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Liposomes for targeted delivery of antithrombotic drugs

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Background: Targeted delivery of antithrombotic (thrombolytic) drugs is expected to increase their efficacy and decrease side effects, especially in the case of thrombolytic enzymes. Liposomes, phospholipid nanosized bubbles with a bilayered membrane structure, have drawn a lot of interest as pharmaceutical carriers for drugs and genes. In particular, several attempts have been made to use liposomes as vehicles for antithrombotic agents. *Objective*: This review analyzes the available data on the application of liposomes, including liposomes targeted by specific ligands, for the delivery of antithrombotic/thrombolytic agents in order to increase their efficacy and decrease side effects. *Methods*: The papers published on the subject of liposomes loaded with antithrombotic agents, mainly over the last 10 – 15 years, will be discussed. *Conclusion*: Liposomes loaded with various antithrombotic drugs, though they have been the subject of a significant number of experimental papers, can hardly be considered as real candidates for clinical application in the near future.

Keywords: antithrombotic drugs, liposomes, targeted delivery, thrombolysis

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1. Introduction

In brief, thrombosis is the formation of a blood clot (thrombus) inside a blood vessel, which obstructs the flow of blood through the circulatory system and causes a hypoxia or even infarction of tissues supplied by the vessel. A thrombus is the final product of the blood coagulation step in hemostasis. It is built via the aggregation of platelets that form a platelet plug, and the activation of the humoral coagulation system (i.e., clotting factors). A thrombus is physiologic in cases of injury, but pathologic in case of thrombosis. It may be occlusive or attached to the vessel wall without obstructing the lumen (mural thrombus). A thrombus in a large blood vessel will decrease blood flow through that vessel; but in a small blood vessel, blood flow may be completely cut off, resulting in death (necrosis) of tissue supplied by that vessel. On the other hand, if a thrombus dislodges and becomes free-floating, it represents an embolus that can finally lodge in and completely obstruct a blood vessel, causing various effects, depending on where it occurs. Most thrombi, however, become organized into a fibrous tissue, and the affected vessel could gradually become recanalized. Certain conditions that elevate the risk of blood clots developing include an atrial fibrillation, heart valve replacement, recent heart attack, extended periods of inactivity (as in cases of deep venous thrombosis), and genetic or disease-related deficiencies in the blood's coagulation system. In general, blood clot prevention significantly reduces the risk of stroke, heart attack and pulmonary embolism [1,2].

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In classic terms, thrombosis is caused by abnormalities in one or more of the following conditions, known as Virchow's triad:

1. Changes in vessel wall morphology (i.e., endothelial cell injury, such as from trauma or atheroma)
2. Changes in blood flow through the vessel (i.e., hemostasis, as with valvulitis or aneurysm)
3. Changes in blood composition (i.e., hypercoagulability, as in leukemia).

The pathogenesis of thrombosis includes an injury to the vessel's wall (such as by trauma, infection, or turbulent flow at bifurcations); slowing down or stagnation of the blood flow past the point of injury; or through blood hypercoagulability. Occasionally, abnormalities of the coagulation system account for thrombus formation. In all cases, an intravascular coagulation occurs, forming a structureless mass of red blood cells, leukocytes, and fibrin.

There are two distinct forms of thrombosis:

1. Venous thrombosis, such as the most common deep venous thrombosis (with or without pulmonary embolism; together classified as venous thromboembolism/VTE), or portal vein thrombosis, renal vein thrombosis, hepatic vein thrombosis (Budd-Chiari syndrome) and cerebral venous sinus thrombosis.
2. Arterial thrombosis, which is a major component of the end stage of atherosclerosis. The rupture of an atherosclerotic plaque can trigger thrombus formation, leading to the rapid occlusion of the artery or blood vessel embolization with a part of the thrombus, which in turn can cause stroke, myocardial infarction or thoracic outlet syndrome [2,3].

In addition, if a bacterial infection is present at the site of thrombosis, the breakdown of the thrombus may spread particles of infected material throughout the circulatory system (pyemia, septic embolus) and set up metastatic abscesses wherever they come to rest [4].

2. Antithrombotic drugs

Within the vascular network, the blood is in a delicate balance between the ability to flow freely throughout all body tissues and coagulate when a blood vessel is injured or ruptured. Thrombogenesis is an intricate process that involves a range of factors, including naturally occurring internal factors and/or external factors. Under normal conditions, vascular integrity is maintained by the process of hemostasis, which ensures that blood remains within the vessel. Careful balance of complex interrelated systems that include platelets, coagulation and fibrinolytic systems, plasma inhibitors, the endothelium and its subjacent structures, flow characteristics of the blood and an overall vascular tone are crucial for the proper function of the hemostatic system.

Coagulation is the process whereby blood loses its fluid consistency and becomes a semisolid mass, or a clot. In response to vessel wall injury or exposure of blood to foreign surfaces, the amount of the blood loss/imbalance is immediately reduced by a thrombogenic cascade of reactions (**Figure 1**) that results in local vasoconstriction, adhesion of specialized blood platelets to the insult area, formation of a platelet aggregate (or 'plug'), and formation of the blood clot, when fibrinogen is converted to fibrin, the fundamental component of the clot [5]. The entire clotting process is well described elsewhere [2,4] and will not be detailed here because of its complexity.

In brief [6,7], there exists a set of chemical reactions resulting in the activation of the enzyme thromboplastin, which converts prothrombin into thrombin. Thrombin, in turn, catalyzes the conversion of fibrinogen to fibrin, which forms a meshwork that reinforces the already-formed platelet plug and produces the final stable clot. The chemical reactions required for the formation of thromboplastin utilize 'factors' that are synthesized in the liver. Although clotting in response to tissue injury is normal and beneficial, inappropriate intravascular clotting is harmful and thought to be involved in a variety of cardiovascular disorders. Thus, anticoagulant drugs are widely used. On the other hand, once the blood clot is formed, fibrinolytic enzymes in the blood are activated that are capable of dissolving blood clots. A precursor substance (pro-enzyme), plasminogen, is activated to fibrinolysin, which can lyse fresh clots with the production of fibrin degradation products (**Figure 1**). Most of the time, a plasminogen activator is used to increase the availability of fibrinolysin and the intensity of clot dissolution [8]. Usually, plasminogin activators are given either intravenously or intra-arterially near the site of occlusion. After the initial clot lysis, clot re-formation should be further prevented by the use of antocoagulants – first heparin, then warfarin.

Antithrombotic agents that prevent or reverse different steps of the coagulation process are generally classified as follows:

1. Anticoagulants; they mostly work upstream to prevent thrombogenesis, like heparin and warfarin and its derivatives;
2. Thrombolytics (plasminogin activators); that work downstream to dissolve the thrombus or slow down or prevent its formation [9], such as streptokinase, urokinase and tissue plasminogin activators (tPA, e.g., alteplase R: recombinant tPA, anistreplase: anisoylated plasminogen streptokinase activator complex, and reteplase R: recombinant non-glycosylated tPA). Mostly, serine proteases activate the endogenous fibrinolytic system by cleaving the arginine 560 – valine 561 bond in plasminogen, which in turn converts enzymatically to active plasmin, initiating fibrinolysis [4,10,11]. Alteplase, reteplase and urokinase cleave the peptide bond directly. On the other hand, anistreplase and streptokinase act indirectly by forming an

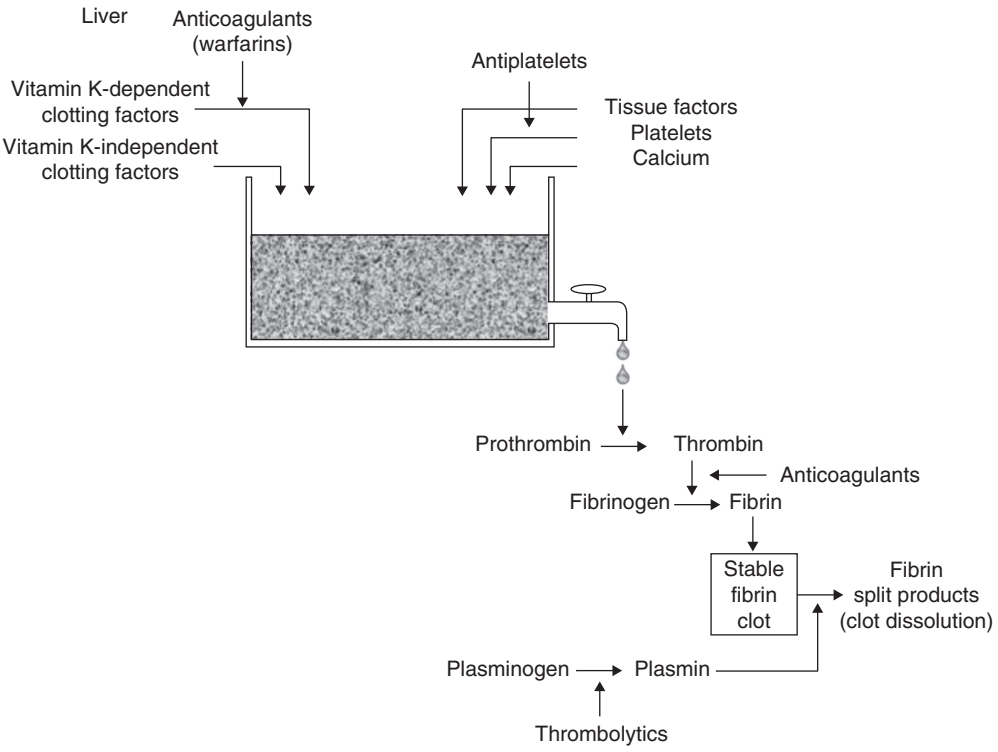

Figure 1. Thrombosis and antithrombotic agents.
Adapted and abbreviated from [5].

equimolar complex in plasma with plasminogen creating an activator complex that converts residual plasminogen to fibrinolysin [12].

3. Problems with conventional parenteral thrombolytic therapy

Anticoagulation is routinely used during various medical/surgical procedures to prevent thrombosis above the level of vessel occlusion (for example, during angioplasty or upon aortic occlusion during endograft placement) and to prevent thrombus formation on or in a catheter upon prolonged catheterization. In addition, many patients requiring vascular disease management may have a hypercoagulable state. For example, up to 10% of patients undergoing peripheral vascular procedures have a hypercoagulable state [13], and there is the evidence that the platelets of patients with peripheral vascular disease are activated [14].

Heparins (either unfractionated, UFH, or low molecular weight heparins, LMWHs) are the most commonly used anticoagulants in clinical practice. They are ineffective against existing thrombus, and have significant biologic variability owing to their various levels of plasma protein binding, often leading to bleeding complications. In addition, the downfall of all heparins is their immunogenicity and the induction of heparin-induced thrombocytopenia (HIT) syndrome, which occurs in roughly 1 – 3% of all patients who receive heparin [15].

In contrast to the indirect inhibition of thrombin offered by heparin, new direct thrombin inhibitors, like hirudin and desirudin, require no preceding interaction prior to their effect on thrombin, and are more specific and active on both soluble and thrombus-bound thrombin. This is distinct from heparin, which has no activity against thrombus-bound thrombin [4,13].

The major side effect of all direct thrombolytic agents is hemorrhage, which results from two factors: i) the lysis of fibrin in 'physiological thrombi' at sites of vascular injury; and ii) a systemic lytic state that results from the systemic formation of plasmin, which produces fibrinogenolysis and destruction of other coagulation factors (especially factors V and VIII) [10,11,16], although there exists also an opinion that the role of the systemic fibrinolysin cannot be really important because of its rapid neutralization by α2-antifibrinolysin [17,18]. The actual toxicity of streptokinase and t-PA is difficult to assess. In early clinical trials, many bleeding episodes resulted from the extensive invasive monitoring of therapy that was required by the protocol. Many studies to evaluate thrombolysis involved concurrent systemic heparinization, which also contributes to bleeding complications. Analysis of more recent clinical trials suggests that heparin confers no benefit in patients receiving fibrinolytic therapy plus aspirin [19,20].

If heparin is used concurrently with either streptokinase or tissue plasminogin activators (tPA), serious hemorrhage will occur in 2 – 4% of patients. Intracranial hemorrhage is by far the most serious problem. Hemorrhagic stroke occurs with all regimens and is more common when heparin is used. Evidence from controlled clinical trials suggests that thrombolytic therapy of occluding the clot with tPA can successfully reperfuse ischemic brain [21] when administered within a narrow window of 1 – 3 h of onset. However, treatment efficacy is associated with an elevated risk of intracerebral hemorrhage [22,23], reaching up to 6% in such treated patients [24]. Oxidative damage to vascular cell membranes was implicated [25-27], in addition to neurovascular cell death due to indirect degradation of extracellular matrix integrity. Combination stroke therapy with liposomes was demonstrated to help reseal compromised cell membranes and restore cellular function in a rat model of focal stroke. Hence, tPA reperfusion strategies involving liposomes could prove useful to restructure vascular damage and reduce hemorrhage associated with thrombolytic stroke therapy [28].

Based on the data from three large trials involving almost 100,000 patients, the efficacies of tPA and streptokinase in treating myocardial infarction are essentially identical. Both agents reduce death and reinfarction by about 30% in regimens containing aspirin [20]. Recent studies suggest that angioplasty with or without stent placement, when feasible, is superior to thrombolytic therapy, although direct comparisons using otherwise identical regimens have not been performed [29-31].

Moreover, the overall pharmacokinetics of thrombolytics is somewhat variable, which requires extremely patient monitoring in order to control the clinical outcome of the thrombolytic therapy. This derives from the fact that the majority of the clinically used thrombolytic drugs are in fact proteins or large peptide molecules that can suffer from various activations within the bloodstream. This can be easily seen from the short plasma half-lives of most of these agents upon intravenous administration. For example, native tPA has a plasma half-life of only about 5 min [32,33], which is extended a little, to approximately 15 – 20 min, for its recombinant forms, alteplase and reteplase [34,35]. In the same way, streptokinase and urokinase (or pro-urokinase), as purified or recombinant enzymes, have estimated principle plasma half-lives of 20 and 10 min, respectively [36,37].

4. Drug delivery approaches for protein-based thrombolytic drugs

4.1 Delivery and distribution issues for peptide and protein drugs

Thrombolytic therapy utilizes several different enzymes – fibrinoiysin (plasmin), streptokinase, urokinase (pro-urokinase), and tissue plasminogen activator [38-41]. Still, the use of proteins and peptides as therapeutic agents is hampered by the whole set of intrinsic properties associated with their nature as complex macromolecules, which are, as a rule, foreign to the recipient organism [42,43]. This leads to low stability of the majority of peptide and protein drugs at physiological pH values and temperatures, particularly when these proteins have to be active in conditions that differ from those of their normal environment. Processes leading to the inactivation of various biologically active proteins and peptides *in vivo* include: protein transformation into inactive conformation from the effect of temperature, pH, high salt concentration, or detergents; the dissociation of protein subunits or enzyme – cofactor complexes, and the association of protein or peptide molecules with the formation of inactive associates; non-covalent complexation with ions or low-molecular-weight and high-molecular-weight compounds, affecting the native structure of the protein or peptide; proteolytic degradation under the action of endogenous proteases; chemical modification by different compounds in solution (for example, oxidation of SH-groups in sulfhydryl enzymes and Fe (II) atoms in heme-containing proteins by oxygen; thiol-disulfide exchange, destruction of labile side-groups like tryptophan and metionine).

Exogenous proteins being administered into the organism undergo numerous changes due to external influences (schematically shown in **Figure 2**), which lead to their rapid inactivation and elimination from the circulation – mostly through renal filtration, enzymatic degradation, uptake by the reticuloendothelial system (RES), and accumulation in non-targeted organs and tissues. At non-targeted sites, a drug is wasted, in the best-case scenario. However, in many cases, the accumulation of protein and peptide drugs in healthy organs or tissues may cause undesirable side effects. In addition, rapid elimination and widespread distribution into non-targeted organs and tissues requires the administration of a drug in large quantities, which is not economical and is often associated with a nonspecific toxicity. Another very important point is the immune response of the macro-organism to foreign proteins containing different antigenic determinants. Specific antibodies against a given protein can rise during the lifespan or as a result of the repeated administration of a therapeutic protein (peptide). In any case, it leads to protein inactivation or even to allergic reactions, which very often make its further application impossible.

4.2 Protection of proteins against denaturation

The protection of a protein against denaturating influences can be achieved via two very general approaches: the steric separation of a protein/peptide and denaturating agent; and the modification of a protein/peptide, which hinders its interaction with denaturating factors [44-46]. The variety of methods has been developed (known under the common name 'protein immobilization') to achieve such protection via either or both of these approaches. Evidently, immobilized proteins are expected to possess a set of properties that are absent in their native precursors. The most important of these properties are: i) increased

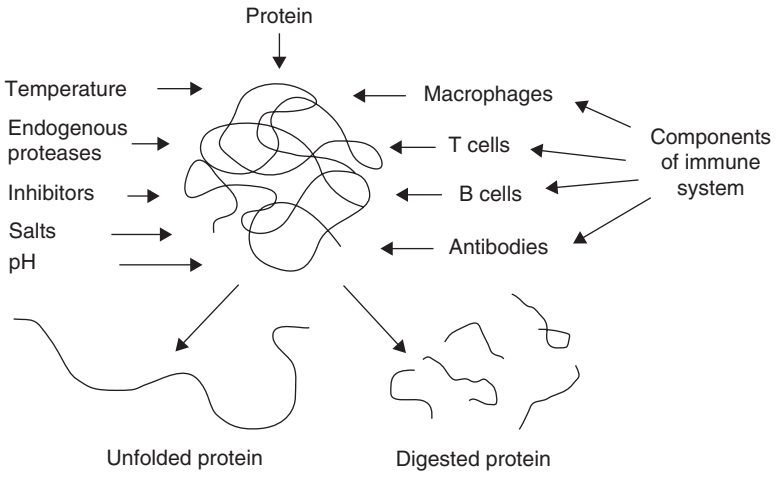

Figure 2. Various factors resulting in the inactivation of protein/peptide drugs in the body.

stability and prolonged activity in the body; ii) decreased immunogenicity and affinity to specific antibodies, which allows repeated administration of therapy; iii) decreased affinity to natural inhibitors; iv) the possibility to administer the whole therapeutic dose of a protein/peptide drug in a single injection, which is simpler and more convenient for both patient and physician; v) a decrease in the total quantity of a protein/peptide drug needed for the treatment, which makes the treatment more economical.

Parenterally administered immobilized/modified protein/peptide drug should exist in the active form for a long period. It should also reach and penetrate an affected site and demonstrate improved pharmacokinetic properties and an increase in bioavailability. In addition to improving upon the biological properties of a protein/peptide drug, it is desirable to obtain a preparation that is easier to handle.

4.3 Liposomes as carriers for protein and peptide drugs

Among many known methods to stabilize protein/peptide drugs and favorably change their bioavailability and pharmacokinetics, the encapsulation of such drugs into artificial microreservoir (microparticulate) carriers is frequently applied. Inside these carriers, a protein (therapeutic enzyme) is protected from many aggressive influences of the external medium and, in turn, does not act on normal tissues and cells. This type of system includes liposomes, micelles, polymer microparticles, and cell ghosts. The use of such carriers allows for a higher active moiety/carrier material ratio compared to many other systems. They also provide a higher degree of protection against enzymatic degradation and other destructive factors upon parenteral administration because the carrier wall completely isolates drug molecules from the environment. An additional advantage of these carriers is that a single carrier particle is capable of delivering multiple drug species. All microparticulates are of a size that excludes the possibility of loss by renal filtration. Certain problems with microreservoir-type carriers are associated with their tendency to be taken up by the RES cells, primarily in liver and spleen [47].

One of the most popular and well-elaborated technologies to immobilize/stabilize protein drugs and improve their pharmacological properties is their incorporation into artificial phospholipid vesicles (liposomes). The encapsulation of proteins and peptides into liposomes has been widely studied over the years. Liposomes are artificial phospholipid vesicles, obtained by various methods from lipid dispersions in water. The problems connected with liposome preparation, their physicochemical properties and possible biomedical application have already been discussed in several monographs [48-52]. By now, many different methods have been suggested to prepare liposomes of different sizes, structure and size distribution. The most frequently used methods are ultrasonication, reverse-phase evaporation and detergent removal from mixed lipid-detergent micelles by dialysis or gel filtration. To increase liposome stability towards the action of an aggressive physiological environment, cholesterol is incorporated into the liposomal membrane (sometimes up to 50% mol). The size of liposomes depends on their composition and preparation method, and can vary from around 50 nm to > 1 μm in diameter. The encapsulation efficacy for different substances is also variable depending on the liposome composition, size, charge, and preparation method. Use of the reverse-phase evaporation method [53] permits inclusion of ≥ 50% of the substance to be encapsulated from the water phase into the liposomes. Besides, a variety of methods have been developed to obtain lyophilized liposomal preparations possessing good storage stability [54]. The *in vitro* release rate of different compounds from liposomes, including proteins of a moderate molecular weight, such as lysozyme or insulin, is usually < 1%/h, under the condition that the incubation

temperature sufficiently differs from the phase transition temperature of a given liposomal phospholipid, since the maximal permeability of liposomes is usually observed at temperatures close to the phase transition temperature of the liposomal phospholipid. *In vivo*, this parameter can vary within wide limits from minutes to hours, and depends on the liposome membrane composition, cholesterol content, and liposome location in the body.

From the biomedical point of view, liposomes are biocompatible, causing no or very few antigenic, pyrogenic, allergic and toxic reactions; they easily undergo biodegradation; they protect the host from any undesirable effects of the encapsulated drug, at the same time protecting any entrapped drugs from the inactivating action of the physiological medium.

Liposomes loaded with protein/peptide drugs (proteoliposome) can incorporate these drugs in a variety of fashions: water-soluble proteins are entrapped into the liposomal inner aqueous space (and, in case of multilammellar liposomes, into the aqueous space between bilayers), while less soluble proteins and peptides may be incorporated into the phospholipid membrane. Intermediate cases also exist (see the scheme in **Figure 3**).

Biodistribution of liposomes, and hence their protein cargo, is a very important parameter from the practical point of view. As with other microparticulate delivery systems, conventional liposomes suffered from raid elimination from the systemic circulation by the cells of the RES [53]. Many studies have shown that within the first 15 – 30 min after intravenous administration of liposomes, 50 – 80% of the dose is adsorbed by the cells of the RES, primarily by the Kupffer cells of the liver. In order to make liposomes capable of delivering pharmaceutical agents to targets other than the RES, attempts were made to prolong their circulation lifetime. This was achieved with the development of surface-modified long-circulating liposomes grafted with a flexible hydrophilic polymer (PEG being the most common example [55]) that prevents plasma protein absorption to the liposome surface and the consecutive recognition and uptake of liposomes by the RES [56,57].

It has been shown with a broad variety of examples that, similar to macromolecules, liposomes are capable of accumulating in various pathological areas with affected vasculature (such as infarcts, and inflammations) via the enhanced permeability and retention (EPR) effect [58,59], and their longer circulations naturally enhance this mode of target accumulation. Evidently, long-circulating liposomes can be easily adapted for the delivery of peptide (protein)-based pharmaceuticals to infarcts and other 'leaky' areas.

4.4 Liposomes for the delivery of thrombolytic agents

Thus, it seems quite natural that liposomes have been successfully used as pharmaceutical carriers for thrombolytic drugs. One of the notable examples in experimental thrombolytic therapy is using the liposome-incorporated tissue-type plasminogen activator (tPA) in rabbits with experimental jugular vein thrombosis in an attempt to improve the therapeutic index of tPA as thrombolytic agent. The following preparations were compared in terms of thrombolytic activity and side effects upon the bolus injection in the ear vein: free tPA, liposome-encapsulated tPA, tPA encapsulated in plasminogen-coated liposomes, and the mixture of free tPA and empty liposomes. Both liposomal tPA formulations (plain and plasminogin-coated) showed a significantly better thrombolysis efficiency than equimolar doses of free tPA: about 0.24 mg/kg of the liposomal tPA showed the lytic activity – equal to that of a dose of 1.0 mg/hg of free tPA, i.e., about fourfold improvement. At the same time, liposome encapsulation did not affect the systemic activation of alpha 2-antifibrinolysin and plasminogen by tPA. Further optimization was proposed by coupling liposomal tPA to plasminogin [60].

Liposomal forms of thrombolytic drug (enzyme) streptokinase have also been prepared. Early work by Bhakta and colleagues [61] investigated the thrombolytic efficacy of liposome-encapsulated streptokinase in a canine model of acute myocardial infarction. The time required to restore vessel patency was reduced by > 50% with the liposomal enzyme, compared with free streptokinase. Additionally, the total dosage of streptokinase required for therapy was lower, and smaller remnant thrombi were observed with the encapsulated agent. These initial results demonstrated significant implications for further reduction in mortality from heart attacks by therapy with liposome-encapsulated plasminogen activators [62].

In later work, streptokinase-bearing pegylated liposomes were prepared by the freeze-thawing method and administered via femoral vein to rats (15,000 IU/kg) [63]. The activity of streptokinase in plasma was determined following the amidolytic activity of the streptokinase – plasminogen complex. Pharmacokinetic parameters of streptokinase incorporated in liposomes were also compared with those of free streptokinase. The half-life and plasma AUC of PEG-liposomes-encapsulated streptokinase increased by 16.3- and 6.1-fold, respectively, compared to those of free streptokinase. Hence, longer thrombolytic activity was predictable with liposomal streptokinase formulation, compared with drug alone [63].

Streptokinase was also encapsulated into interdigitation-fusion liposomes, and tested in both normal rabbit model of thrombolysis and in rabbits immunized against streptokinase [64]. In non-immunized rabbits, the thrombolytic activity of the liposomal streptokinase was comparable to that of free streptokinase. Nevertheless, in rabbits immunized against streptokinase, liposomal (33.8 ± 1.5%) but not free streptokinase (29.3 ± 2.1%) showed significant thrombolytic activity compared with saline (22.4 ± 3.3%). The authors generally favored liposomal streptokinase, and suggested that the liposomal streptokinase would be particularly beneficial to those patients possessing high levels of antistreptokinase antibodies (5% of the population), to

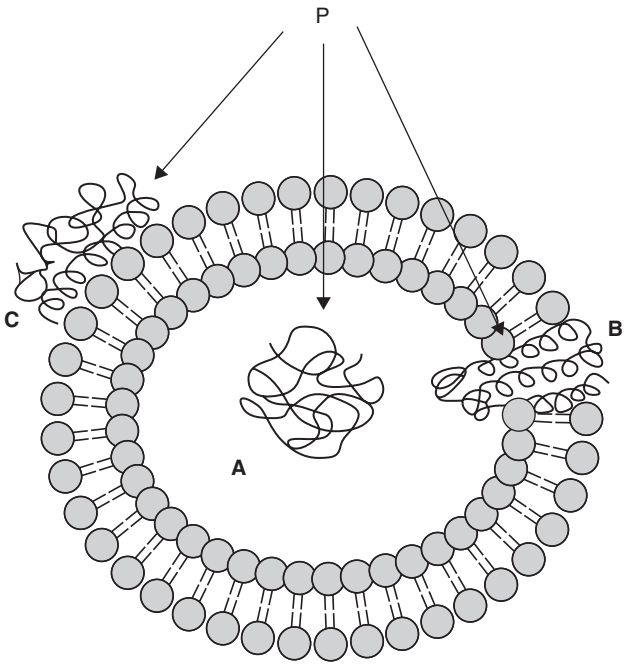

Figure 3. Protein immobilization into liposomes. Protein (P) entrapment into liposomes: **A.** hydrophilic proteins can be encapsulated in the inner aqueous space of the liposome; **B.** hydrophobic proteins incorporate into the phospholipid membrane; **C.** proteins could also be adsorbed on the liposome surface.

provide the equivalent degree of therapy expected from free streptokinase [64].

In one recent study, the efficacy of two formulations of encapsulated streptokinase and free streptokinase was compared in a rabbit model of carotid artery thrombosis [65]. Arterial thrombosis was induced by the injection of thrombin mixed with autologous whole blood into rabbit carotid arteries, then, 30 min after the confirmation of an occlusive thrombus, different streptokinase formulations were infused at a dose of 6000 IU/kg into the jugular vein. Free streptokinase was compared with identical doses of the liposome-encapsulated streptokinase entrapped in a water-soluble polymer, and free streptokinase mixed with blank microparticles. Carotid arterial blood flow was determined by pulsed Doppler flowmetry to confirm clot formation and reperfusion, followed by animal sacrifice 2 h after drug infusion, and assessment of residual thrombus mass. Compared with free enzyme (74.5 ± 16.9 min), both liposomal and polymer-microencapsulated streptokinase demonstrated significantly reduced reperfusion times of 19.3 ± 4.6 min and 7.3 ± 1.6 min, respectively, with no appreciable effect in the case of the free streptokinase + blank microparticles mixture. Moreover, both liposomal and microencapsulated streptokinase resulted in the reduced residual clot mass and greater return of arterial blood flow, confirming that the encapsulation of streptokinase offers a potential approach to improve the fibrinolytic treatment in the patients with clot-based disorders [65].

Another example illustrates the tremendous potential of liposome-entrapped antiplatelet peptides to reduce both intravascular platelet aggregation and thrombosis [66]. In this work, the capacity of CD39-containing liposomes to inhibit platelet activation induced by ADP, collagen, or thrombin was determined *in vitro* by platelet aggregometry. CD39-liposomes caused the decrease in Km by nearly an order of magnitude over the detergent-solubilized CD39 form, with related increase in both ADPase and ATPase catalytic efficiencies. Furthermore, CD39-liposomes effectively inhibited platelet aggregation when platelets were activated by ADP, collagen, or thrombin, and also promoted platelet disaggregation (60.4% ± 6.1%). A murine model of thromboplastin-induced thromboembolism was used to determine the *in vivo* effectiveness of intravenous liposomal CD39 in limiting platelet consumption and mortality. While the treatment with CD39-liposomes did not change the platelet counts after the thromboplastin injection, in survival studies, liposomal CD39 reduced mortality from 73 to 33% [66].

The following parameters are usually considered as a proof of enzyme incorporation into the inner aqueous phase of liposomes or its firm and irreversible association with the liposomal membrane: the possibility of chromatographic separation of liposome-encapsulated and free enzyme; the latency of liposome-encapsulated enzymes; and the correlation between protein incorporation and a change in net charge of the lipid bilayer [67-69]. A good example of the effective tPA incorporation into liposomes can be seen in studies that have used dried-rehydrated liposomes for protein encapsulation in the presence of the cryoprotectant, trehalose. Data demonstrated that the entrapment efficacy of about 50% was achieved for model protein BSA and active tPA. While varying the lipid composition significantly affected the loading efficiency of the proteins, it was shown that tPA encapsulation and release were dependent on both membrane rigidity and the presence of trehalose as cryoprotectant, used to preserve the stability of the formulation during freeze-drying [70].

4.5 Targeting of antithrombotic drugs and drug carriers

Therapeutic targeting of the most prominent components of Virchow's triad is one of the main strategies for both prevention and treatment of thrombotic disorders. It is virtually impossible to consider drug targeting within the bloodstream, separately from the main organs of the vascular system. Heart and lung diseases are closely related to those of the blood vessels. Thus, for example, a heart disease such as myocardial infarction is usually caused by thrombosis or atherosclerosis of the coronary blood vessels; pulmonary embolism is the direct result of the thrombotic occlusion of the pulmonary artery. In fact, the

same irregularities underlie the development (as well as therapy) of thrombi and atheromas in any particular compartment of the circulation. Therefore, many approaches to be considered within this section are of a general character and might also be applied to the diagnostics or treatment of similar pathologies located in the pulmonary or cardiac compartment of the vascular system [71].

The natural and most important intravascular target, which can also be met in the lungs and the heart, is the blood clot. Targeted visualization (imaging) of thrombi and targeted thrombolytic therapy are now among the hottest areas in the fields of both drug delivery and thrombolysis. The most popular construct to be used for targeting thrombi is a conjugate between a diagnostic label (primarily radioactive metal) or thrombolytic enzyme (urokinase, streptokinase, tPA), sometimes free but mostly loaded into protecting nanovesicles, and thrombus-specific monoclonal antibody. The main reason for this approach in thrombolytic therapy, as pointed out in [72], is the fact that the use of some recently developed thrombolytic agents such as single-chain urokinase-type plasminogen activator and tPA was disappointing, mainly due to some of their negative properties *in vivo* (i.e., rapid inhibition and clearance).

Naturally, fibrin – the main non-cellular thrombus constituent that is not present in the blood or in normal tissues – has been considered a promising antigen from the very beginning. However, the structural similarity of the 'pathological' fibrin and 'normal' fibrinogen and the presence of common antigenic determinants in their molecules make it difficult to raise antibodies capable of selective binding with fibrin in the presence of fibrinogen (the real situation existing in the blood). This is why the very first attempts to prove the principal possibility of using enzyme-antibody conjugates for enhanced thrombi dissolution were performed in simplified model conditions *in vitro*. Thus, proteolytic enzymes bound with polyclonal antifibrin antibodies via dextran bridges were shown to adsorb selectively on artificial fibrin clots and enhance their degradation [73]. Plasminogen activator – urokinase – was bound directly to polyclonal rabbit antibodies against human fibrinogen via the heterobifunctional reagent *N*-succinimidyl 3-(2-pyridylthio)-propionate (SPDP) [74]. Both components of the antibody-enzyme conjugate preserved their specific activity. The conjugate also demonstrated an increased stability in human plasma, compared with the native urokinase. Later, the whole family of monoclonal antibodies against various specific fibrin epitopes appeared [75]; see **Table 1** for some examples [71,76].

Further, monoclonal antibodies have been obtained against synthetic polypeptides imitating amino acid sequences specific for fibrin but not for fibrinogen (in particular, the amino-terminal sequence of the human fibrin chain) [77,78]. It was shown that these monoclonal antibodies specifically bind fibrin but not fibrinogen, and preserve their specificity in the presence of fibrinogen in concentrations similar to that of normal human plasma. Haber and co-workers [79,80] demonstrated that antifibrin antibodies can be conjugated with urokinase via SPDP without affecting the specific properties of the enzyme or antibody. Also, tPA was coupled to antifibrin antibody, 59D8, via disulfide bonds at neutral pH values [32], and the tPA-antibody 59D8 conjugate was shown to be 3 – 10 times more potent than free TPA or its conjugate with nonspecific antibody. Echogenic liposomes were covalently linked to fibrin(ogen)-specific antibodies and their F(ab') fragments, and used for the ultrasonic imaging of atherosclerotic plaques and subsequently for targeted delivery of thrombolytic proteins [81]. This approach will be discussed in more detail in the following section.

Platelets themselves have also been used as targets for thrombolytic therapy. The targeting of thrombolytics to platelet-enriched thrombus was achieved by urokinase conjugation with the monoclonal antibody selective towards platelet surface IIb/IIIa glycoprotein [82]. Antibodies against other platelet glycoproteins, such as GPIIIa and GPIIb, have also been conjugated with different plasminogen activators and used for targeted thrombolysis [83].

In several studies, liposomes were surface-modified by fibrinogen-mimetic cyclic RGD motifs that can selectively target and bind integrin GPIIb-IIIa on activated platelets [84,85]. The *in vitro* platelet-binding of RGD-modified liposomes was showed to be superior compared with non-targeted liposomes. RGD-modified liposomes tested *in vivo* in a rat carotid injury model and analyzed *ex vivo* were found to bind activated platelets much better than the control RGE-liposomes. At the same time, the RGD-liposomes did not exhibit any significant effect on platelet activation or aggregation. Hence, this approach is considered a feasible way for the development of a platelet-targeted antithrombogenic drug delivery system, rather than for the targeted treatment of established thromboembolism.

Damaged endothelial cells can also serve as targets for antibody-targeted thrombolytic conjugates/liposomes. In the initial stage of many vessel injuries, including atherosclerosis and thrombosis (coronary among them), a disruption of the integrity of the endothelial cover of the vessel wall leading to subendothelial injury (denudation) serves as a strong stimulator of platelet activation and adhesion [86,87]. Naturally, it is tempting to think of early detection of such disruptions of the endothelium, and direct action at these sites to promote endothelium regrowth or prevent platelet adhesion onto the exposed subendothelial collagen. Thus, urokinase was conjugated to the monoclonal antibody P14G11 against damaged endothelial cells and effectively reduced thrombus formation *in vivo* in the rat vena cava model [88].

To prove the possibility of using targeted immunoliposomes as specific drug carriers to such areas, conjugates have been obtained between liposomes and antibodies against extracellular matrix antigens like collagen, laminin and

Table 1. Thrombus accumulation of some thrombus-specific antibodies and proteins with natural affinity towards the thrombus* [76].

Antibody or protein	Thrombus-to-tissue ratio, times (M ± SEM)	Thrombus-to-blood ratio, times (M ± SEM)
4C1 (antithrombospondin)	6.2 ± 2.1	5.1 ± 1.8
CA1 (antifibrin)	9.5 ± 3.4	7.9 ± 2.3
5D5 (anti-cardiac ATPase, control)	0.9 ± 0.6	1.3 ± 0.6
Fibrinogen	4.2 ± 1.5	3.9 ± 1.1
Thrombin (devoid of clotting activity by chemical modification)	3.8 ± 1.8	3.6 ± 1.6
Heat-inactivated α-chymotrypsin (control)	1.1 ± 0.6	0.9 ± 0.5

*Rabbit experiments, n = 4 for each protein; thrombus was induced by the wool thread method; monoclonals were labeled with ^{111}In via DTPA residues; activity counting of the blood was performed 2 h after injection.

fibronectin [89,90]. Studies have shown that radiolabeled liposomes with coupled anticollagen or antifibronectin antibody can specifically recognize and bind collagen gaps in endothelial cell cultures grown on fibrillar collage, and also showed four- to sixfold higher accumulation on the balloon-denuded vessel areas compared to that of nonspecific liposomes in *ex vivo* bovine, rabbit and human arterial segments. Thus, it was demonstrated that liposomes can be effectively targeted to certain areas of the pathological luminal vessel wall, which opened opportunities for the targeted delivery of diagnostic agents and/or therapeutics to these areas [71].

Antimyosin immumoliposomes were proven as a useful adjuvant to conventional thrombolytic therapy. Because oxidative injury is likely to involve damage to vascular cell membranes, it was hypothesized that a treatment that could 'reseal' membranes may be useful. Such an approach uses targeted immunoliposomes which recognize intracellular antigens that become exposed in cells with damaged membranes, e.g., liposomes with coupled antimyosin monoclonal antibody. These targeted immunoliposomes would then selectively bind to damaged cells and fuse (plug) with the damaged membranes. The validity of this idea has been shown in hypoxic myocytes *in vitro* [71] and in an *in vivo* model of cardiac ischemia [44,91]. It was also shown that antiactin-targeted immunoliposome would significantly reduce tPA-induced hemorrhage in hypertensive rat model of embolic focal stroke [28]. Results indicate that delayed administration of tPA (free or co-administered with non-targeted liposomes) induced intracerebral hemorrhage volumes of 9.0 ± 2.4 ul. On the other hand, in rats treated with tPA plus antiactin immunoliposomes, hemorrhage volumes were significantly reduced to 4.8 ± 2.7 ul. Moreover, fluorescent immunohistochemistry showed that rhodamine-labeled targeted liposomes co-localized with vascular structures in ischemic brain which stained positive for the endothelial barrier antigen, a marker of cerebral endothelial cells. These data suggest that immunoliposomes may ameliorate vascular membrane damage and reduce hemorrhagic transformation after thrombolytic therapy in cerebral ischemia.

Although different antithrombus monoclonal antibodies seem to be very convenient vectors for the targeted delivery of thrombolytics, attempts have been made to use other targeting moieties. Attention was paid to a number of blood proteins and peptides demonstrating increased accumulation in the thrombus. Such proteins participate in thrombus formation, which results in their higher concentration in the thrombus than in the blood. Fibrinogen and thrombin can serve as examples. One of the early examples is the immobilization of urokinase on fibrinogen (UK-FGN) [92,93] via diamine-derived spacer arm to minimize the mutual inactivating effect of two proteins because of steric hindrances. In experiments on dogs with induced arterial or venous thrombosis, it was proven that UK-FGN far exceeds the native enzyme in the prevention of radiolabeled fibrinogin incorporation into the growing thrombus.

When thrombin was used as a carrier for thrombolytic targeting [94], it was first chemically modified to eliminate its clotting activity without affecting its property to bind to activated platelets [95]. Besides, modified thrombin does not cause platelet aggregation. At the same time, its ability to bind to fibrin and to accumulate in the model blood clot remains practically unchanged. Modified inactivated thrombin was used to obtain a urokinase-thrombin conjugate possessing both thrombolytic activity and the ability to accumulate on thrombi *in vitro*, and in *ex vivo* flow models [96].

One unique example is based on a combined delivery system composed of liposomes loaded with the model protein horseradish peroxidase (HRP), encapsulated inside fibrin [97]. In principle, liposomes enable the protein to remain in its preferred aqueous environment and protect it during the polymerization process, while the encapsulation of liposomes inside fibrin was carried out to achieve a depot system with sustained protein release. *In vitro* experiments showed that the protein-loaded liposomes were absolutely stable within the fibrin network. In contrast to 'free' HRP,

enzyme entrapped in liposomes was completely retained by the fibrin network and wasn't released from the device unless the fibrin was degraded by plasmin. Hence, this combined liposomal delivery system shows great potential as both targeted and depot delivery vehicle for thrombolytic enzymes at the site of active thrombus.

4.6 Liposomes for drug delivery and monitoring of thrombolytic therapy

It is interesting to note that many of the monoclonal antibodies mentioned above are already used for the diagnostics of thromboses. Localization and visualization of thrombi are usually performed with radiolabeled antibodies, radioactive γ-emitting isotopes of heavy metals serving frequently as a label that can be bound to the appropriate antibody via the chelating group chemically incorporated into a protein [98]. The localization and visualization of pulmonary emboli with radiolabeled fibrin-specific monoclonal antibody can serve as a good example for this approach [99]. ^{111}In- and ^{99m}Tc-labeled antifibrin antibodies have also been successfully used for diagnosis of deep vein thrombosis [75].

One of the approaches in this area is based on the concept of acoustically reflective liposomes that can be targeted for site-specific acoustic enhancement [100,101]. Liposomes made of phosphatidylcholine, 4-(*p*-maleimidophenyl) butyryl phosphatidylethanolamine, phosphatidylglycerol, and cholesterol were conjugated with antifibrinogen antibodies via the thioether linkage, and shown to acquire the ability to attach to fibrin-coated surfaces and thrombi in cell culture and flow models [102,103]. In addition, antifibrinogen acoustically reflective liposomes were shown to attach to fibrous atheroma and thrombi in a Yucatan miniswine model of induced atherosclerosis, whereas liposomes conjugated to anti-intercellular adhesion molecule-1 (anti-ICAM-1) were demonstrated to target early stage atherosclerotic plaques [104].

This concept was further developed into targeted delivery of echogenic liposomes with thrombolytic loading [81,105], and the effect of ultrasound exposure of thrombolytic-loaded echogenic liposomes on thrombolytic efficacy was investigated. Following 50% tPA entrapment into acoustically reflective liposomes, *ex vivo* porcine clots treated with tPA-loaded echogenic liposomes demonstrated an effective clot lysis with an effect similar to the treatment with free tPA [81]. Ultrasound exposure of tPA-loaded echogenic liposomes resulted in twofold enhanced thrombolysis compared with the case with no ultrasound, and it appears that the majority of the ultrasound effect was related to the increase in the localized drug release from the tPA – liposome complex. In a recent study, these tPA-loaded echogenic liposomes demostrated twice the affinity for fibrin than free tPA, and the evaluation of the clots demonstrated an enhanced highlighting of clots treated with tPA-echogenic liposomes compared to controls [105].

Liposomal vesicles have also been loaded with ^{99m}Tc-radiolabeled fibrinolytic enzymes, urokinase [106] and streptokinase [107], and demonstrated ample enzyme capacity and a slow release profile. The tracking of the biodistribution behavior of these preparations showed an increased thrombus uptake of the liposomal enzymes compared with that of the free drugs, in addition to improved imaging quality of thrombi.

5. Expert opinion

Liposomes have been successfully used as experimental carriers for thrombolytic protein drugs (enzymes) and thrombi-imaging agents. Promising results have been obtained in *in vitro*, *ex vivo* and *in vivo* systems. Still, the total number of publications in this area remains limited and there are no available data on clinical testing of such preparations. Though there is ample industrial experience with the liposomal drugs, liposome-based thrombolytic preparations have still failed industry tests, and many important questions remain unanswered regarding the feasibility of scaling up the corresponding processes, not to mention the final cost of the resulting liposomal thrombolytics. This all contributes to the conclusion that prospects for clinical application of the liposome-based thrombolytic drugs remain dim, although studies in this direction continue to be encouraged by the recognized usefulness of liposomes as drug carriers and the positive clinical experience that has been accumulated with other liposomal drugs.

Declaration of interest

The authors state no conflicts of interest and have received no payment in the preparation of this manuscript.

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