1. Introduction

1.1. Thrombosis

Cardiovascular is a word combining “cardio” implying the heart and “vascular” which means blood vessels. Cardiovascular diseases (CVDs) are chronic disorders of the heart and the vascular systems such as arteries, veins, and blood capillaries. CVDs include angina pectoris, myocarditis, myocardial infarction, pericardial disorder, thrombosis, atherosclerosis, hyperlipidemia, hypertension, pulmonary arterial hypertension, stroke, etc. [1]. Altogether, CVDs are the leading causes of mortality and morbidity in developed countries, owing to their sedentary lifestyles [2]. Furthermore, obesity and diabetes, and related disorders, are linked with CVDs, hence becoming a comorbid relation. A complication such as coronary artery obstruction is among the number one causes of death in adults that lead to thrombus plagues in the intima of the arterial wall, mainly in the carotid artery [3]. Cardiovascular diseases are prevalent in high-income countries such as the United States of America, but they have also become a global issue. In 2016, a report demonstrated that 62.5 and 12.7 million people died due to cardiovascular diseases in India and America, respectively. Out of these deaths, most causes for deaths were observed as ischemic heart disease and stroke, and likely more will be in the future if serious actions are not taken [4]. The formation of thrombi in blood vessels due to the disruption of atherosclerotic plaques is considered as the major reason that triggers their onset [5]. Thrombosis plays a pivotal role in the creation of various cardiovascular events, such as ischemic cardiovascular disease and venous thromboembolism. Thrombosis is defined as localized blood clotting regulated by an activated coagulation cascade due to a myriad of abnormal physiological conditions [6]. In other words, this disease mainly occurs due to the deposition of blood clots in vessels (thrombus) that obstruct blood flow in the circulatory system [7]. A thrombus is an end product of the hemostatic process, resulting

from aggregated platelet plugs and activated clotting factors involved in the coagulation pathway. It plays a dual role; physiological in the case of vascular damage and pathological in the case of thrombotic events [8].

When the thrombus is displaced from its original site and becomes free-flowing, then it is termed as an embolus. According to the shape and size of the thrombi, it can obstruct or block the blood vessels. The blockage of vessels causes rapid necrosis in adjacent tissues due to a lack of nutrients.

1.2. Mechanism of normal hemostasis and their role in targeted therapy of thrombosis

In the coagulation cascade, activation of various factors occurs successively by the proteolytic breakdown and further amplifies the preceding steps, as illustrated in **Figure 1.1**. Therefore, targeting the upstream parts of the coagulation cascade like tissue factors could be more beneficial than the downstream targets such as GP IIb/IIIa, thrombin, fibrin, etc., for the treatment of thrombotic diseases. However, inhibition of tissue factors retards the initiation of the coagulation pathway needed for physiological hemostasis and may lead to severe bleeding manifestations. An experiment done on gene knockout mice demonstrate that tissue factor is needful for hemostasis and life [9]. After vascular injury, tissue factor is expressed on the surface of the injured endothelium that interacts with factor VII, and activates the coagulation pathway leading to the production of thrombin. Thrombin works as a precursor that converts fibrinogen to fibrin as well as an activator of other plasma coagulation factors, which allows more production of thrombin and further activates more platelets [10, 11]. Thrombin is a key enzyme that plays a central role in the coagulation pathway, inhibition of thrombin can be utilized as efficient anti-thrombotic therapy. Some peptide sequences such as (rkrk(LVPRGrkrk)3) [12] and PPACK [13] were reported to target thrombin and inhibit their biological activities at the site of the thrombus. Direct and indirect inhibitors of thrombin are also available as anti-thrombotic drugs. Direct inhibitors

do not require cofactors and bind reversibly with thrombin whereas indirect inhibitors act via antithrombin and inactivate plasma factor Xa [10]. These drugs can be loaded into the nanoparticles and formulated for targeting thrombosis [14].

1.3. Types of thrombosis

There are two key types of thrombosis based on their occurrence in the area of the blood vessels, arterial thrombosis, and venous thrombosis

1.3.1. Arterial thrombosis

Arterial thrombosis is induced by the rupture of atherosclerotic plaque and carries a higher amount of activated platelets. Arterial thrombosis denies oxygen and nutrition to an area of the body. Thrombosis of an artery leading to the heart causes a myocardial infarction and thrombosis of an artery leading to the brain causes a stroke. The thrombus formation process starts with the activation of platelets via different enzymes (p-selectin, thrombin, fibrin) receptors (GPIIb/IIIa, PAR) and clotting factors (Von Willebrand factor, tissue factor, plasma factors) that can be served as a target for the antithrombotic therapy. Arterial and venous thrombosis are unlike each other by means of the composition of the formed thrombus (clot). For this reason, arterial thrombosis is treated by anti-platelet drugs however fibrinolytic agents are used to treat venous thrombosis in most of the cases [15].

1.3.2. Venous thrombosis

Venous thrombus developed due to the “Virchow triad” and is rich in fibrin content with trapped RBCs. Virchow triad mainly involves three basic reasons, first damage in the endothelial layer of a blood vessel (vascular injury or endothelial cell injury); second variation in normal blood flow inside the vessel (like in an aneurysm); and third due to change in blood composition (by a mutation in coagulation protein or hypercoagulability) [16, 17].

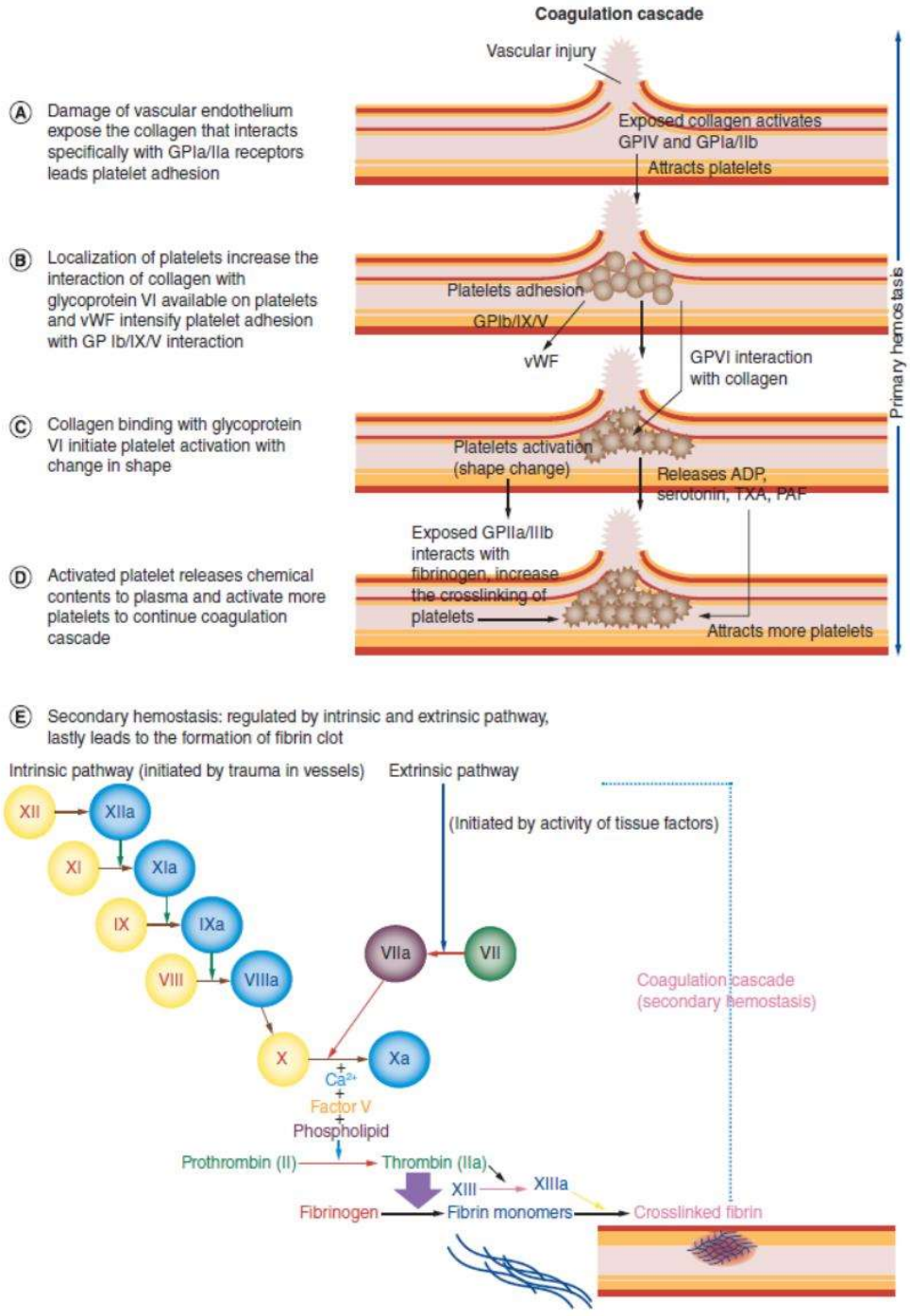

Figure 1.1. Mechanism of the primary hemostasis and secondary hemostasis [18]

1.4. Symptoms of thrombosis

1.4.1. Development of wounds or blisters

In severe cases of thrombotic events sometimes skin tears away or sometimes a cracked wound is formed.

1.4.2. Sloughing of skin

It is a condition in which the skin is separated and falls away from the underneath tissues.

1.4.3. Necrosis

Thrombosis can cause the death of the skin or tissues and turns the affected area black or dark. This dead tissue area gradually can spread outward.

1.4.4. Change in skin color

Areas beyond the obstruction (that means a thrombus is formed between the heart and that pale area), looks typically paler than other area of the skin.

1.4.5. Change in temperature of the affected area

Blockade of blood vessels due to thrombosis affects the blood flow in that area that reduces the temperature and makes the affected area feel cooler than other areas.

1.4.6. Weakness in the affected area

It involves the impairment in voluntary movements of affected body parts.

1.4.7. Tingling and numbness

Thrombosis can induce the feeling of pricking by needles or pins and is sometimes associated with pain in the affected area [17].

1.5. Causes of thrombosis

1.5.1. Cardiovascular diseases

1.5.1.1. Atrial fibrillation

In this physiological condition the upper chambers of the heart beat very fast as a result heart can't able to pump blood properly. The Blood remains left out in these chambers and consequently forms blood clots that can travel to other areas and can lead to stroke.

1.5.1.2. Hypertension and hypercholesterolemia

High blood pressure and cholesterol can able to impair cardiac function and can damage blood vessels. Both of these conditions can limit blood circulation and easily allow clot formation.

1.5.1.3. Cardiac valve disease

Damage in the heart valve is associated with some diseases like mitral valve stenosis cause narrowing of the valves, more likely to allow clot formation.

1.5.2. Impairment in blood clotting

In a blood clotting disorder, blood clots too quickly, and this condition is known as thrombophilia or a hypercoagulable state. The body creates a blood clot to stop the bleeding when you are injured and the liver produces clotting factors (proteins) that bind to blood platelets to form a blood clot (coagulate). Normal coagulation is crucial for controlling bleeding from a cut and starting the healing process. However, excessive clotting can lead to thrombus formation.

1.5.3. Genetically inherited clotting disorders

The most frequently found genetic abnormalities that raise the risk of blood clotting are factor V Leiden and prothrombin gene mutation (G20210A). Other than that, deficiencies in naturally occurring proteins (such as antithrombin, protein C, and pro tein S) that stop blood from clotting, elevated fibrinogen concentrations, or abnormal fibri

nogen, increased levels of factors VIII, IX, XI, and dysfunctional fibrinolytic system are few rarely occurring conditions that inherited hypercoagulable state.

1.5.4. Cancer

Malignancy can express a number of procoagulant proteins, including enhanced tissue factor expression, which is another well-known indicator of hypercoagulability. It is well-recognized that certain cancers, particularly solid tumors (like pancreatic cancer), greatly increase the risk of thrombosis.

1.5.5. Age

The progression of thrombosis is also influenced by gender and age, with an ever higher risk of thrombosis as people get older. For instance, studies have revealed that older people produce more prothrombotic coagulation factors including von Willebrand factor and thrombin. In comparison to younger people, the elderly may also suffer a typical physiological activation of platelets.

1.5.6. Medical conditions

Additional risk factors include underlying connective tissue or rheumatologic diseases (such as vasculitis, and SLE), alongside the rare HITT, myeloproliferative disorders, antiphospholipid syndrome, and PNH (all of which can increase the risk of both venous and arterial thrombosis)

1.5.7. Dearth of action/Other factors

Blood stasis in the arterial or venous system may be caused by inactivity, pregnancy, or reduced blood flow as a result of prior thrombosis (such as a persistent blood clot, blood vessel remodeling or fibrosis, or atherosclerosis). Long journeys with restricted movement can further increase the risk of thrombosis, especially if other risk factors are present at the same time (as mentioned above).

1.5.8. Surgical intervention

A central venous catheter, injury to a vein from surgery, a broken bone, or other trauma can induce thrombosis.

1.5.9. Use of medications

Estrogen-containing birth control pills, hormone replacement therapy for menopause symptoms, and chemotherapy may all trigger blood clots.

1.5.10. Smoking

The risk of myocardial infarction and coronary thrombosis appears to rise with both active and passive cigarette smoke exposure [19].

1.6. Available therapies

1.6.1. Blood thinners

These drugs prevent blood from clotting too quickly. An existing clot cannot be removed, but it can be stopped from growing larger. Blood thinners fall into two categories: anticoagulants and antiplatelet medications.

1.6.2. Thrombolytic therapy

Thrombolytic agents are used to dissolve blood clots. These "clot-busting drugs" are particularly beneficial for clots in vulnerable places. For heart attacks, strokes, and other thrombosis-related problems, they provide emergent medical care.

1.6.3. Surgical intervention

A thrombectomy procedure is one of the most direct methods primarily used by surgeons to access and remove a clot inside the blood vessel. Additionally, numerous endovascular techniques are also used, such as pharmacomechanical catheter-directed thrombolysis, venous balloon dilatation, percutaneous aspiration thrombectomy, and catheter-directed thrombolysis [20].

1.7. The risk associated with antithrombotic therapy

All the direct thrombolytic agents have been reported for severe hemorrhage as a major side effect, which emerges due to a few reasons including disruption of fibrin at the thrombus site and by the increase in systemic plasmin which produces further breakdown and degradation of other clotting factors (Factor V and VIII) [21]. Generally, heparins (both unfractionated and low molecular weight heparins) are the most common anticoagulants used clinically. Their effect is limited for already existing thrombi and they have symbolic systemic variability due to alteration in plasma protein binding in each individual, causing the bleeding risk [22]. Additionally, all heparins produce immunogenicity as well as thrombocytopenia. Combination therapy of heparin with thrombolytic agents such as tissue plasminogen activators or streptokinase leads to severe hemorrhage in 2-4% of individuals. The other downfall includes the farthermost intracranial bleeding. Each of the anti-thrombotic regimens is associated with hemorrhagic stroke when used in combination with heparin [23]. Furthermore, the pharmacokinetics of most of the anti-thrombotic agents are extremely variable for each individual and it requires continuous monitoring in order to maintain the plasma concentration to get the desired clinical outcomes. The reason for this drawback is, most of the anti-thrombotic drugs are peptides or protein that experiences various level of stimulation within the circulatory system of the body. It can be easily examined to look at their short plasma half-life upon administration by an intravenous route such as tissue plasminogen activator has a half-life of about 5 min in plasma, further, it is increased to 15-20 min by using its recombinant forms eg. Alteplase, reteplase, and tenecteplase. Similarly, streptokinase and urokinase also have short plasma half-lives of approximately 10-20 min [8](**Table 1.1**).

1.8. Challenges in anti-thrombotic drug delivery

A major challenge in designing targeted nanoparticulate systems is that the expression of receptors varies according to individual vascular entities. In other words, each of the cellular structures expresses its own range of receptors that regulate its function inside the body. Anti-thrombotic therapy employs various enzymes/proteins (like tissue plasminogen activator(t-PA), streptokinase (SK), urokinase(UK), plasmin) peptides (abciximab, eptifibatide, tirofiban) and polysaccharides (heparins) as therapeutically active agents. The utilization of these agents in anti-thrombotic therapy is limited due to a number of intrinsic properties carried by these macromolecules that are considered as foreign moiety to the recipient system. Thus it leads to the instability of numerous protein and peptide drugs in distinct environmental conditions such as temperature, physiological pH, etc [8, 24]. Activation of proteins and peptides is a crucial step that depends upon their stability in the present environment. Denatured or inactive proteins do not share any therapeutic effect in the biological system.

Different mechanisms that lead to the inactivation of proteins *in-vivo* include 1. Conversion of protein conformation into inactive form by the effect of temperature, pH, solvent, electrolyte concentration, and surfactants; 2. Dissociation of subunits or complexation with cofactors; 3. Association of proteins via non-covalent bonding with surrounding ions and molecules or macromolecules. Besides that proteolytic degradation by various endogenous proteases and chemical modification via a chemical reaction with different entities (such as oxidation of -SH group, and Fe(II) atoms, etc) also influence the original structure of proteins [24].

When the exogenous proteins were administered into the systemic circulation, it undergoes several transformations due to the influence of endogenous surroundings, resulting from instant denaturation and elimination (mainly via enzymatic degradation, reticuloendothelial

system(RES) uptake, renal excretion, and retention in non-targeted tissue and organs). Accumulation of these peptidic drugs at non-targeted sites leads to unwanted side effects as well also wastes the drug dose. Therefore, the requirement of administered dose becomes high, which adds extra cost and in most cases elicits nonspecific toxicity. Additionally, the immune response generated by microorganisms due to the antigenic behavior of foreign proteins leads to protein inactivation and the generation of allergic reactions (by the induction of specific antibodies). That restricts the further use of those proteins in therapeutic applications [25].

The polysaccharide-based anti-thrombotic drug such as heparins, were reported for having temperature-based sensitivity and binding with antithrombin-III protein. Moreover, heparin activity and stability depend on the pH of the media and also electrolyte concentration. Use of 5% dextrose as media for heparin cause a slight reduction in its activity [26]. Affected by these many drawbacks (their stability, efficacy, and safe targeting) formulating protein drug delivery systems thus remains challenging because of their low bioavailability due to high molecular weight, short half-life, low lipophilicity, and interaction with charged molecules [24]. Newer approaches are needed to improve the delivery of anti-thrombotic drugs to thrombus sites by overcoming the said problems. Nanocarrier-based delivery approaches have been widely explored for the delivery of such sensitive drugs. They can enhance the specificity of the treatment and at the same time help to overcome the drawbacks associated with conventional therapy. Applications of nanocarriers to solve risks associated with anti-thrombotic therapy are presented in **Table 1.1**.

Table 1.1. Utilization of drug loaded nanocarriers to solve risk associated with anti-thrombotic therapy

Name of the drug	Half-life	Risks	Used for which stage of thrombosis	Use of nanocarriers to improve half-life or reduction in risks	Ref.
Unfractionated Heparin(UFH)	1 h	Hemorrhage, thrombocytopenia, osteoporosis	Initial and extended treatment for acute and chronic thrombosis	Self-titrated nanocomplexes encapsulated UFH shown initial shorter half-life while after secondary half-life is prolonged for hrs. Stimulus responsive behavior of these also reduces bleeding complications.	[12]
LMWH	3-4 h	Hemorrhage, thrombocytopenia, osteoporosis	Initial and extended treatment for acute and chronic thrombosis	----------	
Urokinase	15 min	Anaphylaxis, gastrointestinal, urogenital, Intracranial bleeding	Initial treatment for high risk thrombotic event	Magnetic nanoparticles loaded urokinase prolonged the half-life by reducing the clearance and minimizing the risk of severe bleeding than the free drug.	[27]
Tissue plasminogen activator (t-PA)	4-5 min	Intracranial hemorrhage, major systemic hemorrhage	Initial treatment for acute and chronic thrombosis	Non-pegylated and pegylated liposomes improved t-PA half-life 103,141 min respectively and reduced hemorrhagic risk.	[28]
				Ultrasound-responsive PEG modified gelatin nanocomplexes prolonged half-life of t-PA by three times with reduction in bleeding complications	[29]
Alteplase	4-8 min	Intracranial bleeding	Initial treatment for acute and chronic thrombosis	PEG-PCL nanoparticles loaded alteplase shown 18 times longer halflife than free drug	[30]
Staphylokinase	6 min	Bleeding, Rethrombosis	Initial treatment for acute and chronic thrombosis	----------	
Reteplase	14-18 min	Blood in urine, nausea, vomiting with blood	Initial treatment for acute and chronic thrombosis	----------	
Tenecteplase	11-20 min	Nosebleeds, coughing of blood, red urine	Initial treatment for acute and chronic thrombosis	----------	
Abciximab	10-30 min	Bleeding at administration site, abdominal pain, nausea	Initial and extended treatment for acute and chronic thrombosis	----------	

1.9. Anti-thrombotic nanomedicines

Thrombosis obstructs the flow of blood, leading to ischemia and myocardial infarction. It happens when an injury occurs at the atherosclerotic plaque, triggering platelet aggregation at the luminal site, resulting in rapid thrombus growth along with the development of a fibrin network that stabilizes platelet-rich thrombus [18, 31]. Thrombus lysis can be induced by thrombolytic agents such as tissue-type plasminogen activators (tPA) that activate plasmin. Additionally, a serine protease could dissolve fibrin contained in clots [32]. An earlier study achieved accelerated thrombolysis in rabbits using streptokinase encapsulated in liposomes with water-soluble polymers. The occlusions were reduced with liposomal formulation than free streptokinase with multiple episodes of reocclusion and subsequent reperfusion. Compared to free streptokinase (74.9 ± 16.9 min), drug encapsulated liposomes (19.3 ± 4.6 min) and water-soluble polymers (7.3 ±1.6 min) have shown lesser reperfusion times [33]. The lipid bilayer vesicles are derived from a platelet bound with active integrin GPIIb/IIIa, P-selectin, GPIb-type receptors, thrombospondin, and C-X-C type chemokine, and thrombin receptors. Specifically, these platelet-derived vesicles can be directed to the thrombus via binding to active platelet integrin GPIIb/IIIa and P-selectin. The chain reaction causes the vesicle to destabilize and relieves the thrombolytic payload that was activated by the clot-relevant enzyme, i.e., phospholipase-A2 [34]. Targeted liposomal nanocarriers have been exploited with RGD peptide decoration that employs a higher affinity towards active platelet integrin GPIIb/IIIa receptors. RGD peptide ligand inactivates integrins GPIIb/IIIa, thus platelet adhesion and aggregation were prevented [35, 36]. Conformation constraint of peptides such as cyclic RGD was reported with liposomes that bonded to activated platelets considerably more than linear RGD-liposomes [37]. Semi-permeant perfluorocarbon core nanoparticles having D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone as a targeting moiety have

been reported to inactivate thrombin. The thrombin is a promoter of procoagulant factors synthesis, leading to endothelial disruption, coagulation, inflammation, and plaque expansion [38]. Another chitosan-coated magnetic theranostic nanoparticle encapsulating tissue plasminogen activator was developed by employing superparamagnetic properties. It ensures that the activity was altered externally by utilizing a magnetic field gradient. Furthermore, magnetization was discontinued once the magnetic field was deactivated. Overall this formulation has proven its efficacy as it requires only one-fifth of the regular dose of tPA to produce its thrombolytic activity [39]. In addition to this, a newer thrombus-targeted fibrinolytic agent was developed with theranostic and multifunctional properties. The study was based on crosslinked dextran-coated iron oxide (CLIO) nanoparticles with recombinant tissue plasminogen activator (r-tPA) conjunction that targets fibrin and activated factor XIII. It was reported to show theranostic capabilities and a high affinity towards clots; hence exhibiting its multipurpose tag [40]. To conserve the integrity of the blood vessels, generally, a thrombus (blood clot) forms in wounded blood vessels. It reduces the blood flow resulting in the death of cells fed by the arterial blood flow, which is a principal cause of various life-threatening cardiovascular disorders. Recently, Kang et al. developed fibrin-targeted theranostic nanoparticles that thrombosed blood vessels and simultaneously inhibit thrombus formation. The developed nanoformulation decreased the synthesis of tumor necrosis factor-alpha (TNF- α) and soluble CD40 ligand (sCD40L) in stimulated platelets. Additionally, it prevented the production of H_2O_2, suggesting its inherent antioxidant, anti-inflammatory, and antiplatelet action. They selectively attacked the obstructed thrombus in an animal model of ferric chloride ($FeCl_3$)-induced carotid thrombosis and significantly increased the fluorescence/photoacoustic signal. The tirofiban-loaded nanoparticles were used at a concentration of 80 µg/kg in mice. They showed 10 and 7 folds higher bleeding time and blood loss, respectively which confirmed

the significant suppression in thrombus development [41]. Research trends and available preclinical and clinical data suggested that theranostic nanomedicines are promising candidates for imaging and treatment of cardiovascular diseases.

1.10. Targeted drug delivery approaches

Currently, for the treatment of thrombosis, rapid revascularization is available as the best emergent therapy, by angioplasty and surgical interventions of the occluded vessel. But it is a time taking process involving the transfer of the patient to a specialized hospital where cardiac surgery can be performed [28, 42]. Clinically, intravenous and oral administration of various anti-thrombotic drugs like anticoagulants, anti-platelets, and thrombolytic agents is also a treatment option of arterial and venous thrombosis for cardiac patients [9]. Many of these anti-thrombotic drugs have short half-lives, require high doses, have poor targeting capability, low therapeutic windows, require constant monitoring, and can easily induce hemorrhage and other side effects [7, 8]. Therefore, effective and safe delivery of these anti-thrombotic agents at the target site is the primary objective of anti-thrombotic treatment [7]. Hence, in order to minimize side effects and maximize targeting efficiency, various approaches have been exploited, such as formulating drug-loaded nanoparticles, liposomes, micelles, nano-vectors, etc. with the addition of targeting peptides on their surface or by using different external forces like a magnetic field, ultrasound (US), heat, light, etc [43-46]. Research on targeted nano drug delivery systems has been explored due to its immense potential to revolutionize the pharmaceutical industry by providing safe and effective delivery of drugs to the target site within the body [47]. Similarly, theranostic approaches have also been exploited in this area, which can facilitate simultaneous diagnosis and treatment, these have drawn extensive attention due to their ability to govern therapeutic outcomes [6]. Many antithrombotic drugs are associated with life-threatening side effects (severe bleeding risk) and also suffer from various challenges during systemic

delivery (due to their proteinous nature). To overcome these issues, passive (shear activated therapeutics), active (ligand targeted), and other miscellaneous targeting approaches, utilizing nanocarriers, have been exploited for antithrombotic drug delivery applications.