slow injection of lipids that have been dissolved in ether (or a diethyl ether/methanol mixture) into an aqueous phase that contains the substances to be encapsulated and is heated to a temperature between 55 and 65 °C (to aid in the solvent's evaporation from the liposomal product). The formation of LUVs is favored by the gradual elimination of the organic solvent (under decreasing pressure). SUVs are created when an ether mixture containing lipids is injected into the aqueous phase and then the ether solvent evaporates (this process is known as the ether vaporization method) [158].

2.6.1.4. Reverse phase evaporation method

This approach involves dissolving lipids in an organic solvent that promotes the development of inverted micelles, such as a 1:1 mixture of diethyl ether and chloroform. After that, a predetermined amount of an aqueous phase (buffer) is added to the solution. A water-in-oil (W/O) microemulsion is produced when the lipids rearrange themselves at the water-oil interface. In order to assist in the production of a homogenous dispersion, the W/O microemulsion can be emulsified using sonication or mechanical methods. A phosphate saline buffer is frequently added to the aqueous phase with the goal of increasing the liposomes' effectiveness. The organic solvent can be eliminated by using continuous rotary evaporation (under reduced pressure) until the production of a thick gel. The rupture of the inverted micelles and the subsequent production of liposomes (LUVs) is facilitated by the delayed organic solvent removal. The production of liposomes occurs when the gel breaks down at a key point where an excess of phospholipids distributes itself surrounding the inverted micelles resulting in a lipid bilayer around the (residual) water droplets [159].

2.6.1.5. Sonication method

Sonication is one of the most widely used techniques to fabricate liposomes. The MLVs liposome solution is subjected to a high (ultrasonic)-energy input based on cavitation while

being placed in an inert, passive environment. An aqueous dispersion of a phospholipid system is sonicated using two different methods: bath sonication and probe sonication. In the probe sonication method, a sonicator tip is dipped into the liposome solution (often used for tiny volumes). To prevent the high energy given by the tip, which results in local heating and deterioration of the lipidic solution, the bath vessel is submerged in a water/ice bath. In the bath sonication approach, typically employed for large quantities, the liposome dispersion is put into a sterile vessel with a temperature-control system or under an inert environment. Low encapsulation efficacy, potential phospholipid (or encapsulated chemical) degradation, and large-size polydispersity are the key drawbacks of this strategy [160].

2.6.1.6. Extrusion method

The extrusion technique involves the extrusion of material through membranes that have pores (with diameters ranging from 1 mm to 25 nm). To perform the extrusion above the phospholipids' phase-transition temperature, a heating block is placed around the extruder. The generation of (narrow-size distribution) LUV liposomes with dimensions that are close to the diameters of the membrane pores is possible after several passes through the polycarbonate membrane filters. Numerous studies conducted on different lipid formulations show that this method enables a reproducible outcome of the final liposomal product [161].

2.6.1.7. Homogenization (high speed and pressure)

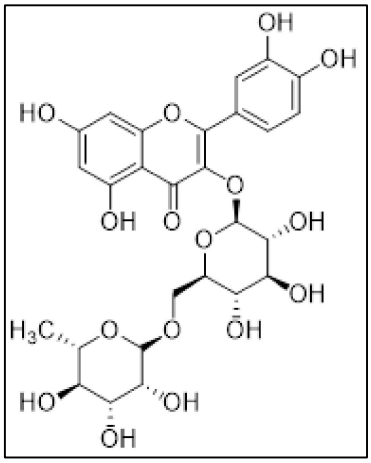
In this technique, the initial liposome suspension, which is made up of multilamellar liposomes, is constantly pumped with high pressure through an aperture, where it collides with a stationary stainless-steel wall and shrinks the liposomes. Cavitation, shear events, and turbulence all play a role in the construction of liposomes. The size distribution of liposomes may still be wide and varied using this strategy [162].

2.6.1.8. Novel methodologies

The main limitations of conventional liposome preparation methods include the challenges of establishing high encapsulation efficiencies and establishing an easily scalable technique for (large-mass) production. Additionally, due to their exposure to organic solvent residues (with sensitive toxicity), detergents, and (high) shear homogenization (or sonication) processes, which may negatively impact the clinical applications, and suggests that the conventional methods may not be suitable for the processing of many (bio-) molecules. Recently, novel methodologies for the production of liposomal nano-formulations have been developed with the intention of resolving those important problems. These techniques are lyophilization, supercritical fluid-assisted dense gas technology (supercritical reverse phase evaporation, supercritical anti-solvent method, rapid expansion of a supercritical solution, SuperLip method, depressurization of an expanded liquid organic solution) microfluidic method, and membrane contactor method etc, [163].

2.6.2. Drug profile (Rutin)

2.6.2.1.Chemical structure:



2.6.2.2. Description: Rutin (RUT, quercetin 3-rutinoside) comes under the category of flavonoids (flavonol glycoside), obtained naturally from Scphora japonica L., Fagopyrum esculentum Moench, Hypericum ascyron L., and Ruta graveolens L., etc. RUT was

investigated to constrain thrombus formation by giving well-tolerated concentration in mice and humans. RUT has been identified as a selective blocker of extracellular protein disulfide isomerase (PI) that involves thrombus formation contrary to intracellular PI which performs the synthesis of proteins [164, 165].

2.6.2.2. IUPAC name: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl] [oxymethyl]oxan-2-yl] oxychromen-4-one.

2.6.2.3. Molecular formula: $C_{27}H_{30}O_{16}$

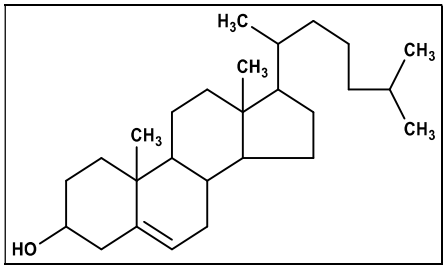
2.6.2.4. Molecular weight: 664.6

2.6.2.5. Elimination half-life: 9.11 ± 1.50 h

2.6.2.6. Solubility: Poorly soluble in water (125 mg/L)

2.6.3. Excipients profile (Cholesterol, CHOL)

2.6.3.1. Chemical structure:



2.6.3.2. Description: Cholesterol is a steroid generally analogous to mammals; the human pathogen Mycobacterium tuberculosis can completely degrade this molecule and contain a large number of genes that are regulated by its presence. Many of these cholesterol-regulated genes are homologs of fatty acid beta-oxidation genes but have evolved in such a way as to bind large steroid substrates like cholesterol [166].

2.6.3.3. IUPAC name: Cholest-5-en-3β-ol

2.6.3.4. Molecular formula: $C_{27}H_{46}O$

2.6.3.5. Molecular weight: 386.66

2.6.3.6. Physical properties

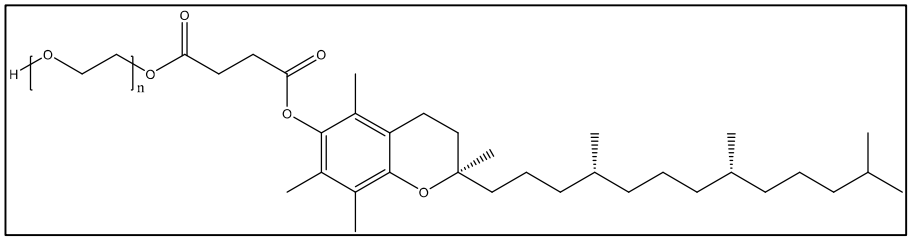
- **Appearance:** White crystalline power
- **Melting point:** 148 to 150^{o}C
- **Boiling point:** 360^{o}C

2.6.3.7. Solubility: Soluble in chloroform, acetone, benzene, ethanol, ether, hexane, isopropyl myristate, ethanol.

2.6.3.8. Metabolism: The liver oxidizes cholesterol into a variety of bile acids. These are conjugated with glycine, taurine, sulfate, or glucuronic acid. Conjugated and non-conjugated bile acids mixture along with cholesterol, is extended from the liver into the bile.

2.6.4. Excipients profile (Tocopheryl polyethylene glycol succinate, TPGS)

2.6.4.1. Chemical structure:



2.6.4.2. Description: It acts as a surfactant/emulsifier in nanoformulation. Recently, some non-ionic surfactants with PEG chains such as TPGS and Tween-80 are commonly employed in the drug delivery systems to enhance their stability and half-life in the blood circulation [167]. TPGS is a vitamin-E derivative and approved by the FDA, so it is often used as an excipient in various pharmaceutical preparations [168, 169]. Moreover, TPGS is the inhibitor of the P-glycoprotein efflux pump, making the anticancer drug delivery system more effective [170, 171]. In addition, TPGS can also be modified with appropriate chemical groups such as TPGS-COOH, TPGS-NH_2, TPGS-SH, etc., so that necessary functional groups can be used to bind ligands over nanomedicine surface [172-174]

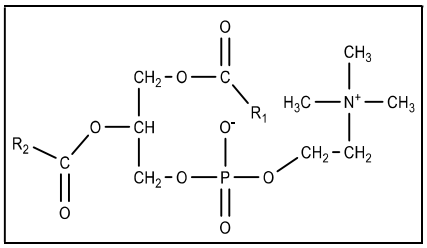
2.6.4.3. IUPAC name: α-Hydro-ω-([4-oxo-4-(((2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydro-2H-1-benzopyran 6yl)oxy)butanoyl]oxy)poly(oxyethylene)

2.6.4.4. Molecular formula: $(C_2H_4O)_{nC33}H_{54}O_5$

2.6.4.5. Molecular weight: 1513 Da

2.6.5. Excipients profile (Phosphatidylcholine, HSPC)

2.6.5.1. Chemical structure:



2.6.5.2. Description: All phospholipids contain choline as a head group and glycerophosphoric acid with a variety of fatty acids. Mainly from saturated fatty acids like palmitic acid and margaric acid, and another unsaturated fatty acid like oleic acid. Phosphatidylcholine is mainly obtained from eggs, soybean, mustard, and sunflower .it is a neutral lipid, but it carries an electric dipole moment of about 10 D.

2.6.5.3. Molecular formula: $C_{44}H_{86}NO_8P$

2.6.5.4. Molecular weight 788.1293

2.6.5.5. Physical properties

- **Appearance**: white to off white
- **Physical form**: Powder
- **Solubility in chloroform**:100 mg/mL