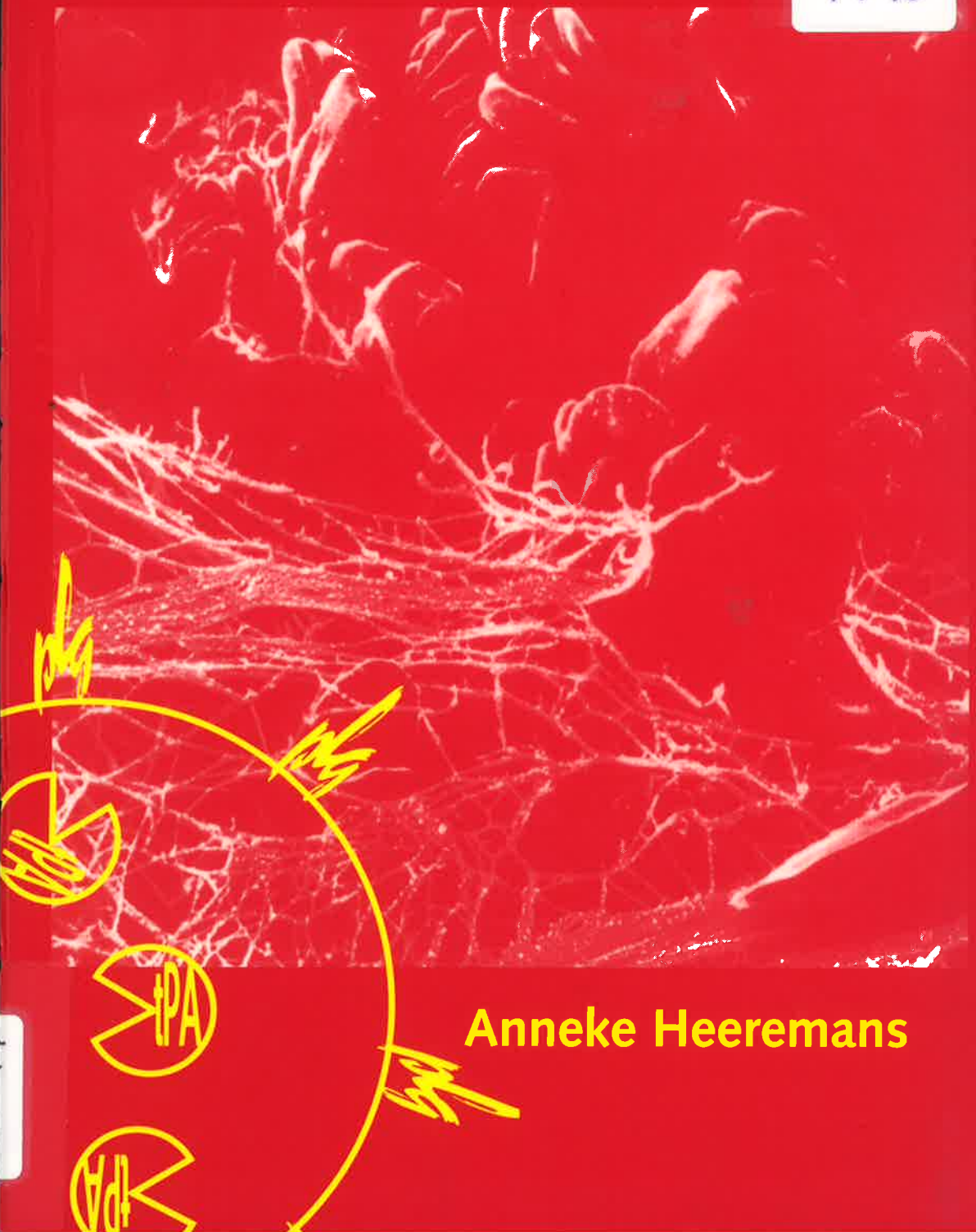
Liposomes in thrombolytic therapy

t-PA targeting with plasminogen-liposomes, a novel concept



Anneke Heeremans

Liposomes in thrombolytic therapy

t-PA targeting with plasminogen-liposomes, a novel concept

Liposomen voor thrombolytische therapie

t-PA targeting met plasminogeen-liposomen, een nieuw concept

(met een samenvatting in het Nederlands)

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Johanna Louise Maria Heeremans

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Prof. Dr. C. Kluft

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Life gives everything that is needed to be happy:
all you need to do is reach out for it

Aan mijn ouders

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Contents

Chapter 1

General introduction

Liposomes

Liposomes are vesicular structures consisting of hydrated bilayers. They are formed when certain amphiphatic phospholipids are exposed to an aqueous environment. Defined polar and nonpolar regions exist in the bilayer, since the phospholipids direct their polar headgroups towards the water phase, while the hydrophobic acyl chains self-aggregate at the inner side of the bilayer. Liposomes can differ considerably with respect to their behaviour in vivo and in vitro, depending on the selected bilayer components and on the way they are prepared. Informative reviews and textbooks, partly authored by our research group, are readily available and the reader is referred to those for detailed information about these versatile carrier systems [1-7]. The major phospholipid forming the backbone of the bilayer is usually phosphatidylcholine, a neutral lipid. Charged phospholipids, like phosphatidylglycerol or phosphatidylserine are often included in the bilayer. These charged phospholipids tend to improve the stability of liposomes because electrostatic repulsion forces hamper close contact between neighbouring vesicles. Bilayer rigidity depends strongly on the selected bilayer components. Phospholipids from natural sources containing unsaturated fatty acids (such as phosphatidylcholine, phosphatidylglycerol and phosphatidylserine) form loosely packed 'fluid state' bilayers at room temperature. These bilayers can be rigidified (made less permeable) by inclusion of cholesterol. Another approach to rigidify liposomal bilayers is the use of saturated long acyl chains in the apolar parts of the phospholipids. Examples are dipalmitoyl-phosphatidylcholine and distearoylphosphatidylcholine.

Liposomes have drawn much interest in their role of drug carriers, in particular of bioactive molecules such as peptides and proteins. Their use as drug carrier is based on several characteristics, which make them unique among colloidal carrier systems. The liposomal carrier can protect the drug against metabolic degradation and may favourably change its disposition. Moreover, the liposomal structure with both lipophilic and aqueous regions makes them suitable for entrapment of a wide variety of drugs. The internal liposomal aqueous milieu can protect encapsulated proteins from (irreversible) structural changes often observed when a protein is transferred into a water-free medium. Furthermore, liposomes have been shown to be relatively safe [8]. They can be formulated as parenteral pharmaceutical products, successfully overcoming problems like reproducibility, scale up, sterility and stability. Liposomes are easy to manipulate, and can be used for controlled release of the drug. 'Homing devices' can be attached to liposomes to improve their targeting potential to reach certain target sites. As a homing device (monoclonal) antibodies or other tissue-selective molecules may be used, enabling specific interaction of the liposomes with the biological environment, leading to site-specific accumulation.

Acute myocardial infarction, thrombolytic treatment, t-PA

Acute myocardial infarction is one of the main causes of death and disability in the adult population of Western societies. During an acute myocardial infarction a thrombus, stabilized by fibrin, usually causes the obstruction of one of the coronary arteries, which leads to myocardial ischemia and cell death. For treatment aggressive thrombolytic therapy is the medical approach of choice [9]. Early, complete and sustained reperfusion of the coronary artery is the ultimate goal in the treatment of acute myocardial infarction [10].

Thrombolytic therapy involves the fibrinolytic system: pharmacological dissolution of the thrombus is initiated via intravenous infusion of plasminogen activators that activate the natural enzyme system in the blood and thrombus. Plasminogen activators convert the fibrinolytic pro-enzyme plasminogen to the active enzyme plasmin, which then digests fibrin to soluble degradation products [11]. Currently, several thrombolytic regimens, often combinations of thrombolytic agents with conjunctive antithrombotic therapy, have been tested for their efficacy in treatment of myocardial infarction. Many clinical studies report on (usually small) differences between these regimens [e.g. 12]. An important drawback of thrombolytic treatment is the occurrence of systemic effects, concerning degradation of haemostatic plugs or effects on circulating components in the blood [8]. Bleeding episodes during lytic therapy can be life-threatening when located intracranially (in up to 1% of the patients) [13]. Besides, all fibrinolytic treatments are associated with increased thrombin generation in blood [14-18] which potentially favours reocclusion. The natural thrombolytic agent t-PA (tissue-type Plasminogen Activator) has the benefit of showing fibrin-specificity under physiological conditions [19]. However, at the high dosages required in thrombolytic treatment in clinical practice, thrombus specificity of t-PA is far from realized and adequate prevention of systemic side-effects can not be guaranteed [9, 20].

Another drawback associated with the use of t-PA is its short plasma half life of 3-5 minutes in humans, which not only calls for high doses to be administered [20], but which could also favour reocclusion [17, 21]. Therefore, intravenous administration of t-PA over an extended period of time has been considered [21] and novel recombinant plasminogen activators with prolonged half lives are being tested (e.g. [22]).

Concept

In this thesis the following concept is discussed: Improved therapeutic index of t-PA by targeted delivery through liposomes exposing glu-plasminogen.

This concept is schematically depicted in Figure 1. The goal is to minimize systemic side-effects of t-PA by liposome encapsulation and accumulation of t-PA laden liposomes at the target site (fibrin thrombi). This targeting is envisioned to be accomplished by attaching glu-plasminogen to the external leaflet of the liposome bilayer.

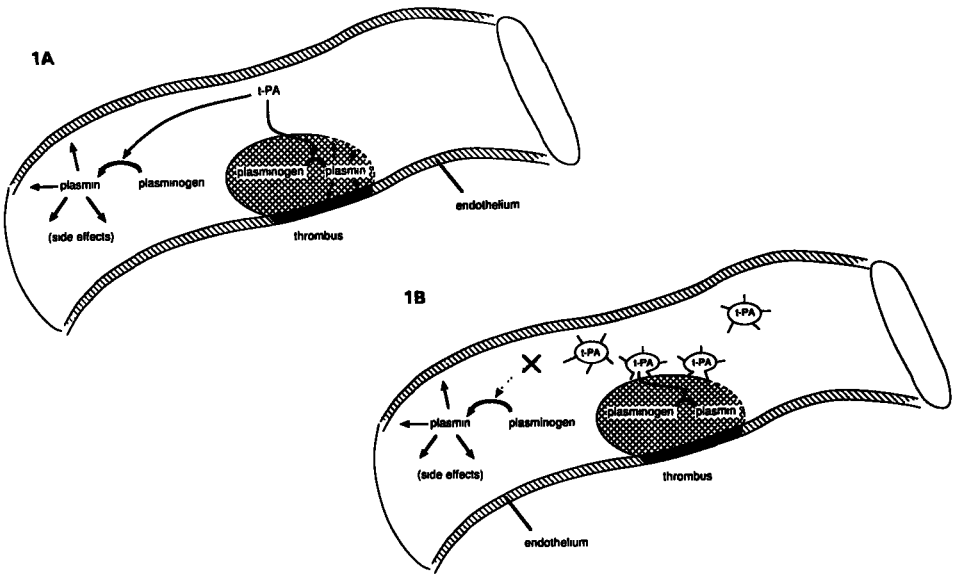

Figure 1 Schematic representation of the concept of this thesis.
While administration of free t-PA may cause systemic side-effects by activation of circulating Plg (A), t-PA laden liposomes which are transported specifically to the target tissue will only be active at the site of the thrombus (B).

t-PA, some basic physical and chemical information

Tissue-type Plasminogen Activator consists of 527 amino acids and has a molecular weight of about 70 kDa. A comprehensive overview of its structural and physicochemical properties can be found in a review by Nguyen et al. [24]. It is a domainally structured molecule, with a finger domain, an epithelial growth factor domain and two kringle structures in the heavy (N-terminal) chain, while the active site triad of amino acids (serine, histidine, aspartic acid) resides in the light (C-terminal) chain. At positions 117, 184, 218 and 448 sugar residues can be attached to the t-PA backbone. Its solubility in neutral aqueous solutions is usually below 0.2 mg/ml, but can be influenced by pH, ionic strength and the presence of basic amino acids like arginine or lysine. The endogenous level of t-PA in human blood is between 1 and 5 ng/ml (between 0.01 and 0.07 nM). Under normal haemodynamic conditions t-PA is relatively inactive, but its ability to activate plasminogen is markedly enhanced in the presence of fibrin [e.g. 9, 20, 24-26].

The fibrin selectivity of t-PA (partly) derives from its pronounced affinity for fibrin rather than for fibrinogen. Published Kd-values for the binding of t-PA to fibrin vary from the μM range [27] to the nM range [28, 29], depending on the different experimental conditions and the assumptions made.

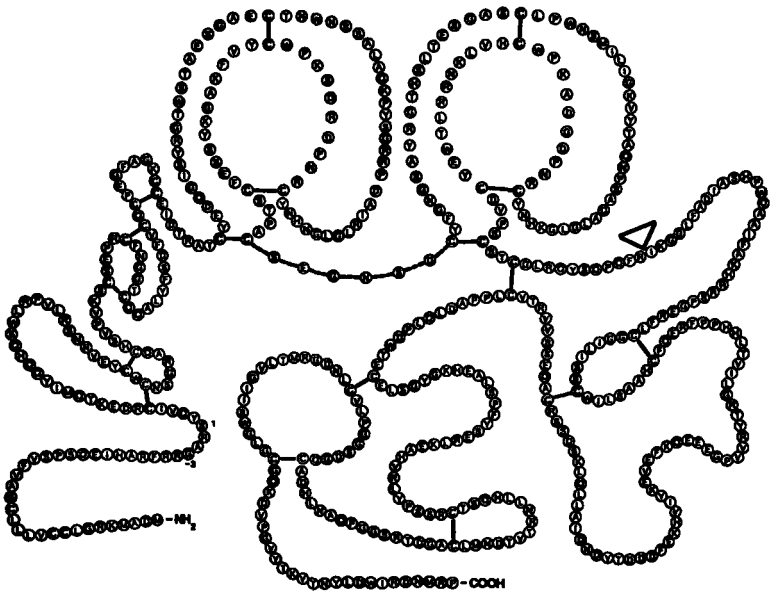

Figure 2 Covalent structure of t-PA.
(Reprinted with permission from [23]. Copyright 1984, American Chemical Society.)

Glu-plasminogen, some basic physical and chemical information

Native plasminogen consists of 790 amino acids and has a molecular weight of about 89 kDa. It is called more specifically glu-plasminogen, since it has a NH_2-terminal glutamic acid residue. Detailed structural and physicochemical information can be found in reference [30]. It is composed of an N-terminal peptide region, five kringles and a C-terminal serine protease domain. Positions 289 and 346 are carbohydrate attachment sites. By plasmic digestion the N-terminal peptide region of 77 residues can be cleaved. The derived degraded form of plasminogen is called lys-plasminogen, since the predominant NH_2-terminal amino acid is lysine (valine and methionine also occur). Both glu- and lys- plasminogen are enzymatically inactive. The glu-plasminogen plasma concentration is about 180 μg/ml (2 μM). The transition from plasminogen to active plasmin (by t-PA or another plasminogen-activator) involves cleavage of the Arg560 - Val561 bond. By this cleavage plasmin is formed, a two-chain molecule held together by two disulphide bridges. The heavy A-chain (N-terminal)

contains the kringles, the light B chain (C-terminal) the catalytically active SP domain [e.g. 30-32]. Plasminogen has different kinds of binding sites. Lysine binding sites (LBS) can bind carboxy-terminal lysine-analogues such as lysine and ε-amino-caproic acid. The so called AH (amino-hexyl) binding sites only bind amino-hexyl ligands without a carboxy-terminus, such as benzamidine. Three conformations of plasminogen have been postulated: the compact α-form, with a LBS and an AH intramolecular interaction, the semi-opened β-form, with only the intramolecular lysine binding, and the open γ-form, devoid of the above mentioned intramolecular interactions. Binding of benzamidine to native α glu-plasminogen induces conformational changes in glu-plasminogen to generate the β-form, which is similar to the structure of lys-plasminogen; it is more easily activated by t-PA than the more compact α glu-plasminogen. The same α to β conversion is thought to occur upon binding of glu-plasminogen to (intact) fibrin. β to γ conversion can take place upon binding of lysine or ε-amino-caproic acid, or upon further binding of plasminogen to lysines on a partially degraded fibrin surface [26, 30, 33, 34]. Kd values for binding of glu-plasminogen to intact fibrin between 1 and 40 nM have been published, whereas partially degraded fibrin binds plasminogen more strongly [26, 35].

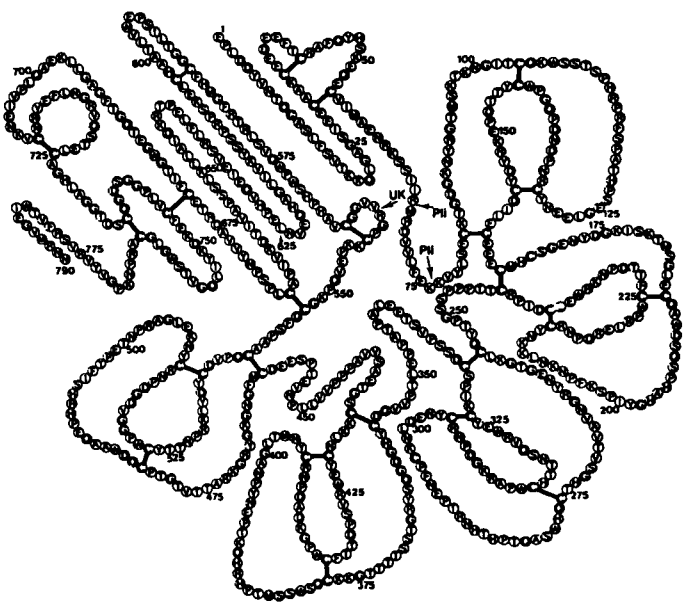

Figure 3 Covalent structure of glu-plasminogen.
(Reprinted with permission from Seminars in Thrombosis and Hemostasis Vol. 8, p. 4, 1982, Thieme Medical Publishers Inc.)

Aims and outline of this thesis

This thesis reports on the concept where liposomal encapsulation of t-PA is combined with coupling of glu-plasminogen -homing device- to the outside of the liposomes [36] (Figure 1). Combination of these two aspects (encapsulation and targeting) is expected to improve the therapeutic index of thrombolytic treatment relative to free t-PA for two main reasons: a) a reduction of systemic activity by t-PA is obtained by the encapsulation and, consequently, the physical separation of t-PA from plasma plasminogen, b) liposomes are targeted -by glu-plasminogen exposed on the surface of the liposome- to fibrin deposits. This will increase the fraction of t-PA actually reaching the thrombus compared to non-targeted delivery.

One may wonder whether liposomes will be able to reach ischemic myocardial tissue under reduced blood flow conditions. In vivo studies where liposomes were used under these circumstances indicate that, indeed, (non-targeted) liposomes were able to reach the ischemic site [e.g. 37-40]. Tang et al. [37] even showed that ischemia of the myocardium significantly increases the liposome uptake. This effect is probably related to ionic disturbances and membrane lipid peroxidation of the injured tissue.

Besides, the described concept may not only be applicable to targeted administration of thrombolytic agents during myocardial infarction, but also in case of pulmonary artery thromboembolism or deep venous thrombosis.

Glu-plasminogen was chosen as the preferred homing device for several reasons. First of all, it has affinity for fibrin, a first prerequisite. Secondly, it is a human endogenous protein, with minimal induction of immunological effects upon liposomal administration. Thirdly, fibrin-affinity is expected to increase during thrombolysis when nicked (partially degraded) fibrin is produced. This could increase target site delivery.

The implementation of this concept calls for special precautionary measures, since enzyme (t-PA) and substrate (glu-plasminogen) are present in one formulation. In order to create these complex liposomes, various novel technologies were developed separately and eventually combined.

Aim of this thesis was to study different elements leading to a new concept for targeted delivery of thrombolytics. Some of these elements have a technological character, some are more pharmacokinetically / pharmacologically oriented. Thereby, it elegantly exemplifies the multidisciplinary nature of drug targeting research. In this thesis the different aspects of the described concept were examined following a "target-oriented approach". Choices had to be made in order to gain insight into critical success factors for this complicated concept: time did not allow us to elaborate on all details. Each chapter of this thesis addresses a specific issue. They are not necessarily presented in chronological order. Therefore, discrepancies between chosen experimental conditions per chapter can arise.

In *Chapter 2a* a procedure for covalent coupling of the homing device glu-plasminogen to the surface of liposomes is described. *Chapter 2b* reports on the interference of ε-amino-caproic

acid with this coupling procedure. The in vitro fibrin binding (targeting) characteristics of the obtained glu-plasminogen-bearing liposomes are presented in *Chapter 3*.

In *Chapter 4* the reader is informed about efforts to optimize the preparation procedure for tissue-type Plasminogen Activator (t-PA) containing liposomes. *Chapter 5* deals with the challenge of providing stable conditions for the preparation and storage of liposomes containing both t-PA and glu-plasminogen (substrate to t-PA).

The thrombolytic efficacy and the systemic side-effects of (non-optimized) t-PA containing liposomes were tested and compared to the effects observed after administration of free t-PA in rabbits, as described in *Chapter 6*.

Finally, *Chapter 7* shows some preliminary results on the fibrin binding properties of glu-plasminogen-bearing liposomes in a flow-through system and evaluates the results presented in this thesis. Suggestions are made for further research.

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Chapter 2a

Development of a procedure for coupling the homing device glu-plasminogen to liposomes

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Biochimica et Biophysica Acta 1117, 258-264 (1992)

Summary

The aim of this study was to find a suitable way of coupling the homing-device glu-plasminogen to the outside of liposomes. The described procedure is based on the reaction of thiol groups introduced into the protein with thiol-reactive groups of the liposome.

Details on the thiolation of proteins with the reagent N-succinimidyl S-acetylthioacetate (SATA) were studied for a model-protein, amylase. Increasing the incubation ratio SATA : amylase resulted in a gradually growing number of introduced thiol groups, until a maximum of about 5 mol SH per mol amylase was reached. The enzymatic activity of the derivatized protein was even higher than that of native amylase.

The thiol introduction was then applied to glu-plasminogen itself. After activation with SATA, the protein was incubated with liposomes containing the thiol-reactive anchor maleimido-4-(p-phenylbutyrate)-phosphatidylethanolamine (MPB-PE). Under the chosen conditions, incubation of 0.5 - 2.5 mg/ml protein with 6.0 - 7.5 μmol/ml phospholipid for 30 - 120 minutes resulted in coupling ratios of 20 to 94 μg glu-plasminogen per μmol phospholipid. This corresponds with about 165 to 780 protein-molecules per liposome. SATA-derivatization of glu-plasminogen brought about a loss of its enzymatic activity induced by streptokinase. This activity of liposomally coupled glu-plasminogen was about 52 to 74% of the activity of native glu-plasminogen (depending on the coupling ratio). Although this may seem a significant loss of activity, it was shown that the capacity of liposomal glu-plasminogen to bind to its target, fibrin, was not reduced but several-fold higher under the used conditions than that of the free protein.

Therefore, the described method for thiol introduction is an effective way to thiolate amylase without loss of activity, and to bind the homing-device glu-plasminogen to liposomes without critically interfering with its fibrin binding / homing capacity.

Introduction

Liposomes (lipid bilayers surrounding an aqueous compartment) can be used for drug-targeting. Encapsulation of the drug in liposomes should minimize unwanted systemic effects during transportation through the body. In order to achieve site specific targeting, a homing device with affinity for the target-tissue should be coupled to the outside of the liposome. The aim of the present study was to provide liposomes with a homing device with affinity for fibrin-clots: glu-plasminogen. These liposomes are to be used in the future for specific delivery of a thrombolytic agent at the site of a thrombus such as with myocardial infarction.

A variety of methods for the covalent coupling of proteins to liposomes has been described [1-12]. The reaction of thiol groups of proteins with thiol-reactive maleimide groups of liposomes is well-known [e.g. 5]. In many proteins, however, no unpaired, reactive thiol groups are available and, consequently, they have to be introduced. Glu-plasminogen, for

instance, lacks a free thiol group. For the introduction of reactive thiol groups at the site of primary amino-groups of the protein, the SATA-reaction has been proposed by Duncan and co-workers [13]. The method is described as a convenient and mild way of obtaining oxidation-protected thiol groups within the protein [14]. Therefore, this was the method of choice for this study.

In the present study the Ellman reaction [15-17], generally used to assess the number of (introduced) thiol groups in proteins, was validated for the derivatized protein. An adjustment of the standard procedure was necessary to determine the thiol group content upon activation. The SATA thiol introduction in proteins was tested in a preliminary investigation on a model-protein, amylase. Amylase was chosen as a model-protein because it is cheap and its enzymatic activity can be measured relatively easily. α-Amylase (from Bacillus subtilis) has a molecular weight of about 48.7 kDa; it is constituted of 406 amino acid residues, 25 of which are lysines (ε-amino-groups). It contains neither sulphydryl groups nor disulphide-linkages [18, 19]. Information was collected on the influence of the molar incubation ratio SATA : amylase on the number of introduced thiol groups per mol protein. Besides, attention was paid to the effect of the pH during incubation. Finally, the stability of the obtained thiol containing protein was examined and the enzymatic reactivity of the derivatized amylase was compared to that of the native protein as a criterion for the mildness of the SATA-method.

The experience obtained above was utilized to modify glu-plasminogen (glu = N-terminal amino acid). Human glu-plasminogen (shortly referred to as Plg) has a molecular weight of about 89 kDa; it is constituted of 790 amino acid residues, 47 of which are lysines. The molecule contains 48 cysteine-groups (contains -SH), all expected to be involved in disulphide-pairings and as a consequence not available for the reaction with the liposomal maleimide-groups [20]. On the basis of the results obtained, one molar incubation ratio SATA : glu-plasminogen was chosen and the thiol containing reaction product was coupled to liposomes. The composition of the liposomes used was phosphatidylcholine : phosphatidylglycerol : cholesterol : MPB-PE (a maleimide-containing anchor-molecule) = 38.5 : 4 : 16 : 1.5 (molar ratio). The maleimide-groups of the anchor-molecule are known to react with thiol groups, forming a stable thio-ether bridge [5]. The enzymatic activity of the derivatized protein and of liposome-coupled glu-plasminogen was measured. Finally, the capacity of liposomal Plg to bind in vitro to its target, fibrin, was established.

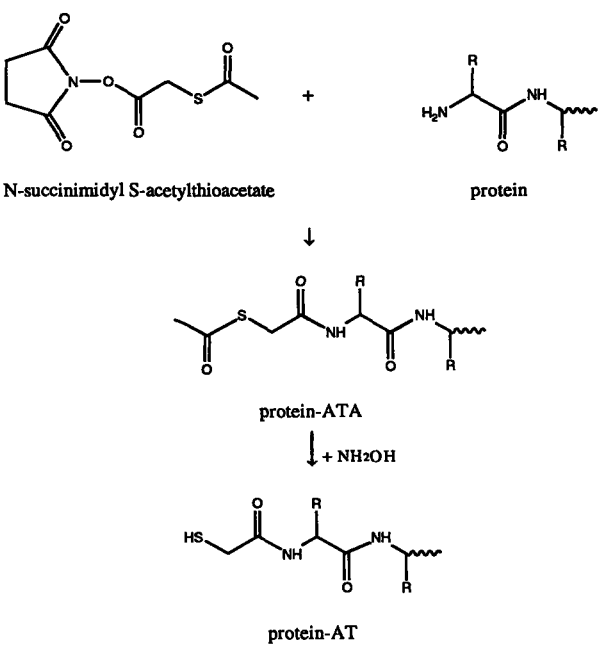
Materials and methods

α-Amylase (1,4-α-D-glucan glycanohydrolase) and a kit to assay its activity (procedure no. 576) were obtained from Sigma Chemicals (St. Louis, MO, USA).

Glu-plasminogen was a gift from Kabi Pharmacia (Stockholm, Sweden); courtesy of Prof. L.O. Andersson; the chromogenic plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide.2 HCl

was obtained from Chromogenix (Mölndal, Sweden). SATA (N-succinimidyl S-acetylthioacetate) was purchased from Pierce Chemical Co. (Rockford, USA) and DTNB (5,5'-dithiobis(2-nitrobenzoic acid) from Janssen (Beersse, Belgium). PC (phosphatidylcholine), cholesterol and N-ethyl-maleimide were obtained from Sigma; egg-PG (phosphatidylglycerol) was a gift from Nattermann GmbH (Cologne, FRG).
Maleimido-4-(p-phenylbutyrate)-phosphatidylethanolamine (MPB-PE) was synthesized from succinimidyl-4-(p-maleimidophenyl)butyrate (SMPB) (Pierce Chemical Co., Rockford, USA) and egg-phosphatidylethanolamine (egg-PE) (Lipid Products, Nutfield, UK) as described by Martin et al. [5]. Human fibrinogen was prepared as described [21] and kindly provided by Dr. W. Nieuwenhuizen. Bovine thrombin was from Leo Pharmaceuticals (Denmark), streptokinase (Kabikinase) was from Kabi Pharmacia (Woerden, The Netherlands).
All other chemicals were of analytical quality and were used without prior purification.

Preparation of thiolated protein
Basically, the procedure to thiolate proteins with SATA (N-succinimidyl S-acetylthioacetate) as described by Duncan et al. [13] was used. This reaction is shown in Scheme 1.



Scheme 1 Thiol introduction into a protein by the SATA-reaction.
The reaction takes place with primary amino-groups of the protein, i.e. lysines and (as shown in the Scheme) the terminal amino-group of the protein.

Circumstances were chosen taking into consideration the results obtained by other authors as well [22-24].

Amylase was dissolved in a standard concentration of 250 μM in 10 mM Hepes buffer, containing 135 mM NaCl and 1 mM EDTA. SATA was dissolved in DMF in varying concentrations. The solutions were mixed in a volume ratio of DMF (SATA) : buffer (amylase) = 1 : 100. They were incubated at room temperature for 20 minutes with a SATA : amylase mol ratio varying from 1 : 1 to 50 : 1 and a standard pH of 7.5; some tests were performed at pH 6 or 10.

For the thiol introduction into glu-plasminogen only one incubation-condition was used: 584 μM SATA and 73 μM glu-plasminogen (8 : 1) were incubated at a pH of 7.5. The same buffer as mentioned above was used; the volume ratio of DMF : Hepes was 1 : 100.

Separation of protein-ATA from unreacted reagent

Immediately after the incubation, the reaction-mixture was separated on a Sephadex G-50 column. The protein (amylase or glu-plasminogen) fractions in the eluate were detected by monitoring the absorption at 280 nm. The fractions were collected, combined and stored at -20°C.

Protein determination

For the determination of the concentration of amylase the method according to Bradford was used [25]. Glu-plasminogen-determinations were performed according to Wessel and Lowry [26-28].

Measurement of the number of introduced thiol groups

Acetylthioacetyl-protein (amylase-ATA or glu-plasminogen-ATA) was deacetylated by adding a freshly prepared 0.5 M hydroxylamine-HCl solution containing 0.5 M Hepes, 25 mM EDTA and pH 7.5. The incubation lasted for an hour; the volume ratio was amylase-ATA solution : NH_2OH solution = 10 : 1.

Subsequently, the free sulphydryl groups were assayed with 5,5'-dithiobis(2-nitrobenzoic acid) according to Ellman; cysteine was used for the calibration curve [15].

Preparation of liposomes

Small unilamellar vesicles consisting of phosphatidylcholine, phosphatidylglycerol, cholesterol and MPB-PE in a molar ratio of 38.5 : 4 : 16 : 1.5 were prepared by the 'film'-method as described by Szoka et al. [29]. The liposomes were extruded through polycarbonate membrane filters with pores of 0.6 μm (once), 0.4 μm (once) and 0.2 μm (three times); after extrusion their average diameter [as determined by dynamic light scattering with a Malvern 4700 system, Malvern Ltd, UK] was between 0.23 and 0.25 μm. The

liposomes were prepared in 10 mM Hepes buffer pH 7.5, containing 135 mM NaCl and 1 mM EDTA.

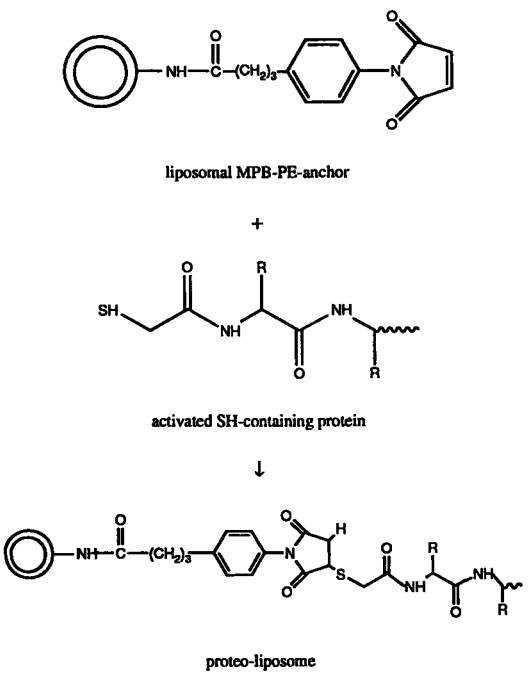
Phospholipid-determination

Phospholipid concentrations were assessed (after perchloric acid destruction) by a phosphate-assay according to Fiske-Subbarow [30].

Coupling of thiolated glu-plasminogen to liposomes

After deacetylating glu-plasminogen with hydroxylamine (as described above), varying amounts were added to the liposomes and allowed to react for 30, 75 or 120 minutes at room temperature (reaction scheme shown in Scheme 2). The phospholipid incubation concentration was around 6.7 µmol per ml and the protein incubation concentration ranged from 0.5 to 2.5 mg/ml, in a total volume of 0.5 ml.

Subsequently, the coupling-reaction was stopped by adding 50 µl of N-ethylmaleimide (8 mM in Hepes buffer) and the liposomes were separated from free protein by two ultracentrifugation steps (Beckman Instr. Inc., California, USA) for 45 minutes, at 80,000 x g at 4°C.



Scheme 2 Coupling of thiolated protein to anchor-containing liposomes.

Measurement of amylase activity

For the determination of the activity of amylase a kit was used. α-Amylase hydrolyses the substrate, which eventually leads to formation of p-nitrophenol, of which the extinction is measured after 15 and 75 seconds at 405 nm. The difference in extinction between these two points of time is directly proportional to the activity of the amylase-sample [31, 32].

Measurement of glu-plasminogen activity

The activity of free, derivatized or liposomal glu-plasminogen was determined by activation with streptokinase according to Friberger et al. [33]. The Plg samples were incubated with a molar excess of streptokinase and the activity of the streptokinase-Plg complex was determined by the conversion of the synthetic substrate H-D-Val-Leu-Lys-pNA, which was measured by monitoring the absorbance of the reaction-product pNA (para-nitro-aniline) at 405 nm.

Measurement of glu-plasminogen binding to fibrin

96-wells plates were coated with a fibrinogen layer and then activated with thrombin, giving rise to fibrin-coated plates containing fibrin monomer at a density of around 3.6 x 10^{-4} nmol/cm^2. The obtained fibrin-plates were treated with Tween 20 in order to prevent aspecific binding of Plg to non-coated parts of the plate.

Glu-plasminogen samples at a concentration around 15 nmol/l (1% of the Plg plasma concentration) were incubated overnight at room temperature at pH 7.4. Supernatant was harvested and the bound portion was washed three times with buffer. The concentration of Plg in both fractions was determined by conversion of the synthetic substrate as described above. The percentage of bound Plg could then be calculated. When incubating with liposomal glu-plasminogen, the same concentrations were used as for the free protein. The buffer used contained 0.05 M Tris-HCl and 0.01% (w/w) Tween 20. At this Tween 20 concentration there was no extensive breakdown of liposomes.

Results

Amylase

Validation of the thiol group measurement according to Ellman

When performing the Ellman thiol assays on SATA-derivatized proteins, a blank sample was required to correct for the absorption of the hydroxylamine-reagent, used to activate the protein-ATA to protein-AT. Cysteine used for calibration does not require activation by hydroxylamine. Therefore, calculating the samples from this calibration-curve without correction for the hydroxylamine-extinction introduces an error; the results depend on the NH_2OH-solution used: its concentration and "freshness". The sample-blank consists of all

components of the sample itself, the only difference being that, instead of protein-ATA, the same concentration of underivatized protein is used. Since the influence of hydroxylamine varies with the concentration of protein, the blank must contain protein and hydroxylamine for proper correction. This correction gave rise to lower values for the introduced number of thiol groups (to around 50% of the uncorrected value). These values were, however, reproducible and no longer dependent on the hydroxylamine-solution used.

The introduction of thiol groups into amylase

Figure 1 demonstrates that initially at low mol ratios SATA : amylase an increase in this ratio induced an increase in the number of introduced thiol groups (expressed as mol SH per mol amylase). However, upon reaching an incubation ratio of 50 : 1, a maximum introduction of around 5 mol SH per mol amylase was seen. This phenomenon could however be (partially) due to a SATA solubility-problem at these high levels.

The incubation of the 10 : 1 ratio was repeated at pH 6 and pH 10. The results are shown in Table 1: the coupling ratio was not influenced by raising the pH from 7.5 to 10; a decrease in pH from 7.5 to 6, on the other hand, hampered the thiol introduction considerably. The number of introduced (protected) thiol groups slowly dropped upon storage at -20°C. After 8 weeks the number of thiol groups was reduced to about 65% (duplicate experiment, data 8% apart) of the original value.

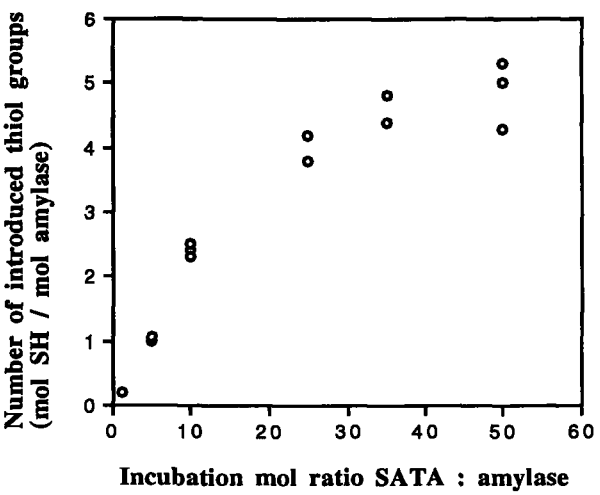


Figure 1 Thiol introduction into amylase as a function of the molar incubation ratio SATA : amylase.

Table 1 Thiol group introduction efficiency for amylase: dependence on pH.
SATA : amylase (mol ratio) is 10 : 1.
± : standard deviation for n ≥ 3; deviation from average value for n = 2.

Incubation pH	Number of introduced thiol groups (mol SH / mol amylase)
6	0.5 ± 0.1 (n=2)
7.5	2.4 ± 0.1 (n=4)
10	2.6 ± 0.3 (n=3)

The enzymatic activity of thiolated amylase

The influence of thiol group introduction with SATA on the enzymatic activity of amylase is shown in Table 2. There was a clear tendency to an increase in activity, which depended on the number of thiol groups introduced. The maximum observed increase was 2-fold (taking the activity of the native protein as 100%).

Table 2 The influence of thiol group introduction on the activity for amylase.
Duplicate experiments, data at most 13% apart.

Number of introduced thiol groups (mol SH / mol amylase)	Activity of derivatized amylase (percentage of native protein activity)
0	100
0.2	142
2.5	190
5.0	208

Glu-plasminogen

The introduction of thiol groups into glu-plasminogen

The thiol group introduction into glu-plasminogen was performed using a standard incubation ratio SATA : protein of 8 : 1 (as described by Duncan et al. [13]). This incubation gave rise to a mean introduction of 0.5 - 1.8 mol SH per mol glu-plasminogen.

The enzymatic activity of thiolated glu-plasminogen

Upon thiol group introduction with SATA, the enzymatic activity of glu-plasminogen tended to decrease (results not shown). For a proper evaluation of these data it should be taken into consideration that measurements were done on the non-separated mixture of derivatized and non-derivatized glu-plasminogen.

The coupling efficiency of thiolated glu-plasminogen to liposomes

The results of coupling glu-plasminogen to liposomes are shown in Figure 2. The protein concentration was determined colorimetrically according to Wessel and Lowry [26, 28]. The phospholipid concentration at the start was kept at a constant value. Because of variable phospholipid losses during extrusion, there was a slight fluctuation of the concentration in the incubation medium within the range of 6.0 to 7.5 μmol/ml PL. The glu-plasminogen concentration was varied from 0.5 to 2.5 mg/ml, and incubation-times of 30, 75 or 120 minutes were chosen.

Increasing the protein concentration (incubation ratio) gave rise to higher coupling ratios (expressed as μg Plg coupled per μmol PL). There was no indication of a plateau value being reached within the protein concentration-range used. Prolonging the incubation-time from 30 minutes to 75 minutes tended to increase the coupling ratio; incubation for 120 minutes showed the reverse effect: it tended to be less effective than 75 minutes. These results were consistently found in each series of experiments, performed with one particular batch of liposomes and modified glu-plasminogen. In Figure 2 the results with a number of different batches are shown. Because of inter-experimental variation in coupling-efficiency data scattering was considerable and only a trend could be observed.

Non-specific binding of thiolated Plg to control vesicles (lacking MPB-PE) was less than 1.5 μg per μmol PL; no binding of non-derivatized glu-plasminogen to anchor-containing vesicles could be detected (data not shown).

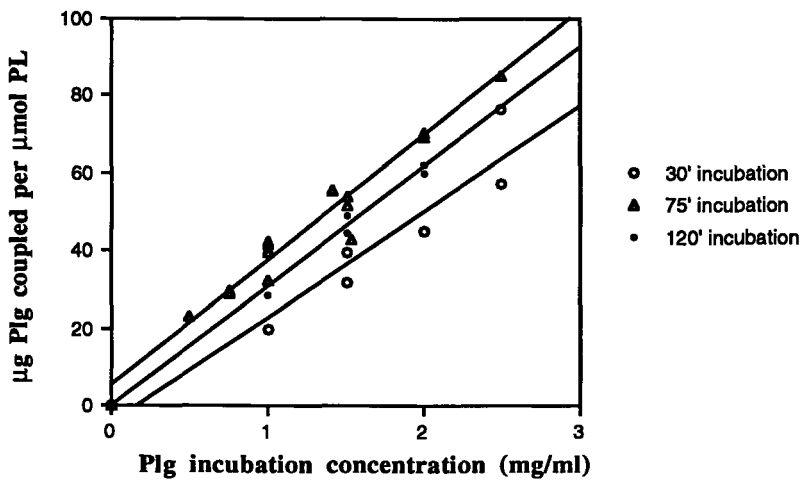


Figure 2 Plg-coupling to liposomes as a function of the Plg incubation concentration, for three incubation times.

The enzymatic activity of glu-plasminogen upon coupling to liposomes

The enzymatic activity of glu-plasminogen decreased upon coupling to liposomes (Figure 3). Between 52 and 80% of the original activity (e.g. before SATA-modification) was retained. The mean activity of liposomal glu-plasminogen was found to be 68% (± 6%) compared to the native protein. There was no clear relationship between the protein density at the liposome surface and loss of activity. Prolonging the incubation time did not enhance loss of activity.

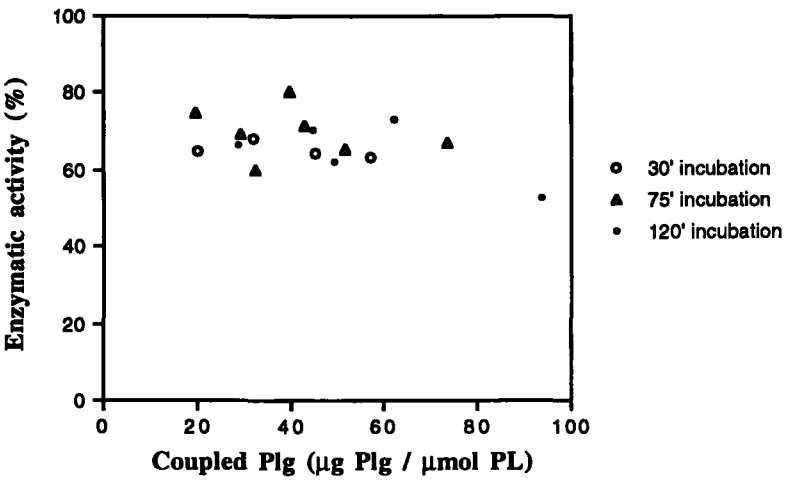


Figure 3 Enzymatic activity of liposomal Plg as a function of the Plg density at the liposome surface.
Enzymatic activity is expressed as the percentage of the activity of underivatized, free Plg.

The fibrin binding capacity of liposomal glu-plasminogen

The fibrin binding capacity of two of the obtained proteo-liposome dispersions was compared to that of native glu-plasminogen, in order to see whether the homing-capacity of the protein was effected by the binding to liposomes.

The ratio of fibrin bound protein / total protein times 100% was calculated for free and liposomal glu-plasminogen at a fixed low dosage of about 15 nM Plg. The two liposome samples concerned both had a protein-density of 50 µg per µmol phospholipid. The fraction of liposomal protein that was bound was considerably higher, almost six fold, than for the native protein. The actual data were 29% for liposomal Plg and 5% for native Plg. When empty liposomes were added to the native protein, no interference with the binding to fibrin was seen (data not shown).

Discussion

Amylase

The introduction of thiol groups into protein molecules by reaction with N-succinimidyl S-acetylthioacetate (SATA) was found to be a convenient and mild method. The generated SATA-amylase conjugate is rather stable if stored at -20°C. Upon activation with hydroxylamine (a mild treatment) reactive thiol groups are obtained, ready to couple to, for example, maleimide-groups anchored to liposome bilayers.

For the assessment of the number of (introduced) thiol groups in a protein the reaction with Ellman reagent is regularly used. An important finding in this study was that in our hands hydroxylamine, required to generate the SH-groups after SATA coupling, interfered with the assay unless proper blank readings were taken. Since this problem may occur under other circumstances as well, it should be tested for when using the Ellman-reaction.

Under the chosen incubation conditions a maximum of 5 mol of introduced SH-groups per mol protein was found. As amylase contains 25 groups which can, in principle, react with SATA, only a minor fraction is actually derivatized.

Results of SATA thiol group introduction in other proteins were published before. Hutchinson et al. [23, 24] also found a maximum in the introduction of thiol groups in WGA (wheat germ agglutinin), namely 3 mol of introduced thiol groups per mol WGA. WGA has 14 to 16 lysine and N-terminal residues available for SATA-derivatization. Similar efficiencies (of 4-6 mol SH per mol protein) were found [13, 22] for SH-group introduction via the SATA-method in IgG. Duncan et al. [13] reported even lower values for the SH-group introduction efficiency in thyroglobulin and peroxidase. However, these authors used a maximum SATA : protein mole ratio of 20 : 1 (in the present study 50 : 1). Under those circumstances they did not find a maximum in the introduction for IgG and thyroglobulin.

Alternative methods, especially the one with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) described by Carlsson et al. [34], are also effective in introducing thiol groups in proteins. Carlsson was even able to introduce up to 11 2-pyridyl disulphide structures into amylase. However, the disadvantage of this method is the need for a reducing agent (such as dithiothreitol) to obtain free thiol groups. Traces of this agent may interfere with the coupling reaction [13]. Deprotection of SATA lacks this disadvantage: it is accomplished by hydroxylamine which does not interfere with the sulphydryl-maleimide conjugation. Hydroxylamine has the additional advantage to increase the stability of the liberated thiol group (by preventing oxidation) [13].

Introduction of thiol groups with the SATA-method tends to increase the activity of amylase under the chosen experimental conditions. A 2-fold higher activity is observed for amylase containing 5 mol SH per mol protein compared to the original amylase. This increase in activity upon thiol group introduction with the SATA-procedure was not described by any of the investigators [using the SATA-method] cited in this study. Duncan [13] reported that little

or no loss of activity or function of IgG was caused by introduction of 6 acetylthioacetate-groups. Schwendener [14] also found no loss of specific binding activity of the monoclonal Ab's used in his study upon incubating with SATA at molar ratios [SATA:Ab] of 6:1, 12:1 and 24:1 (the number of SATA-molecules linked was not determined).
For other protein derivatizing methods (non-SATA) usually a slight decrease in activity or no change in activity is reported. However, this often concerns the antigen binding capacity of antibody-conjugated liposomes [2-5, 11], which does not reflect all activities of the antibody-derivative (e.g. complement activation, Fc receptor interaction).
Increasing the pH to 10, a value above that used under standard conditions (pH 7.5) did not enhance the SH-group coupling with SATA; dropping the incubation pH reduced SH-group introduction. At pH 6, complete protonation of primary (terminal) amine-groups may account for hampering the reaction with SATA. Investigators using the SATA-reaction selected pH 7.5 as a standard incubation condition [13, 14, 22-24].
On the basis of these results, the SATA-procedure was considered to be suitable for introduction of thiol groups into the homing-device glu-plasminogen.

Glu-plasminogen
At the standard incubation ratio of SATA : protein = 8 : 1, 0.5 - 1.8 mol SH per mol Plg was introduced. There was no clear indication of the cause(s) for this variability. This SH-group density turned out to be sufficient to induce coupling of Plg to the liposomes. The obtained coupling ratio glu-plasminogen : phospholipid increases with glu-plasminogen incubation concentration (at constant PL concentration) until the highest concentration Plg which was used: 2.5 mg/ml.
The coupling efficiency (calculated by dividing the final coupling ratio by the ratio of protein to lipid during incubation, x 100%) is a rather constant value for one coupling time, without any correlation to the protein incubation concentration used in this experiment.
An incubation time of 75 minutes was sufficient for optimal coupling. Depending on these circumstances the coupling efficiency varied from 20 to 94 μg glu-plasminogen per μmol phospholipid; this corresponds with about 165 to 780 molecules of protein per liposome.
It is unlikely that a higher density of MPB-PE groups in the bilayers will increase Plg attachment as only about 2 to 10 % of the MPB-PE groups (exposed to the external milieu) reacts with Plg. Similar coupling efficiencies were found when F_{ab} fragments were attached to MPB-PE liposomes [35].
In contrast to the model protein the enzymatic activity of glu-plasminogen tended to decrease upon the SATA-reaction and hydroxylamine-activation. A possible explanation for this phenomenon is that the introduced thiol groups contribute to the enzyme-substrate-binding for amylase, whereas they tend to interfere with the reaction schedule in the enzymatic assay for Plg. The enzymatic assay of Plg involves a two step reaction: (1) formation of the

streptokinase-Plg complex with enzymatic activity and (2) conversion of the substrate. At the present time it is not clear whether ATA-incorporation affects only 1, or 2, or both.
It is important to note that SATA-modified protein was not separated from non-modified protein. Only a part of the protein-mixture has been modified whereas another part is not derivatized. Presumably, the section that is converted solely accounts for the decrease in activity. Therefore, one can conclude that the actual decrease in activity of SATA-derivatized glu-plasminogen (and hence of the liposome-coupled fraction) is in fact more than the slight decrease that was seen in the non-adjusted experimental data.
The intrinsic enzymatic activity of glu-plasminogen dropped upon binding to liposomes. Apart from the two reasons for a drop in activity as mentioned above, in this case a third reason can be added: sterical hindrance can make it difficult for streptokinase and/or substrate to approach the bound Plg. Under the chosen conditions no effect of the Plg-density on liposomes on its enzymatic activity was found. However, this finding does not rule out a partially blocked access of the individual, liposome bound Plg molecule to streptokinase and/or substrate as a reason for the observed drop.
Despite the discouraging decay in enzymatic activity (at the low dosage conditions used), liposome-coupled glu-plasminogen is still quite capable of binding to its target, fibrin. The percentage of binding of liposomal protein was found to be about 6 times higher than that of free Plg (per mol Plg). Multivalent interaction (as described by Heath et al. [36]) between liposomal Plg and fibrin may (partially) account for the increase in binding of liposomal Plg.
In conclusion, the results obtained in this study show that the described method provides a convenient and suitable procedure for coupling the homing-device glu-plasminogen to liposomes.

Acknowledgements

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Chapter 2b

Interference of ε-amino-caproic acid with [N-succinimidyl S-acetylthioacetate derived] thiol introduction into glu-plasminogen

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Summary

In this study the importance of the absence of ε-amino-caproic acid (EACA) for the thiol introduction into glu-plasminogen (Plg) with the SATA-reaction (SATA = N-succinimidyl S-acetylthioacetate) was demonstrated. It was shown that under the chosen conditions the presence of variable amounts of EACA reduced the resulting number of thiol groups introduced by the SATA-reaction considerably: only 0 to 1.8 mol thiol groups were found per mol of commercially available, high concentration glu-plasminogen formulation, whereas the maximum amount of 8 to even 9 mol thiol groups per mol were introduced into highly purified glu-plasminogen. It became clear that the SATA thiol introduction (performed with 73 μM Plg) was dependent on the concentration of EACA within the range of 0 to 100 mM EACA.

The most probable mechanism of interference of EACA with the SATA thiol introduction (considering the large amounts of EACA encountered) is the competitive reaction of the primary amino-group in EACA with SATA, inducing a diminished SATA / glu-plasminogen interaction. However, additionally one has to consider the possibility of changes in conformation induced in the glu-plasminogen molecule by EACA. This may cause a reduction in the number of reactive groups for coupling.

Introduction

The SATA-reaction (SATA = N-succinimidyl S-acetylthioacetate), as described by Duncan et al. [1], is a regularly used method for the introduction of thiol groups into protein molecules. It is also the first step in a scheme for coupling proteins that do not expose SH-groups via a thio-ether linkage to liposomes [2-6]. In a previous publication (Chapter 2a; [7]) we described the use of this method for thiol introduction into the fibrinolytic compound glu-plasminogen (referred to as Plg), to make this protein capable of binding to thiol-reactive groups attached to the external liposome surface. The coupling-procedure yielded liposomes which were to be targeted to fibrin-clots by the coupled homing device Plg. In this publication we demonstrate the critical importance of proper removal of ε-amino-caproic acid (EACA) from commercially supplied Plg batches to achieve acceptable thiolation levels.

Materials and methods

Several batches of glu-plasminogen were obtained from Pharmacia (Stockholm, Sweden), courtesy of Prof. L.O. Andersson.

The chromogenic plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide.2 HCl was obtained from Chromogenix (Mölndal, Sweden). SATA (N-succinimidyl S-acetylthioacetate) was

purchased from Sigma Chemicals (St. Louis, MO, USA) and DTNB (5,5'-dithiobis(2-nitrobenzoic acid) from Janssen Chimica (Beersse, Belgium). Human fibrinogen was prepared as described [8] and kindly provided by Dr. W. Nieuwenhuizen. Bovine thrombin was obtained from Leo Pharmaceuticals (Ballerup, Denmark) and streptokinase (Kabikinase) from Kabi Pharmacia (Woerden, The Netherlands).
All other chemicals were of analytical quality and were used without prior purification.

Preparation of thiolated glu-plasminogen
Glu-plasminogen, purified by means of affinity chromatography with immobilized lysine and elution with EACA [9], was obtained in several batches, with varying Plg concentrations and differing with respect to the degree of removal of EACA.
When a high concentration of glu-plasminogen was required, the protein was precipitated with 0.31 mg/ml ammonium sulphate, centrifuged for 30 min at 15.000 g at 4°C, whereupon the precipitate was redissolved in a 10 mM Hepes buffer containing 135 mM NaCl and 1 mM EDTA at pH 7.5.
Basically, the procedure to thiolate proteins as described by Duncan et al. [1] was used. Circumstances were chosen taking into consideration the results obtained by other authors as well [2-4], as described previously (Chapter 2a; [7]).
Briefly: final concentrations of 584 μM SATA (in dimethylformamide, DMF) and 73 μM glu-plasminogen (in Hepes buffer, pH 7.5) were incubated at room temperature for 20 min. The volume ratio of DMF (SATA) / Hepes buffer (Plg) was 1 : 100.

Separation of protein-ATA from unreacted reagent
Immediately after the incubation, the reaction-mixture was separated on a Sephadex G-50 column. Putatively modified glu-plasminogen fractions in the eluate were detected by monitoring the absorption at 280 nm. These fractions were collected, combined and stored at -20°C.

Protein determination
For the determination of the concentration of glu-plasminogen an activity-measurement was used: the obtained concentrations therefore refer to activatable Plg.
The activity of glu-plasminogen was determined by activation with streptokinase according to Friberger et al. [10]. The Plg samples were incubated with a molar excess of streptokinase and the activity of the streptokinase-plasminogen complex was determined by the conversion of the synthetic substrate H-D-Val-Leu-Lys-pNA, which was measured by monitoring the absorbance of the reaction-product pNA (para-nitro-aniline) at 405 nm.

Measurement of the number of introduced thiol groups

Acetylthioacetyl-glu-plasminogen (Plg-ATA) was deacetylated by adding a freshly prepared 0.5 M hydroxylamine-HCl solution containing 0.5 M Hepes, 25 mM EDTA and pH 7.5. The incubation lasted for an hour. The volume ratio was Plg-ATA solution : NH_2OH solution = 10 : 1. Subsequently, the free sulphydryl groups were assayed with 5,5'-dithiobis(2-nitrobenzoic acid) according to Ellman; cysteine was used for the calibration curve [11].

When performing the Ellman thiol-assay on SATA-derivatized proteins, a blank sample was required (as was described in Chapter 2a; [7]) to correct for the absorption of the hydroxylamine-reagent, used to activate the protein-ATA to protein-AT. The sample-blank consists of all components of the sample itself, the only difference being that, instead of protein-ATA, the same concentration of underivatized protein is used.

Identification of the contaminant

The identification of the contaminant in the eluate, collected during purification of Plg on a Sephadex G-25 column, was performed by gas chromatography in combination with mass spectrometry (GC-MS). To this end, the contaminant had to be converted into its volatile derivative.

N-methyl-N-(tert.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was used for the one-step derivatization procedure, as described by Chaves das Neves et al. [12]. The obtained tert.-butyldimethylsilyl (TBDMS)-derivative was assayed using a Finnigan MAT Ion Trap Detector 700 coupled to a Carlo Erba HRGC 5160 Mega series gaschromatograph (GC) with an on-column injector. The GC was equipped with a fused silica capillary column J&W DB-5, 17 m long, 0.32 mm i.d. and 0.25 μm film thickness. A deactivated fused silica column, 40 cm long, 0.53 mm i.d. was used as a retention gap to protect the analytical column from contamination. Helium was used as a carrier gas at a pressure of 42 kPa. The temperatures of the ion trap and the interface were 207 and 260°C, respectively. Electron impact (EI) spectra were measured at 70 eV and at an electron multiplier voltage 150 V above the value of the automatic tuning program. Chemical Ionization (CI) spectra were run with all parameters set according to the manufacturers manual using isobutane as a reagent gas.

Determination of the concentration of EACA

The total elution-volume was concentrated prior to derivatization, which induced the presence of very high concentrations of the buffer components at the time of conversion. Since the presence of Hepes buffer disturbed the quantitative conversion of EACA into its volatile derivative, the GC-MS determination method could not be used for EACA-quantification in the Plg-purification-eluate (which was collected from the Sephadex G-25

column). Therefore, once EACA had been identified unequivocally, its concentration was measured using a determination method for primary amines with TNBS, as described by Lentz et al. [13].

Results

With various batches of glu-plasminogen different results were obtained with respect to the efficacy of thiolation: the number of introduced thiol groups ranged from 0 to 1.8. We suspected our preparations to be contaminated with variable amounts of low molecular weight SH-acceptors, notably EACA, causing thiolation-efficacies on Plg to be relatively low.

Thiol introduction into glu-Plasminogen after removal of low molecular weight contaminants

To verify the hypothesis of the presence of low molecular weight contaminants, a Plg batch showing very poor response to thiol introduction (the number of introduced thiol groups was too low to be detectable) was taken. In order to remove all low molecular contaminants as quantitatively as possible, volumes of 3 ml Plg solution, containing 80 mg of protein, were purified over a Sephadex G-25 medium column of 1 meter length and with a 100 ml content. This set up provides a large overcapacity of desalting. Runs were performed at a low elution rate (around 132 ml/h).

After this purification step the SATA-reaction appeared to be very successful, showing the highest SH-introduction seen so far: 9 thiol groups per mol glu-plasminogen.

Identification of the contaminant

The fractions that were collected after the Plg-containing peak had eluted from the Sephadex G-25 column were pooled and concentrated to one Plg-eluate and subsequently analysed by a combination of gas chromatography and mass spectrometry. Comparison of the profile of pure EACA with the Plg-eluate unambiguously demonstrated the presence of EACA.

Determination of the concentration of EACA

The concentration of EACA in the Plg-eluate was determined by the method as described by Lentz et al. [13]. A substantial amount of EACA could be identified; quantification demonstrated the presence of a total amount of 5.1 mg EACA per 3 ml of Plg solution (corresponding with an EACA concentration of 13 mM). Expressed as a molar ratio, this amount corresponds with 45 mol EACA per mol of glu-plasminogen being removed by this purification step.

Presence of other contaminants in the glu-plasminogen batches

To test whether EACA was the only contaminant in the glu-plasminogen solution to interfere with the SATA-reaction, the influence of the eluted material from the column on the SATA-reaction was compared to that of pure EACA. The purified Plg was 'enriched' with either eluate or pure EACA at a similar EACA concentration, mimicking the original degree of contamination of 45 mol EACA per mol Plg. These samples were allowed to incubate for 45 minutes after which the SATA-reaction was performed. The resulting thiol introduction for both enriched samples was similar: 1.4 mol thiol groups per mol glu-plasminogen.

This indicates that EACA is the predominantly interfering substance.

Thiol introduction as a function of the EACA concentration

To study the effect of the presence of EACA on the SATA-reaction in more detail, the reaction was performed with (Sephadex G-25 column-) purified glu-plasminogen samples to which various amounts of pure EACA were added. The resulting EACA concentrations (in the Plg solution of 73 μM) varied from 0 to 100 mM, corresponding with 0 to 1400 mol EACA per mol Plg. As shown in Figure 1, the thiol introduction is strongly dependent on the EACA concentration.

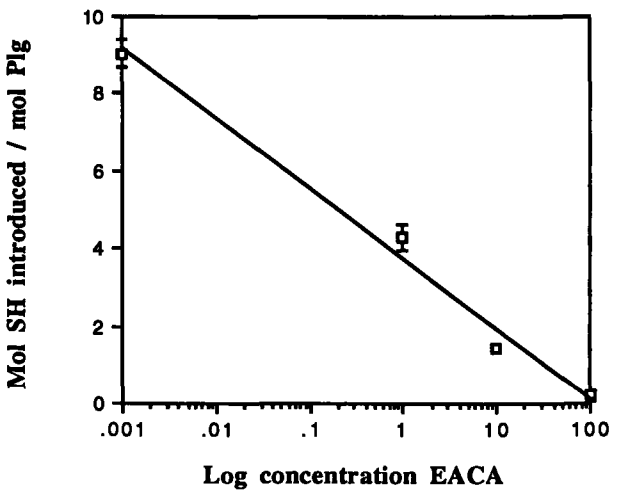

Figure 1 Thiol introduction into glu-plasminogen in the presence of ε-amino-caproic acid.
The number of (SATA-)introduced thiol groups per mol Plg was measured in the presence of varying concentrations of ε–amino-caproic acid added to the purified Plg (n=3). The Plg concentration during thiol introduction was 73 μM in each experiment.

Discussion

In this study it was shown that for effective SATA thiol introduction into glu-plasminogen the presence of EACA should be reduced to a very minimum. Our purification-method caused a reduction of the amount of EACA corresponding with a ratio of about 45 mol EACA per mol Plg. Upon this purification (which probably left the Plg solution with little or no remaining EACA), the SATA-reaction led to an increased introduction of 8 to 9 mol SH per mol Plg.

The SATA-reaction is based on the introduction of an acetyl-thio-acetate-group at the site of primary amino-groups of a protein [1]. Thus, lysines and the N-terminal residues of glu-plasminogen are potentially capable of reacting in the SATA-reaction. The primary structure of glu-plasminogen harbours 47 lysines. However, not all of them are expected to be accessible to the reaction. Our original observations of a maximum of two introduced thiol groups per glu-plasminogen molecule suggested a very limited accessibility of lysines, which was not very likely because of the hydrophilic nature of the molecule. The results with purified Plg as described in the present manuscript suggest accessibility of at least eight to nine lysines (on the average), which would be more in line with the hydrophilic nature of this amino-acid and its expected presence on the outside of the molecule.

It is an interesting coincidence that lysines are of importance both for the SATA-reaction and for the function and conformation of glu-plasminogen [15]. This coincidence provided us with the problems described in the present manuscript.

Lysine binding sites in the glu-plasminogen molecule play an important role in its mechanism of action, and EACA is frequently used as a lysine-like analogue to interfere with the functions of the lysine binding sites. Since the original description by Deutsch and Mertz [9], Plg is nearly exclusively purified on immobilized lysine, and frequently eluted with EACA. In retrospect, it can be concluded that in the various batches of glu-plasminogen that were used in our studies, the removal of EACA from the Plg preparation was incomplete, as EACA has a tendency to co-elute with Plg in standard gel-permeation chromatographic protocols. Such contamination can go unnoticed in most functional tests on Plg performed at concentrations of 1-2 μM or lower. However, the SATA-reaction is performed on concentrated Plg (in our procedure 73 μM).

In our experiments low molecular weight contaminants were carefully removed with a high overcapacity gel permeation technique. The reduced introduction of thiol groups into purified glu-plasminogen after the addition of pure EACA was similar to the introduction obtained upon adding the pooled low molecular weight fraction derived from a Plg purification process (conditions selected to contain the same concentrations of EACA). Therefore, the presence of other substances besides EACA that might contribute to the interference with the SATA-reaction, is considered unlikely. Since EACA was present in excess compared to SATA (at least 5.5 times), the mechanism of interference of EACA with

the SATA-reaction can most probably be attributed to its role as a low molecular weight SH-acceptor.
However, the possibility can not be excluded that lysine and lysine-analogues, such as EACA, affect the conformation of the glu-plasminogen molecule. EACA is considered to have antifibrinolytic properties, as it binds to the lysine binding sites of the glu-plasminogen molecule [14]. Since there is a lysine-dependent interaction within the glu-plasminogen molecule between the kringle (1-2-3) portion and (probably) kringle 4 [15], one could ascribe the interaction of EACA with glu-plasminogen to its binding to a lysine binding site in kringle (1-2-3) or 4, thereby disturbing the intramolecular interaction, leading to a conformational change. This conformational change may subsequently affect the number of lysines that are accessible for the thiol introduction and thereby influence the number of sites for coupling.

In conclusion: when performing the SATA-reaction (and possibly also other reactions involving NH_2-groups) with glu-plasminogen, one should be aware of the possible presence of interfering low molecular weight agents that may be present, such as EACA. In this study, EACA was able to reduce the SATA thiol introduction to negligible levels. Taking into account the relatively high amounts of EACA being present, this effect is most probably brought about by the SH-accepting properties of EACA, competing with glu-plasminogen for the reaction with SATA. However, the effect may (in part) also be caused by EACA-induced conformational changes, which may render glu-plasminogen unreceptive to a reaction previously considered appropriate. Thorough purification of the protein regenerates accessibility of glu-plasminogen for SATA and stabilizes the conformation of the protein introduced into the reaction.

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Chapter 3

Fibrin binding of glu-plasminogen coated liposomes in vitro

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Summary

In this study, the fibrin binding properties of liposomes containing a number of glu-plasminogen (Plg) molecules on the outside were compared to those of free (non-liposomal) Plg in an in vitro model system. Fibrin monolayer coated 96-wells plates were used, containing fibrin monomer at a density of around 3.4 to 3.9 x 10^{-4} nmol/cm^2. These densities are similar to liposomal Plg-densities, thus allowing multivalent interactions to occur.

In the panel of experimental conditions that was chosen, binding of free Plg and liposomes with Plg showed three main differences in characteristics. Firstly, in the fibrin binding of Plg-liposomes not all Plg may be involved, but on the average 40% of the total amount of liposomal Plg. This was shown by lysing the liposomes after binding to the fibrin and estimation of truly bound Plg. With Plg-densities on the liposomes below the fibrin binding sites density the maximal number of bound Plg molecules remains below the amount of available fibrin binding sites. Secondly, a higher binding rate by at least one order of magnitude was observed for liposomes with Plg compared to free Plg. Thirdly, liposomes with Plg exhibit a fibrin binding affinity which increases with Plg-density, because of the multivalent character of interaction. Liposomal Plg can successfully compete for fibrin binding sites with a 100 fold higher concentration of free Plg.

These in vitro findings indicate that in view of avid and rapid fibrin binding, liposomes with attached glu-plasminogen may be suitable for in vivo targeting to fibrin based thrombi.

Introduction

Liposomes (lipid bilayers surrounding an aqueous compartment) can be used for drug-targeting. Encapsulation of the drug in liposomes should minimize unwanted systemic effects during transportation through the body. In order to achieve site specific targeting, generally a homing device with affinity for the target tissue should be coupled to the outside of the liposome. Several reviews have been published describing different targeting strategies with liposomes [1-3].

In a previous article (Chapter 2a; [4]) the production of liposomes with a homing device with affinity for fibrin clots: glu-plasminogen (glu = N-terminal amino-acid) (referred to as Plg) was described. In that study thiol groups were introduced in Plg via SATA (SATA = N-succinimidyl S-acetylthioacetate) [5]. Subsequently the thiol-bearing protein was coupled to anchor molecules in the liposome bilayer. The obtained Plg-bearing liposomes were designed to be used in the future for specific delivery of a thrombolytic agent at the site of a thrombus, e.g. for a myocardial infarction.

The aim of the present study was to investigate the binding behaviour of liposomal Plg to its envisioned target, fibrin. This fibrin binding was measured in an in vitro model system, using a fibrin monolayer, coated onto the wells of a 96-wells plate. A comparison was made between the fibrin binding behaviour of free (non-liposomal) Plg and that of Plg attached to liposomes in different densities. ^{125}I-labeled Plg-liposomes were used for fibrin binding competition with free Plg.

Materials and methods

Glu-plasminogen was a gift from Kabi Pharmacia (Stockholm, Sweden); courtesy of Prof. L.O. Andersson. SATA (N-succinimidyl S-acetylthioacetate) was purchased from Sigma Chemicals (St. Louis, MO, USA), the chromogenic plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide.2 HCl from Chromogenix (Mölndal, Sweden). Egg-PC (egg-phosphatidylcholine) was a gift from Lipoid KG (Ludwigshafen, Germany). Cholesterol and N-ethylmaleimide were obtained from Sigma Chemicals (St. Louis, MO, USA); egg-PG (egg-phosphatidylglycerol) was a gift from Nattermann GmbH (Cologne, FRG).

Maleimido-4-(p-phenylbutyrate)-phosphatidylethanolamine (MPB-PE) was synthesized from succinimidyl-4-(p-maleimidophenyl)butyrate (SMPB) (Pierce Chemical Co., Rockford, USA) and egg-phosphatidylethanolamine (egg-PE) (Lipid Products, Nutfield, UK) as described by Martin et al. [6].

Human fibrinogen was prepared as described [7] and kindly provided by Dr. W. Nieuwenhuizen. Bovine thrombin was obtained from Leo Pharmaceuticals (Ballerup, Denmark) and streptokinase (Kabikinase) from Kabi Pharmacia (Woerden, The Netherlands). All other chemicals were of analytical quality and were used without prior purification.

Coupling of glu-plasminogen to liposomes

Before coupling, Plg was extensively purified over a Sephadex G-25 column, in order to remove EACA (epsilon-amino-caproic acid) (Chapter 2b; [8]). Subsequently, the procedure as previously described (Chapter 2a; [4]) was used. The coupling-reaction is based on the reaction between thiol groups, introduced into the protein, and maleimide-groups of liposomes and is briefly outlined below.

Glu-plasminogen was derivatized by the method of Duncan and co-workers [5], using SATA (N-succinimidyl S-acetylthioacetate) for the introduction of thiol groups. The obtained acetylthioacetyl-glu-plasminogen (Plg-ATA) was deacetylated with hydroxylamine-HCl, which created free thiol groups (assayed with 5,5'-dithiobis(2-nitrobenzoic acid) according to Ellman [9]), available for coupling to liposomes.

Small unilamellar liposomes, consisting of egg-phosphatidylcholine, egg-phosphatidylglycerol, cholesterol and MPB-PE (a maleimide-containing anchor-molecule), molar ratio

39.5 : 4 : 16 : 0.5, were prepared by the "film-method" (first described by Bangham et al. [10]). The liposomes were prepared in 10 mM Hepes buffer pH 7.5, containing 135 mM NaCl and 1 mM EDTA. They were extruded through polycarbonate membrane filters with pores of 0.6 μm (once) and 0.2 μm (three times); after extrusion their average diameter [as determined by dynamic light scattering with a Malvern 4700 system, Malvern Ltd, UK] was between 0.20 and 0.22 μm.

For the liposomes used in this study, the actual coupling was performed by incubating 0.1 - 1.5 mg/ml of deacetylated protein (Plg-AT) with liposomes at room temperature for 75 minutes; the phospholipid concentration was about 6.7 μmol/ml. After stopping the reaction with N-ethylmaleimide, the liposomes were separated from free protein by two ultracentrifugation steps (Beckman Instr. Inc., California, USA) for 45 minutes, at 80,000 x g at 4°C. In order to mask possible unreacted anchor molecules, a large excess of cysteine of 1 mg/ml was added to the buffer during the first ultracentrifugation step.

Immobilization of the fibrin-layer

Solid-phase fibrin monolayer coated plates were prepared as follows:

96-wells plates (PVC) (Flow Laboratories, Irvine Ayrshire, Scotland) were incubated with 600 ng of fibrinogen in 150 μl of a 0.1 M sodium carbonate buffer solution (pH 9.6) per well. The incubation took place overnight, at 4°C. Subsequently, the plates were emptied and incubated (after-coating procedure) with the carbonate buffer solution, containing 0.2% (w/v) Tween 20, for 2 hours at room temperature (see results section). Then the plates were rinsed three times with assay buffer, consisting of 0.05 M Tris-HCl, 0.85% (w/v) NaCl, 0.01% (w/v) Tween 20, pH 7.4, and once with an aqueous preservation solution, containing 100 g/l mannitol and 20 g/l sucrose. Finally, the plates were stored at -20°C until use.

Before use, the fibrinogen-coated plates were rinsed (once) with assay buffer and afterwards treated with 0.1 NIH/ml thrombin in assay buffer, 150 μl per well. The incubation was performed at 37°C, for 30 minutes. This procedure provided a fibrin monolayer, stable on storage and ready for incubation with glu-plasminogen after rinsing with assay buffer (three times).

Glu-plasminogen binding to fibrin

A 96-wells plate containing a solid-phase fibrin surface prepared as described above was used. (Liposomal) Plg samples (in assay buffer) at various concentrations up to 2.5 μg Plg per well were incubated for 2 hours, under gentle mixing conditions, at room temperature at pH 7.4. Supernatant was harvested and the wells were washed three times with assay buffer. For some experiments, a subsequent washing procedure with a 4% w/v Tween 80 solution was introduced (see Results section). Fibrin bound Plg was incubated with the 4% Tween solution for 30 minutes after which three washing steps with assay buffer were performed.

The amount of Plg in both the supernatant and the bound fraction was determined as described below. On each plate duplicate samples were incubated and measured. Because of inter-experimental differences in the obtained liposomal Plg surface-densities and in the obtained amount of bound fibrin per plate, the liposomal fibrin binding curves in this study usually refer to typical examples. Binding characteristic trends as presented in the figures were verified for several Plg-liposome batches on several different plates.

Measurement of glu-plasminogen activity

The activity of bound and unbound glu-plasminogen (free or liposomal) was determined by activation with streptokinase according to Friberger et al. [11]. The Plg samples were incubated with a molar excess of streptokinase and the activity of the streptokinase-Plg complex was determined by conversion of the synthetic substrate H-D-Val-Leu-Lys-pNA.2 HCl, which was measured by monitoring the absorbance of the reaction-product pNA (para-nitro-aniline) at 405 nm.

With this assay an estimate of the binding capacity of glu-plasminogen to fibrin monolayers is obtained. It should be noted that the results also reflect the possible influence of the coupling (-procedure) on the activity of liposomal Plg (Chapter 2a, [4]).

Binding of ^{125}I-labeled glu-plasminogen(-liposomes) to fibrin: competition with free Plg

Plg was trace-labeled with ^{125}I according to the Iodogen-method. When liposomal Plg was to be labeled, the labeling reaction was performed before the SATA (and coupling-)reaction. Incubations were executed on a fibrin monolayer as described above. Simultaneous incubations of radio-labeled liposomal Plg and unlabeled free Plg were performed. Radio-active liposomal Plg was incubated in concentrations up to 2.5 µg/well. Its fibrin binding in the absence of free Plg was compared to the binding in the presence of a 25-fold, a 50-fold and a 100-fold excess of free (unlabeled) Plg.

Results

Surface-densities of glu-plasminogen and fibrin.

The standard incubation ratio of SATA / protein = 8 (molar ratio) results in introduction of 0.5 - 1.8 mol thiol groups per mol Plg. For the different Plg-liposome batches tested in this study 8 to 60 µg Plg per µmol phospholipid was coupled, which corresponds with about 65 to 500 protein molecules per liposome, assuming unilamellarity and uniform particle size. These densities are in good agreement with those found in our previous study (Chapter 2a, [4]). After the coupling procedure, the average particle size of the liposomes was between 0.23 and 0.25 µm. The Plg surface-density is then between 0.4 to 2.8 x 10^3 molecules glu-plasminogen per square µm.

Coating of the 96-wells plates with a fibrinogen layer (as described in Materials and methods) resulted in binding of 13 to 15% of the incubated amount of fibrinogen, corresponding with about 78 to 90 ng of fibrinogen per well. The fibrin-coated plates resulting after thrombin treatment (assuming full fibrinogen to fibrin conversion) contain fibrin monomer at a density of around 3.4 to 3.9 x 10^{-4} nmol/cm^2, corresponding with 2 to 2.5 x 10^3 fibrin molecules per square μm.
It can be concluded that glu-plasminogen and fibrin densities are in the same range thus allowing multivalent interaction.

Specificity of the binding of glu-plasminogen
a) binding to uncoated plates
When the immobilized fibrin monolayer as described above does not cover the complete surface of the well, the possibility of aspecific binding of Plg and liposomes to non-coated parts of the plate should be considered. In a control experiment it was shown that aspecific binding of Plg and of Plg-liposomes to 96-wells plates devoid of fibrin occurred, when buffer solutions without Tween 20 were used. Especially for liposomes the bound fraction could not be neglected. Expressed as a percentage of the optimal amount bound to fibrin-coated wells, aspecific binding could make up to 4% (free Plg) and 13% (Plg-liposomes) of the total binding.
Negligible aspecific binding of Plg and liposomal Plg (both ≤ 1%) was observed after coating the 96-wells plate with carbonate buffer containing 0.2% (w/v) Tween 20 and dilution of the Plg- and liposomal samples in Tris buffer containing 0.01% (w/v) Tween 20. This low concentration of Tween 20 was shown not to influence liposomal properties such as size.

b) binding to fibrin-coated plates
Fibrin binding of free Plg and Plg-bearing liposomes was monitored in the presence of varying concentrations of epsilon-amino-caproic acid (EACA), a synthetic antifibrinolytic amino acid, known for its ability to specifically displace Plg from the fibrin surface [12]. Figure 1a shows how increasing concentrations of EACA (up to 5 mM) were able to inhibit the binding of high concentrations of free Plg to fibrin. Binding of liposomal Plg was also reduced by EACA, but to a lesser extent than free Plg (Figure 1b). Obviously, binding of free Plg was much more sensitive to EACA effects than that of Plg-bearing liposomes. The presence of EACA in the concentrations used in this experiment did not hamper the enzymatic assay of glu-plasminogen, as only minor differences were observed in measured total activity between Plg samples with increasing concentrations of EACA (data not shown).

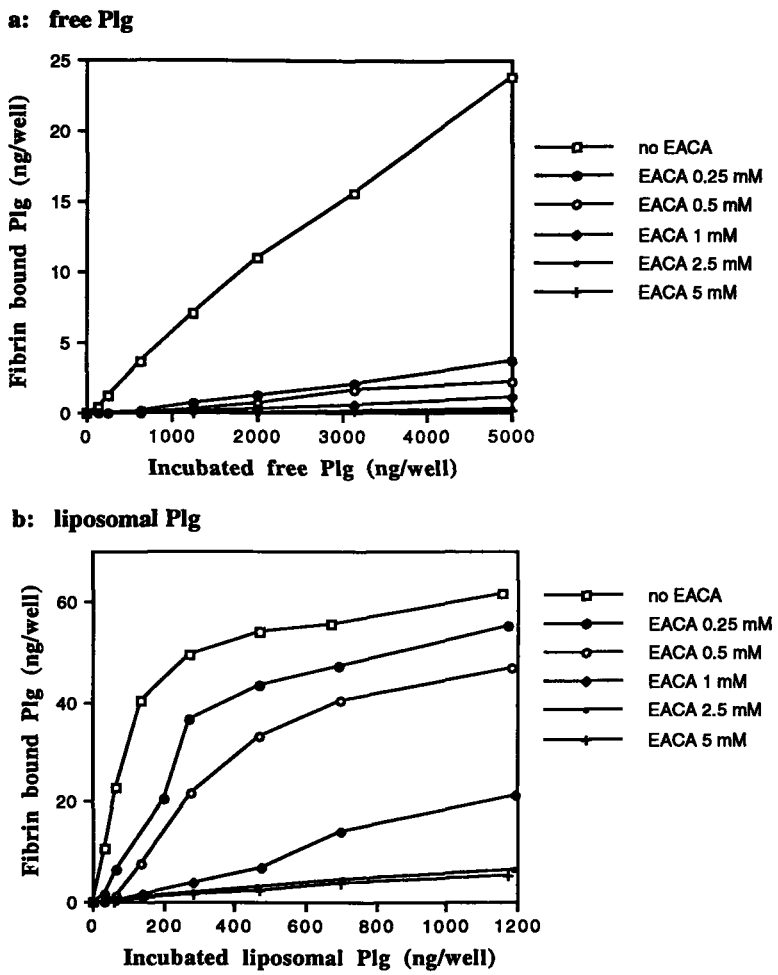


Figure 1 a, b Fibrin binding of free and liposomal Plg in the presence of EACA. Increasing amounts of free (a) or liposomal (b) Plg were incubated for 2 hours with a fibrin-coated plate, in the absence or presence of -varying concentrations of- epsilon-amino-caproic acid (EACA). The amount of fibrin bound Plg was measured following 3 washing steps. Liposomal Plg surface-density (b): 20 µg Plg per µmol PL.

Distinction between fibrin-interacting and non-interacting Plg on fibrin bound Plg-liposomes

We were interested to characterize the fibrin binding of Plg-bearing liposomes also with respect to the question how many of the Plg molecules of a liposomes were involved in the binding. To measure the non-binding portion of Plg we used a high detergent concentration to dissolve bound liposomes. Therefore, after the standard procedure to bind Plg-liposomes and washing three times with assay buffer, an additional washing procedure with a 4% (w/v) Tween 80 solution was introduced (see Materials and methods).

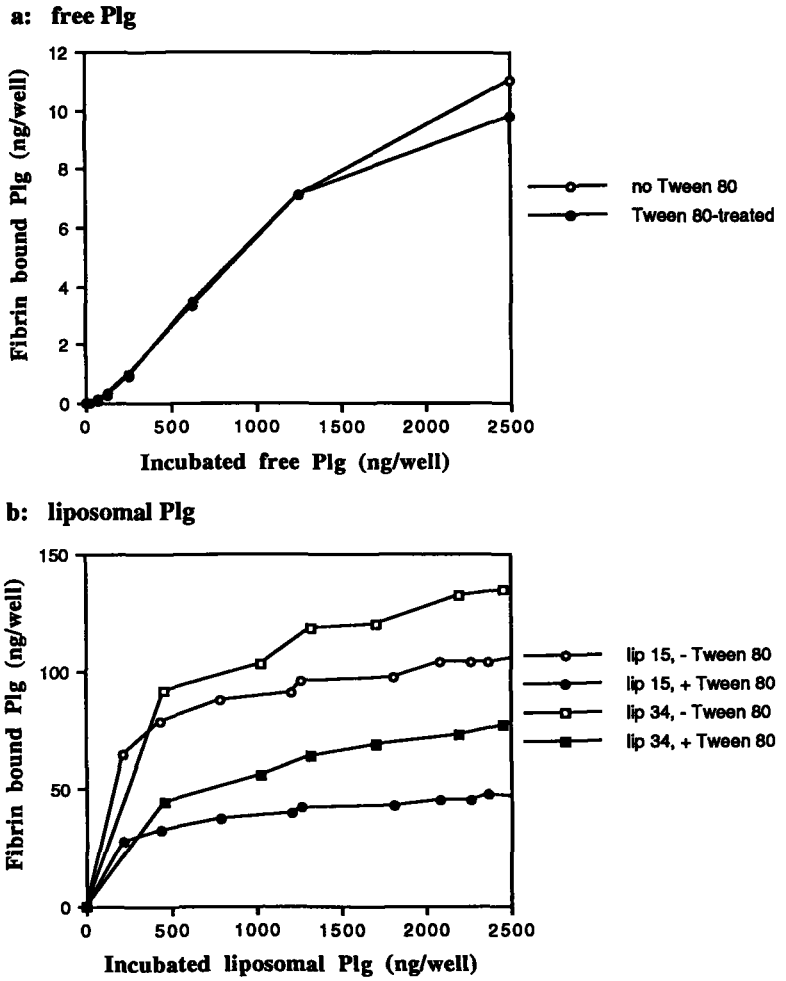
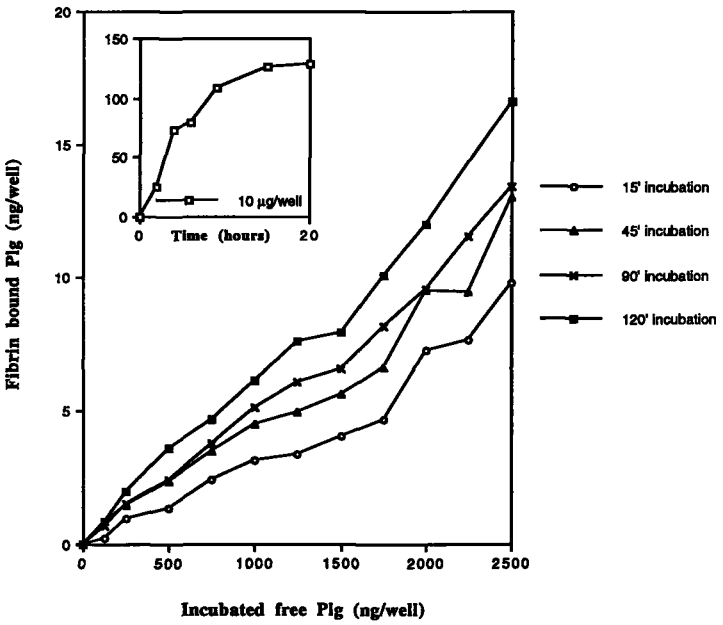


Figure 2 a, b Fibrin binding of free and liposomal Plg and the influence of a subsequent incubation with Tween 80.
Increasing amounts of free (a) or liposomal (b) Plg were incubated for 2 hours with a fibrin-coated plate. The amount of fibrin bound Plg was measured directly (following 3 washing steps) or after a 30' incubation with 4% (w/v) Tween 80 (followed by 3 washing steps). The liposome numbers (b) refer to their liposomal Plg surface-density in µg Plg per µmol PL.

As can be seen from Figure 2a, washing with Tween 80 did not affect the fibrin binding behaviour of free Plg. However, the amount of fibrin bound Plg for liposomal Plg samples (Figure 2b) was substantially reduced by treatment with Tween 80: between 35 and 45% of the original amount was still found to be fibrin bound afterwards (tested for 7 independent Plg-liposome batches with variable Plg surface-densities). Interestingly, no relation between liposomal Plg surface-density and remaining percentage of binding was observed.

a: free Plg



b: liposomal Plg

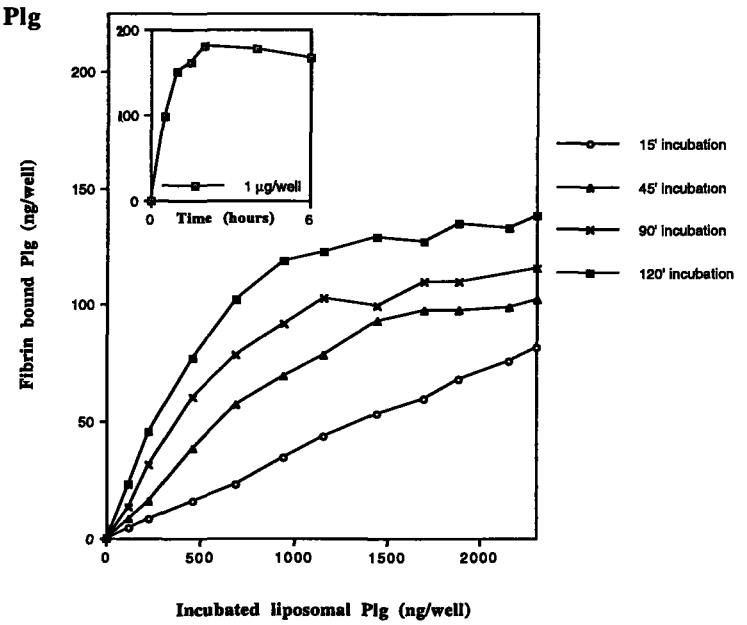


Figure 3 a, b Fibrin binding of free and liposomal Plg after various time intervals.
Increasing amounts of free (a) or liposomal (b) Plg were incubated with a fibrin-coated plate for different periods of time. The amount of fibrin bound Plg was measured following 3 washing steps. Liposomal Plg surface-density (b): 40 µg Plg / µmol Plg.
The inserted figures show the fibrin binding (in ng/well) of 10 µg/well free Plg (a) and 1 µg/well liposomal Plg (b) as a function of -extended periods of- time.

Time dependence of the binding

Comparison of the concentration dependence of fibrin binding of free Plg and Plg-bearing liposomes showed for the range of 0.13 to 2.5 μg Plg per well only for the liposomes the expected pattern of saturation for incubations up to 2 hours (Langmuir curve, Figure 3b) while with free Plg 18-20 hours incubation seems to be required (insert Figure 3a). The rate of binding is obviously much faster for the liposomes which show equilibrium within 2 hours (insert Figure 3b). The initial rate of binding for comparable incubated amounts of Plg and liposomal Plg (not shown) differed by a factor 15. With respect to the 20 hour incubations for free Plg we performed control experiments to exclude artefacts and stability problems. In such control experiments Plg was incubated overnight under identical conditions, but in the absence of fibrin, followed by a standard 2 hour fibrin binding incubation. The same results were obtained as with fresh Plg. These experiments showed that an overnight incubation procedure did not impair the Plg enzymatic activity nor the fibrin binding properties (not shown).

Variation in glu-plasminogen density on liposomes

The fibrin binding behaviour of liposome coupled Plg in varying densities at the liposome surface was compared to that of free Plg. Figure 4a shows that binding of liposomal Plg to fibrin exceeded that of free Plg after 2 hours of incubation (three independent experiments were taken together). Upon incubation of similar Plg concentrations, increasing liposomal Plg-densities led to enhanced binding (higher plateau values) within the range of Plg-densities used. The obtained plateau levels of fibrin binding varied between 35 to 140 ng Plg per well.

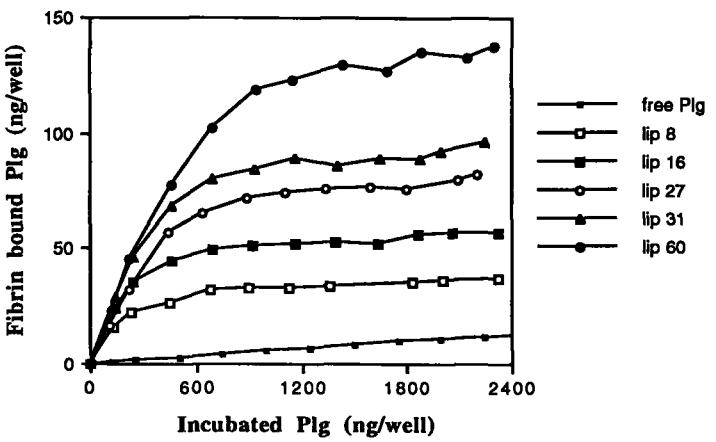


Figure 4a Fibrin binding of free Plg compared to liposomal Plg varying in Plg surface-density.
Increasing amounts of (liposomal) Plg were incubated for 2 hours with a fibrin-coated plate. The amount of fibrin bound Plg was measured following 3 washing steps. The liposome-numbers refer to their liposomal Plg surface-density in μg Plg per μmol PL.

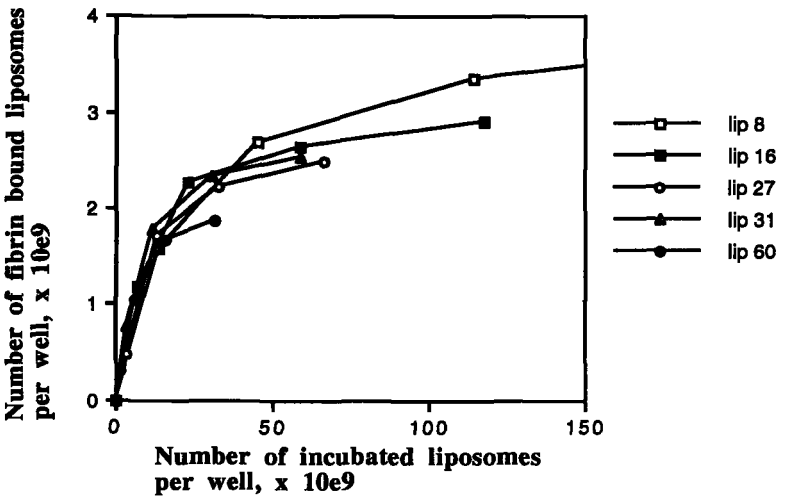


Figure 4b Fibrin binding of liposomes with varying Plg surface-densities.
Derived from figure 4: the number of fibrin bound liposomes is plotted against the number of incubated liposomes. The liposome numbers refer to their liposomal Plg surface-density in µg Plg per µmol PL.

Calculation of the number of fibrin-interacting liposomes which corresponds with the maximal Plg values shown in Figure 4a, leads to Figure 4b. Maximum values for the number of bound liposomes per well between 1.9 and 3.8 x 10^9 were obtained.
When "empty' liposomes (liposomes without Plg) were added (up to 0.2 µmol/well phospholipid) to either native protein or to Plg-liposomes, no interference with the binding to fibrin was seen (data not shown).

Competition experiments
Upon radio-active labeling of either free or liposomal Plg, simultaneous incubation of fibrin with free and liposomal Plg could be performed. In a control experiment it was shown that the interaction between fibrin and free Plg was not affected by radio-active labeling with ^{125}I. Figure 5 shows that even the presence of a 100-fold excess of free Plg could not compete successfully with the binding of liposomal Plg (2.5 µg/well).

Discussion

Relevancy and specificity of the in vitro model
For targeting to fibrin containing thrombi of our Plg-liposomes, it is essential to be able to evaluate the fibrin binding properties of such liposomes in a simple in vitro system. To this end the known system with fibrinogen coated onto PVC and subsequent -thrombin induced-

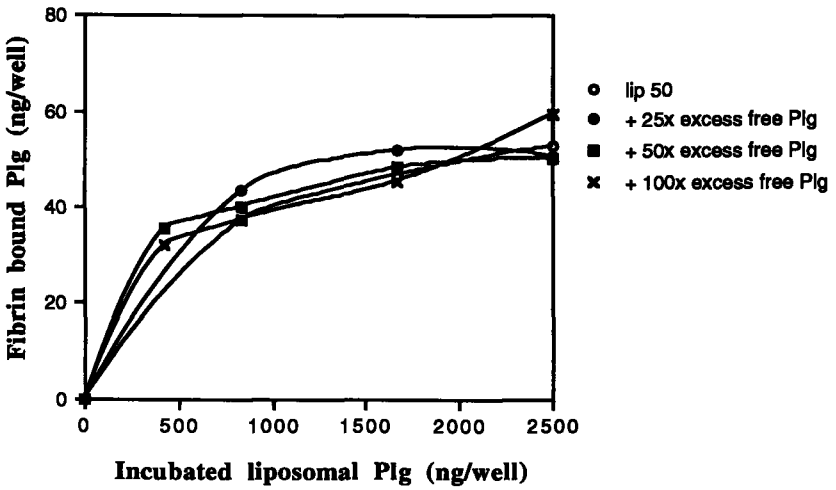


Figure 5 Fibrin binding of ^{125}I-labeled liposomal Plg in the presence of different amounts of unlabeled free Plg.
Increasing amounts of ^{125}I-labeled liposomal Plg were incubated for 2 hours with a fibrin-coated plate, in the absence or presence of -different fold excess of- unlabeled free Plg. The amount of fibrin bound Plg was measured following 3 washing steps. Liposomes with a Plg surface-density of 50 μg Plg per μmol PL were used.

conversion to fibrin appeared suitable. We used carefully purified fibrinogen, avoiding proteolytic degradation in vitro, thus obtaining a fibrin coat representing a fresh fibrin clot, prior to any degradation. Such proteolytic degradation has been shown to produce new plasminogen binding sites [13]. In vivo, thrombi will show partial degradation in most cases, thus our analysis represents a worst case scenario from that point of view. Future studies should also take defined degradation of fibrin into account.

The used surface densities of fibrin on the plate and of glu-plasminogen on the liposome surface were closely similar, allowing for multivalent interaction. The average densities ranged from 0.4 to 2.8 x 10^3 molecules per square μm. With the highest densities the average repeat distances between fibrin molecules were about 20 nm, which is close to the highly ordered longitudinal repeats of 22.5 nm for fibrin molecules in fibrin bundles in vivo [14]. The in vitro system thus has dimensions similar to fibrin bundles on a molecular level. However, the in vitro fibrin monolayer may not represent in vivo fibrin in all respects, since it is expected not to display the highly ordered structure and the high lateral density of plasminogen binding sites of fibrin bundles in vivo.

In the in vitro system aspecific binding of Plg to uncoated parts of the plate formed a problem, especially since Plg-bearing liposomes showed an even more pronounced aspecific binding than free Plg. The use of low concentrations of Tween 20, which did not affect liposome size, solved this problem. The binding of (liposomal) Plg to fibrin was shown to be reduced to a

minimum by epsilon-amino-caproic acid. It has been described in literature [15-17] that the ability of epsilon-amino-caproic acid to bind specifically to lysine binding sites of glu-plasminogen most probably induces a conformational change in Plg. This conformational change inhibits (amongst others) the binding of Plg to fibrin. Interestingly, Plg-bearing liposomes need higher concentrations of epsilon-amino-caproic acid to block fibrin binding than free Plg. This finding suggests an enhanced binding strength (discussed below).

Specific aspects of liposomes

Next to the Plg surface-density, another important parameter is the dimension of the liposomes. It can be envisaged that there is only room for a limited number of liposomes in a fibrin-well. Dividing the available surface per well by the projected surface per liposome provides us with a rough estimate of the maximal number of liposomes that can be bound per well. Assuming that the liposome diameter is 0.24 μm and that the interaction area between liposomes and fibrin-layer is flat, a maximal binding of about 1.5×10^9 liposomes per well is expected (without correction for liposomal space-filling). For liposomes with 65 to 500 Plg-molecules per liposome, this would result in a bound amount (plateau-level) of Plg per well of 14 to 110 ng Plg per well. These numbers are close to the measured experimental values, i.e. between 35 and 140 ng/well for liposomes with a similar range of coupled Plg.

We could make a distinction between fibrin-interacting and non-interacting Plg of fibrin bound liposomes by desintegrating bound liposomes with a high concentration of detergent. This analysis is based on the assumption that when Tween 80 causes the liposomes to fall apart, released non-interacting Plg does not have the opportunity to interact with fibrin. The observation that the binding velocity of free Plg was relatively low compared to liposomal Plg (cf. insert Figures 3a and 3b) seems to justify this assumption. If one hypothesizes that a homogeneous distribution of Plg over the bilayer exists, our findings suggest that 40% of the liposome surface "interacts" with fibrin. However, partial rearrangement of the Plg-molecules may be induced upon binding of the liposomes to the fibrin monolayer, leading to a relatively high surface-density of Plg-molecules at the site of interaction: 'contact capping' [18-20]. For liposomes with a relatively low liposomal Plg surface-density one would expect contact capping to play a larger role, leading to a higher percentage of bound Plg. The -limited number of- data in Figure 2b do not support this hypothesis. Using a liposome composition with a more fluid bilayer character might result in enhanced contact capping. This aspect will be seriously considered in future design of these liposomes.

As expected from the above considerations, the maximal amount of Plg bound with liposomes with low Plg-density remained below the maximal available amount of fibrin binding sites. As can be seen from Figure 2b, liposomes with different Plg-densities showed different plateau levels. Upon treatment with Tween 80, the amounts of "truly' fibrin bound Plg were still

different. Since the limiting amount of 130 ng/well (as seen in insert Figure 3a) was not reached after Tween 80 treatment of liposomal Plg, there was apparently no saturation of all available fibrin binding sites.

Assessment of the maximum number of liposomes binding to the fibrin surface was not performed by direct measurement of liposomes themselves. An indirect approach to such analysis was performed by assuming monodispersity of the liposome dispersion and an even distribution of Plg over all liposomes. Upon calculation of the number of incubated Plg-liposomes and the number of bound Plg-liposomes, Figure 4b is obtained. There appears to be little difference between the binding curves for different liposomal Plg-densities. From this figure estimates of the relative Kd values for liposomes with variable Plg-densities were derived, from the 'concentration' at half maximal binding. The relative Kd for 8 μg/μmol Plg-liposomes was then found to be about four times higher than for 60 μg/μmol Plg-liposomes (observed upon expansion of the x-axis).

Improved rate and affinity of binding by multivalency

The time to reach equilibrium for binding was obviously longer for free Plg, requiring overnight incubation, than for Plg-bearing liposomes which reached equilibrium within a few hours. The rate of binding was not analysed in detail, but estimated to be at least one order of magnitude faster for the liposomes than for free Plg. For the eventual use of the liposomes in thrombolytic therapy, the increase in the rate of binding is an advantage as fast accumulation at the target site is desired. Assuming equilibrium conditions to exist after 18 h, a rough estimate for the Kd for Plg under these equilibrium conditions was derived from the linearized expression of the Langmuir adsorption equation $1/[b] = Kd/[f][Fbt] + 1/[Fb^t]$, where b = bound Plg, f = unbound Plg (supernatant), and Fb^t = total concentration of fibrin binding sites. We used a double reciprocal plot of 1/[b] versus 1/[f] which does not require to include an assumption on the Fb^t. From this plot a straight line was calculated with a y-intercept of $1/[Fb^t]$ and a slope of $Kd/[Fb^t]$. An estimated value for the Kd of about 0.1 μM was obtained. This value differs from the 0.99 μM described by Fleury et al. [21], probably due to differences in the fibrin monolayer: although the fibrin-density of our monolayer is very similar to the one Fleury et al. described, we did not use the covalent fibrinogen linkage-procedure that was performed in their study which might lead to a more flexible fibrin coat and a higher binding affinity.

In the experiments with ^{125}I-labeled Plg it was shown that the fibrin binding of liposomal Plg was not affected by the presence of high concentrations of free Plg (Figure 5). This indicates that the binding of liposomal Plg is strongly enhanced, most probably because of its multivalent character. The interaction with fibrin is based on a multitude of bonds, which increase the interaction energy and therefore reduce the dissociation constant [22-24].

We refrained from a similar analysis of liposomal binding constants as made for free Plg since

only partial participation of liposomal Plg in binding occurred and heterogeneity in the liposomal population was expected. However, a rough comparison between dissociation constants among liposomes could be obtained by evaluating the position of the point of half maximal binding in the curves of Figure 4b. Although there appears to be little difference in binding between varying liposomal Plg-densities, upon expansion of the x-axis the relative Kd for 8 μg/μmol Plg-liposomes was found to be about four times higher than for the 60 μg/μmol Plg-liposomes. This finding supports the assumption that liposomes with a relatively high Plg surface-density have a relatively low Kd.

Conclusions

In conclusion: by coupling glu-plasminogen to liposomes improved fibrin binding characteristics (compared to free Plg) can be obtained. Liposomal Plg exhibits an increased fibrin binding rate and can not be displaced by a 100-fold excess of free Plg. The multivalent character of liposomal Plg is probably responsible for the improved binding characteristics. Fibrin binding of liposomal Plg is thought to be limited by geometrical restrictions, dictated by the available space per well and by the dimensions of the liposomes. These findings indicate that Plg-liposomes indeed may be used in vivo for targeted delivery of fibrinolytics to fibrin based thrombi.

Acknowledgements

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Chapter 4

The preparation of tissue-type Plasminogen Activator (t-PA) containing liposomes: entrapment efficiency and ultracentrifugation damage

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Summary

In this study, a method was developed for the efficient entrapment of active tissue-type Plasminogen Activator (t-PA) into liposomes. Experimental conditions were varied to optimize t-PA entrapment: different buffer solutions were used (pH 4 and 7.5), the effect of the incubation concentrations of phospholipid (PL) and t-PA was monitored and the influence of liposome-size was examined. Furthermore, the effect of ultracentrifugation on t-PA containing liposomes was determined in the presence and absence of Tween 80.

t-PA entrapment strongly depended on experimental conditions and ranged from 30 up to 90%. Almost quantitative (90%) entrapment (entrapment percentage defined as absolute entrapment (IU t-PA / μmol PL) divided by total incubation ratio (IU t-PA / μmol PL), times 100%) was obtained in Hepes buffer pH 7.5, devoid of arginine, with low ionic strength.

Ultracentrifugation, used for removal of non-entrapped t-PA, was shown to have a damaging effect on the liposomes (especially in the presence of 0.05% Tween 80), leading to t-PA loss. However, because acceptable alternatives were not available, ultracentrifugation was used during this study. Therefore, the encapsulation-percentage values shown in this study are in fact underestimates for the true entrapment of t-PA.

In conclusion: almost quantitative t-PA entrapment in liposomes can be achieved by selecting the proper milieu and inducing a strong interaction between t-PA and bilayer.

Introduction

Nowadays, aggressive thrombolytic therapy is the medical approach of choice for the treatment of myocardial infarction [1]. In an earlier study (Chapter 6, [2]) we showed in a rabbit jugular vein thrombosis model the beneficial effects of liposome entrapment on the therapeutic index of tissue-type Plasminogen Activator (t-PA), a potent thrombolytic agent. The liposomes used in that in vivo study were not optimized in terms of their in vivo or in vitro behaviour. A further increase of the therapeutic index was considered conceivable by optimizing the procedure for liposomal t-PA entrapment*.

Studies on the interaction of globular proteins with liposomes are so far mainly confined to the behaviour of a limited number of specific proteins. They show that each protein has its own characteristics (e.g. [3-8]). The association of t-PA with liposomes was described before by Soeda et al. [9], who found a t-PA entrapment of about 60% in several liposome compositions upon detergent dialysis. However, the overall t-PA recovery after dialysis was only 20%. A similar detergent removal method was described for entrapment of plasminogen activators, in particular streptokinase, into phosphatidylcholine-liposomes by O'Rear et al. [10], who reported an entrapment of 30% of the original amount of SK.

Considering the limited number of data and the lack of mechanistic insight for a rational approach to optimize loading efficiency and stability, the present study on the interaction of t-

PA with liposomes was performed. Our aim was to promote t-PA-bilayer interactions in order to find a suitable method to efficiently entrap t-PA into liposomes.
Moreover, this study reports on the stability-interfering effect of ultracentrifugation (especially in the presence of 0.05% Tween 80) on t-PA containing liposomes. This remarkable feature might occur with other protein-liposome systems as well.

*As appears in the course of this study, liposomal t-PA 'entrapment' should be interpreted as a combination of t-PA-bilayer association plus true encapsulation of t-PA in the liposomal aqueous phase.

Materials and methods

Recombinant t-PA (Actilyse) was obtained as a freeze-dried product from Boehringer Ingelheim GmbH (Frankfurt, West Germany).
Egg-phosphatidylcholine (egg-PC) was a gift from Lipoid KG (Ludwigshafen, Germany), cholesterol was obtained from Sigma (St. Louis, MO, USA), egg-phosphatidylglycerol (egg-PG) was a gift from Nattermann Phospholipid GmbH (Cologne, FRG).
The chromogenic plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide.2 HCl was from Chromogenix (Mölndal, Sweden). Tween 80 was obtained from Merck (Darmstadt, Germany); triton X-100 from BDH (Lab. Supplies, Poole, England). Plasminogen for the t-PA-assay was purified in our lab (Leiden, The Netherlands).
All other chemicals were of analytical quality and were used without additional purification.

Preparation of t-PA-containing liposomes, general method

Small unilamellar vesicles consisting of egg-phosphatidylcholine, egg-phosphatidylglycerol and cholesterol in a molar ratio of 10 : 1 : 4 were prepared. Basically, the "film-method" (first described by Bangham et al. [11]) was used. After resuspending the lipid film in buffer, t-PA was added. The liposomes were subsequently freeze-thawed 5 times to increase the encapsulation capacity of the liposomes [12, 13]. In separate experiments we found that the introduction of more than 5 freeze-thawing steps did not improve entrapment (data not shown). Non-entrapped t-PA was removed by ultracentrifugation (Beckman Instr. Inc., California, USA) for 45 minutes, at 150,000 x g and 4°C. The pellet was subsequently redispersed in buffer.

Variations in the t-PA entrapment procedure

In order to find an optimal entrapment procedure the following experimental conditions were varied. First of all, different buffer solutions were used: the entrapment of t-PA in (1) standard buffer pH 7.5: 0.01 M Hepes, 0.058 M NaCl, 0.1 M arginine, 1mM EDTA and

0.05% Tween 80 was compared to (2) entrapment at pH 4 (0.033 M citrate, 0.135 M NaCl, 0.05% Tween 80), and to (3) entrapment in a pH 7.5 buffer of 0.01 M Hepes, 7.5% (w/v) lactose, 1 mM EDTA, 0.05% Tween 80. Buffers (1) and (2) have a relatively high ionic strength (0.17 M and 0.18 M, respectively), buffer (3) has a relatively low ionic strength of 0.01 M. Arginine was added to buffer (1) for optimal solubility of t-PA. It was left out in buffer (2), since the lower pH of that buffer already increases t-PA-solubility, and also in buffer (3), in view of the minimal ionic strength condition that was pursued.

As free t-PA (in contrast to liposomal t-PA) has a strong propensity to adsorb to surfaces, 0.05% (w/v) Tween 80 was included in all buffers to prevent loss of non-encapsulated t-PA from the supernatant. During ultracentrifugation of the obtained liposomes, one pH 7.5 buffer was used -irrespective of the original buffer used for entrapment-, consisting of 0.01 M Hepes, 0.135 M NaCl, 1 mM EDTA, 0.05% Tween 80. Samples were diluted 12.5 times (400 µl to 5000 µl) with this buffer prior to ultracentrifugation. The pellet was redispersed in the original buffer used for entrapment (buffer (1), (2) or (3) as described above).

Furthermore, the phospholipid (PL) concentration was varied from 15 to 150 µmol/ml; 100 µmol/ml was considered as the standard concentration.

The standard t-PA concentration was 2.0×10^5 IU/ml, corresponding with about 300 µg/ml. 2.5 and 5 times higher concentrations of t-PA were used as well.

In order to gain insight into the adsorption of t-PA to the outside of intact liposomes, t-PA was added after freeze-thawing of the liposome dispersion. After 30 minutes of incubation the sample was ultracentrifuged as described above. The liposomal t-PA fraction presumably represented t-PA which was adsorbed to the outside bilayer of the liposomes.

The influence of the particle size of the liposomes on the encapsulated amount of t-PA was also examined. For this purpose, part of the liposome dispersion was extruded (prior to the freeze-thawing-entrapment step) through polycarbonate membrane filters with pores of 0.6 µm (once) and 0.2 µm (three times); after extrusion their average diameter was around 0.23 µm, as determined by dynamic light scattering with a Malvern 4700 system, Malvern Ltd., UK. Electrophoretic mobility data were obtained with a Malvern zeta-sizer 2C unit (Malvern Ltd., UK) using the PC-4 cell at 25°C.

The effect of repeated ultracentrifugation on liposomal t-PA stability in vitro

In order to examine the effect of ultracentrifugation alone on the stability of the t-PA-liposomes, liposome samples (in standard Hepes buffer or in Hepes buffer with low ionic strength) were ultracentrifuged 7 times in a row, using a standard dilution factor of 33.3 before each ultracentrifugation step (150 µl to 5000 µl). The standard time span between two ultracentrifugation steps was 2 hours. However, the possible influence of a longer time interval (± 20 hours) was also studied. Furthermore, the effect of the dilution step prior to ultracentrifugation was examined (for standard Hepes only) by comparing samples diluted

standard 33.3 times to samples which were diluted 200 times (25 µl to 5000 µl) before each ultracentrifugation step.

The effect of Tween 80 on liposomal t-PA stability in vitro

The possible influence of the presence of 0.05% Tween 80 in the buffers on the in vitro stability of liposomal t-PA was also examined. To this end, the repeated ultracentrifugation experiment as described above was also performed with t-PA liposomes which were prepared and ultracentrifuged in buffers devoid of Tween 80 (e.g. these liposomes never were in contact with Tween 80).

Phospholipid-determination

Phospholipid concentrations were assessed (after perchloric destruction) by a phosphate-assay according to Fiske-Subbarow [14].

Measurement of t-PA activity

t-PA concentrations were assessed by measuring the enzymatic activity of t-PA, e.g. all measurements represent enzymatically active t-PA. As a consequence, irreversible loss of activity during the entrapment procedure would result in a recovery (in supernatant plus pellet) of less than 100%.

The t-PA-activity in supernatant samples was determined in the presence of 0.05% w/v Tween 80 (reducing adsorption of t-PA to surfaces); when measuring liposomal t-PA 1% w/v triton X-100 was used for destruction of the liposomes. In general, t-PA containing samples were diluted at least 10^4 times to reach the appropriate range of t-PA concentrations.

The activity of free (supernatant) or liposomal (pellet) t-PA was determined by assessing the amount of plasmin formed, following an incubation with a molar excess of plasminogen, using the reaction as described by Verheyen et al. [15]. In short: the plasmin concentration is determined by measuring its ability to convert the synthetic substrate H-D-Val-Leu-Lys-p-nitroanilide.2HCl. The absorbance of the reaction-product para-nitro-aniline at 405 nm is proportional to the concentration of t-PA in the sample.

Calculations and denominations

The total incubation ratio is defined as the measured value for the total incubated amount of t-PA per µmol PL. This parameter is obtained by measuring the concentration of t-PA and PL in the incubation-mixture after freeze-thawing, before ultracentrifugation, in the presence of 1% w/v triton X-100.

The (absolute) entrapment, expressed as IU t-PA per µmol phospholipid, is obtained by dividing the concentration of entrapped (i.e. associated) t-PA (IU/ml) by the concentration of

phospholipid (µmol/ml), both measured in the liposomal fraction (again in 1% w/v triton X-100) after ultracentrifugation.
In order to provide more information about the efficiency of the entrapment procedure, the entrapment percentage is calculated by dividing the obtained entrapment (IU t-PA / µmol PL) by the total incubation ratio (IU t-PA / µmol PL), times 100%.
It was shown that the measured value for the total incubated amount of t-PA per µmol PL (total incubation ratio) varied per experiment, despite similar starting-conditions. This caused corresponding variations in the obtained values for the absolute entrapment. Therefore, we consider the entrapment percentage (which is corrected for this discrepancy: each value is divided by its own corresponding 'total incubation ratio') a more justifiable way of expressing and comparing the t-PA entrapment values than the (absolute) entrapment values.

Statistics
The statistical significance of differences between two groups was evaluated by student t-tests. P values < 0.05 were considered significant. More than two groups were evaluated by one-way ANOVA. P values < 0.05 were considered significant.

Results

t-PA entrapment using different buffer solutions
As shown in Table 1, t-PA entrapment under standard conditions (standard 100 µmol/ml PL, 2.0×10^5 IU/ml t-PA) in (1) standard Hepes buffer, pH 7.5, led to an entrapment percentage of about 70%. The values for the entrapment percentage under these standard conditions were similar throughout all experiments performed in this study: 69 ± 8% (mean ± standard deviation, n=26). In (2) citrate buffer, pH 4, the obtained entrapment percentage was

Table 1 t-PA entrapment as a function of the buffer medium used.
Standard conditions (with exception of the buffer medium) are used: PL incubation concentration 100 µmol/ml, t-PA incubation concentration 2.0×10^5 IU/ml, unsized liposomes (n=3).

Buffer medium	Total incubation ratio IU t-PA/µmol PL $\times 10^3$ (mean ± SD)	Entrapment (abs.) IU t-PA/µmol PL $\times 10^3$ (mean ± SD)	Entrapment percentage entrapment / tot. inc.ratio $\times$ 100% (mean ± SD)
1) Hepes, pH 7.5[a]	1.3 ± 0.1	0.92 ± 0.03	70 ± 9
2) Citrate, pH 4[a]	1.2 ± 0.2	0.41 ± 0.08	34 ± 2
3) Hepes, pH 7.5[b]	1.2 ± 0.1	1.10 ± 0.06	92 ± 4

[a]high ionic strength, about 0.18 M
[b]low ionic strength, about 0.01 M

significantly (about twofold) lower. Entrapment in (3) Hepes buffer with lactose, pH 7.5, low ionic strength, induced a surprisingly high entrapment percentage of about 90%.
One way ANOVA proved the presence of significant differences in entrapment percentage between groups ($p < 0.01$). Comparison of the separate groups showed significant differences between all groups ($p < 0.01$).

t-PA entrapment using different phospholipid concentrations

Variation of the phospholipid incubation concentration within the range from 50 to 150 µmol/ml did not cause pronounced changes in the obtained t-PA entrapment percentages. On the other hand, reducing the PL concentration to 30 and 15 µmol/ml resulted in a significant reduction of the obtained t-PA entrapment percentage compared to the higher PL concentrations ($p < 0.01$) (Figure 1).

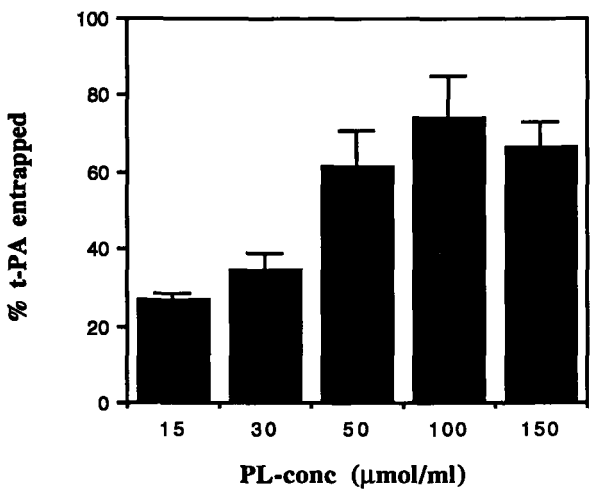

Figure 1 PL-dependence of t-PA entrapment percentage.
Conditions (except PL conc) were standard: Hepes buffer with high ionic strength, pH 7.5, t-PA incubation concentration 2.0 x 10^5 IU/ml and unsized liposomes were used (n=6 for PL conc. 50, 100 and 150 / n=3 for PL conc. 15 and 30 µmol/ml). Bars indicate standard deviations.

t-PA entrapment: t-PA concentration-dependency

Upon raising the t-PA concentration 2.5- or 5- fold (in standard Hepes buffer, pH 7.5, at a fixed PL concentration of 100 µmol/ml) the absolute amount of entrapped t-PA significantly increased (2- and 4-fold, respectively), whereas the entrapment percentage tended to decrease. With respect to the entrapment percentage one way ANOVA proved the presence of

significant differences between groups ($p < 0.05$). Comparison of the separate groups showed that the entrapment percentage of the [1 x t-PA] group was significantly higher than for the 2.5 and 5 x t-PA groups ($p < 0.05$) (Table 2).

Table 2 t-PA entrapment as a function of t-PA incubation concentration in standard Hepes buffer, pH 7.5, high ionic strength.
Standard conditions (except the t-PA concentration) were used: standard Hepes buffer pH 7.5, high ionic strength, PL incubation concentration 100 µmol/ml, unsized liposomes (n=3).

Incubation conc. t-PA (IU/ml x 10^5)		Total incubation ratio IU t-PA / µmol PL x 10^3 (mean ± SD)	Entrapment (abs.) IU t-PA / µmol PL x 10^3 (mean ± SD)	Entrapment percentage entrapment / tot. inc.ratio x 100% (mean ± SD)
(1x)	2.0	3.0 ± 0.3	1.8 ± 0.3	60 ± 8
(2.5x)	5.0	7.8 ± 0.4	3.7 ± 0.2	47 ± 2
(5x)	10.0	16.6 ± 2.4	7.3 ± 0.9	44 ± 5

Adsorption of t-PA : addition of t-PA after freeze-thawing

When t-PA is added after freeze-thawing of the liposomes, the adsorption of t-PA to the outer surface of the liposomes can be measured, as t-PA is unlikely to pass through the bilayer within the time span of the experiment. The adsorption percentages are shown in Table 3.

Table 3 t-PA-adsorption to the outside of liposomes as a function of the buffer medium.
Standard conditions (with exception of the buffer medium) were used: PL incubation concentration 100 µmol/ml, t-PA incubation concentration 2.0 x 10^5 IU/ml, unsized liposomes (n=3).

Buffer medium	Total incubation ratio IU t-PA / µmol PL x 10^3 (mean ± SD)	Adsorption (abs.) IU t-PA / µmol PL x 10^3 (mean ± SD)	Adsorption percentage adsorption / tot. inc.ratio x 100% (mean ± SD)
1) Hepes, pH 7.5[a]	1.6 ± 0.1	0.28 ± 0.02	18 ± 2
2) Citrate, pH 4[a]	1.9 ± 0.2	0.26 ± 0.02	14 ± 2
3) Hepes, pH 7.5[b]	1.7 ± 0.1	0.48 ± 0.03	28 ± 1

[a]high ionic strength, about 0.18 M
[b]low ionic strength, about 0.01 M

In standard Hepes buffer the value of ± 18% corresponds with about one quarter of the entrapment percentage of t-PA when the freeze-thawing protocol is used. In Hepes with low ionic strength the obtained value of 28% suggests that about one third of the total 'entrapped' t-PA (after freeze-thawing) was present in adsorbed form at the outside leaflet of the bilayer exposed to the external aqueous phase. In citrate buffer (pH 4), the value of 14% does not seem very high. However, compared to the total entrapment percentage in this buffer, its

contribution is high: about 40%. One way ANOVA proved the presence of significant differences between groups ($p < 0.01$). Comparison of the separate groups showed significant differences in adsorption percentage between buffers (1) and (3) and between buffers (2) and (3) ($p < 0.01$).

t-PA entrapment as a function of the particle size of the liposomes

Standard t-PA entrapment (100 μmol/ml PL, 2 x 10^5 IU/ml t-PA, Hepes buffer, pH 7.5) was performed in unsized liposomes. They had a wide particle size distribution and their mean diameter before t-PA entrapment was > 1 μm. However, after freeze-thawing their average diameter decreased to about 0.5 μm. The entrapment percentage in extruded liposomes (mean diameter after extrusion, before entrapment around 0.23 μm / after freeze-thawing still around 0.23 μm) was shown to be significantly lower than the standard ($p < 0.01$ in student t-test): around 48% compared to 68%, respectively (Table 4).

Table 4 t-PA entrapment as a function of liposome size.
Standard conditions (with exception of the liposome size) were used: standard Hepes buffer pH 7.5, high ionic strength, PL incubation concentration 100 μmol/ml, t-PA incubation concentration 2.0 x 10^5 IU/ml (n=4).

Type of liposomes	Total incubation ratio IU t-PA / μmol PL (mean ± SD)	Entrapment (abs.) IU t-PA / μmol PL (mean ± SD)	Entrapment percentage entrapment / tot. inc.ratio x 100% (mean ± SD)
Unsized	2.0 ± 0.1	1.4 ± 0.1	68 ± 4
Sized	2.2 ± 0.2	1.1 ± 0.1	48 ± 3

The effect of repeated ultracentrifugation on liposomal t-PA stability in vitro

In order to examine the effect of mere ultracentrifugation on the stability of the t-PA-liposomes, liposome samples were ultracentrifuged several times in a row. Figures 2a and 2b show the significant loss of t-PA from the liposomes upon each ultracentrifugation step, under sink conditions (fresh buffer after ultracentrifugation). After 7 sequential ultracentrifugation runs, the t-PA entrapment percentage for liposomes in standard Hepes buffer decreased from 70 to about 25%. t-PA liposomes in Hepes buffer with low ionic strength showed an even more dramatic loss; from 90 to about 25%.

With respect to Figure 2a (standard Hepes), it should be noticed that, instead of the regular time interval of 2 hours, there was an interval of about 20 hours in between steps 3 and 4 and between steps 6 and 7. No influence of this prolonged period of time on the pattern of loss of liposome-entrapped t-PA was observed (Figure 2a). The same time schedule applied to the low ionic strength Hepes buffer + Tween (Figure 2b, closed symbols). In the absence of

Tween (Figure 2b, open symbols) the prolonged time interval was introduced between steps 4 and 5. No difference in pattern of t-PA loss upon prolonging the time interval was seen for this buffer either. Furthermore, in standard Hepes buffer no significant difference in pattern of t-PA loss occurred upon diluting the samples 200 times instead of the regular 33 times before each ultracentrifugation step (data not shown).

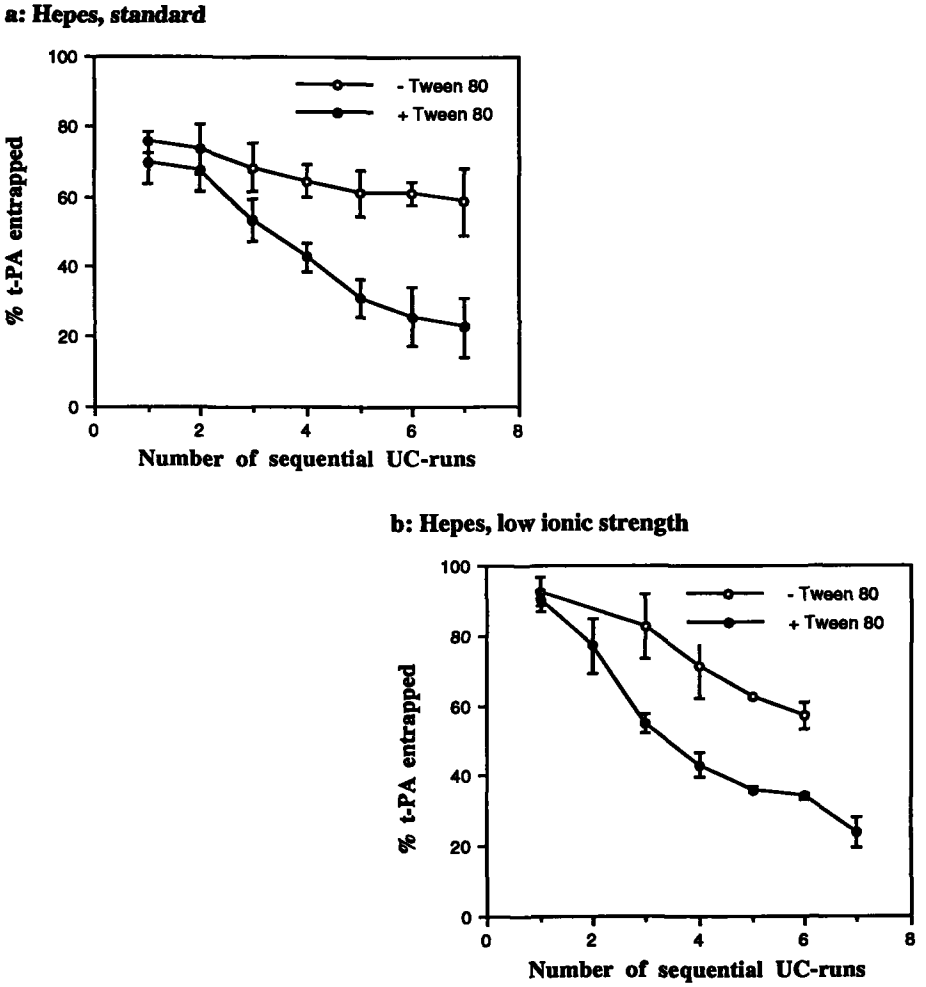

Figure 2a, b Liposomal t-PA loss after repeated ultracentrifugation (UC) runs in (a) standard Hepes buffer pH 7.5, high ionic strength and (b) Hepes buffer with low ionic strength, pH 7.5.
A comparison was made between liposomes which were prepared and ultracentrifuged in the presence of 0.05% Tween 80 and liposomes which were prepared and ultracentrifuged in the absence of 0.05% Tween 80. PL incubation concentration 100 µmol/ml, t-PA incubation concentration 2.0 x 10^5 IU/ml, unsized liposomes. Bars indicate standard deviations. (a): First ultracentrifugation step: n=7, others: n=5. (b): First ultracentrifugation step: n=5, others: n=3.

The effect of Tween 80 on liposomal t-PA entrapment and stability to ultracentrifugation

The contribution of Tween 80 to the damaging effect of ultracentrifugation was investigated by performing the same repeated ultracentrifugation experiment with liposomes, prepared and ultracentrifuged in the absence of Tween in the buffers. From Figures 2a and 2b it is clear that the t-PA loss from the liposomes upon ultracentrifugation was reduced, but still substantial in the absence of Tween 80. Besides, Table 5 indicates that the original t-PA entrapment percentage was not dependent on the presence of Tween 80 in these buffer solutions. There was no significant difference in t-PA loss between samples diluted 33.3 times or 200 times prior to ultracentrifugation in standard Hepes buffer without Tween 80 (data not shown).

Table 5 t-PA entrapment in the presence and absence of Tween 80.
A comparison was made between t-PA entrapment into liposomes which were prepared and ultracentrifuged in the presence of 0.05% Tween 80 and liposomes which were prepared and ultracentrifuged in the absence of 0.05% Tween 80. (PL incubation concentration 100 µmol/ml, t-PA incubation concentration 2.0 x 10^5 IU/ml, unsized liposomes.)

Buffer medium	Entrapment percentage (entrapment / tot. inc.ratio x 100%)	
	+ Tween in buffer	- Tween in buffer
	(mean ± SD)	(mean ± SD)
1) Hepes, pH 7.5[a]	70 ± 6 (n=7)	75 ± 3 (n=7)
3) Hepes, pH 7.5[b]	92 ± 4 (n=3)	93 ± 4 (n=5)

[a]high ionic strength, about 0.18 M
[b]low ionic strength, about 0.01 M

Discussion

The described method leads to substantial t-PA entrapment: about 70% under 'standard'-conditions (2 x 10^5 IU/ml t-PA, 100 µmol/ml PL, Hepes buffer pH 7.5, unsized liposomes). Since "passive" encapsulation via the water phase of the liposomes could only account for roughly 20% of the entrapment (as was measured under these standard-conditions with calcein, a water soluble, non-bilayer interacting probe, data not shown), it can be concluded that an interaction between t-PA and the lipid bilayer of the liposome takes place and significantly contributes to the entrapment. The presence or capability of formation of amphipatic α-helices has been linked with the penetration of proteins into phospholipid bilayers [16]. Therefore, one might consider the possibility that t-PA, which is usually devoid of α-helices at pH 4 and 7.5 (it predominantly shows β-sheets and β-turns) [17], undergoes in (one of) its domains a conformational change into a specific α-helix-containing conformation, upon partitioning in lipid bilayers.

The efficiency for the entrapment of active tissue-type Plasminogen Activator into liposomes depends on the buffer solution used. The very high entrapment value of about 90% that we

found for the Hepes buffer with low ionic strength and containing lactose (Table 1) implies that apparently the interaction of t-PA with the lipid bilayer is promoted in the absence of arginine and NaCl. Under these circumstances the solubility of t-PA is reduced, which may force the relatively hydrophobic protein to interact with the lipid phase, rather than to stay in the hydrophilic water phase. This interaction is apparently not affected by incubation with the buffer used for ultracentrifugation (0.01 M Hepes, 0.135 M NaCl, 1 mM EDTA, 0.05% Tween 80.) It should also be born in mind that the presence of lactose may affect the bilayer characteristics of these liposomes and thereby the extent of t-PA association with the liposomes [7].

Taking into account the negative charge of the liposome-bilayers used, one might expect at pH 4 electrostatic forces to enhance the interaction (e.g. [6,18,19]) between the lipid bilayer and the t-PA-molecule, as t-PA has a higher positive charge at this low pH than at pH 7.5 [17]. Nguyen and Ward report a pI range for t-PA between 6.5 and 8.5. At pH 7.4 by far not 100% of the t-PA molecules is positively charged. A substantial part is negatively charged. Still, 90% of t-PA associates with the negatively charged liposomes. As expected, ζ-potential measurements under low ionic strength conditions at pH 7.5 (0.01 M Hepes, pH 7.5, 7.5% (w/v) lactose, 1 mM EDTA, 0.05% Tween 80) did not show a pronounced difference between the ζ-potential of liposomes with and without associated t-PA (-43 and -40 mV, respectively). Clearly, interactions are not limited by neutralization of the PG-negative charges in the liposomes (Table 3).

Apparently, the average charge on the molecule is not a dominating parameter with respect to liposome - t-PA interactions. But this finding does not exclude the possibility that a local positive charge on parts of the molecule (e.g. on the kringles) might contribute to the interaction process between t-PA and the negatively charged bilayers. The observation that more t-PA is liposome associated upon lowering the ionic strength provides support for the role of electrostatic forces. But, other suggestions can also be made to explain this interaction enhancement when lowering the ionic strength. Lowering the ionic strength might increase intramolecular repulsion, exposing previously hidden, more hydrophobic parts of the t-PA molecule. This would also lead to an increased tendency to interact with the liposome bilayer.

The reduced t-PA entrapment at pH 4 may be partly ascribed to pH-induced conformational changes under acidic circumstances. Although the secondary structure of t-PA is known not to be affected by changing the pH from 7.5 to 4, the tertiary structure does change, probably by unfolding of the molecule [17]. This conformational change may reduce the hydrophobic interaction seen at pH 7.5, perhaps by exposure and concurrent protonation of certain basic amino acid residues that were masked in the native state, as was also described for myoglobin [20]. This mechanism, inducing a decrease in hydrophobicity for t-PA, also offers an explanation for the higher solubility of t-PA at pH 4. One might assume that the possibility of α-helix formation at pH 7.5 as postulated above was no longer possible under these conditions.

Furthermore, apart from pH-introduced conformational changes, the different solubility of t-PA forms may also have affected results. Alteplase (e.g. Actilyse) consists of a mixture of at least four proteins, differing in glycosylation pattern (type I or II) and/ or by being in the one- or two chain form [17]. In our analysis of the amounts of t-PA in several fractions we always measured t-PA by an activity analysis after extensive dilution of the sample in a standard buffer. Under the experimental conditions of the entrapment with high concentrations of t-PA it is, however, possible that solubility differences played a role. At pH 4 both type I and II t-PA dissolve equally well, but at pH 7.5 the poor solubility of type II, and the improvement of this solubility by arginine may have affected the results.

Our experiments do not give a clear mechanistic picture of the factor(s) that limit t-PA encapsulation efficiencies. Reducing the phospholipid incubation concentration to 30 µmol/ml or even lower introduced a lower entrapment percentage. The molar phospholipid incubation concentration at 30 µmol/ml still exceeded that of t-PA by a factor 10^3. Considering the surface area of the bilayer compared to the dimensions of the t-PA molecule, geometric restrictions because of full coverage of the lipid surface area by a t-PA monolayer are highly unlikely.

Increasing the incubation concentration of t-PA indeed increased the absolute t-PA entrapment. However, there was a tendency to a drop in entrapment percentage for the standard Hepes buffer (60 to 44%). In other words: it is possible (under the chosen conditions) to raise the t-PA concentration in the liposomes, but a certain reduction in entrapment percentage should be taken into account.

The influence of particle size of the liposomes on the entrapment percentage was found to be significant: after extrusion a value of only 48% was found, compared to 68% in (standard) unsized liposomes. A reduction in encapsulated aqueous volume might be partly responsible for this drop. Additionally, the t-PA-bilayer-interaction may be altered under these conditions. As the total bilayer area available for t-PA interaction does not depend on particle size, the interaction between t-PA and bilayer might have become less efficient as a consequence of the reduction in particle size. On the basis of our experiments, the possibility that t-PA loss occurs, because of the high shear forces the liposomes are exposed to during extrusion, can not be ruled out.

t-PA containing liposomes were ultracentrifuged after their formation to remove non-entrapped t-PA. A subsequent ultracentrifugation run was shown to cause a surprisingly big drop in t-PA content of the liposomes. Dilution induced loss of that part of liposome-associated t-PA that was merely adsorbed to the exterior lamellae of the liposomes could be given as an explanation for this observation. Stripping of the external bilayer by dilution was described before in the literature for several liposome-associated compounds (e.g. interleukin 2 [7]). However, in our study, upon ultracentrifuging t-PA liposome samples several times in a row, the drop in t-PA content exceeds the relative contribution of externally adsorbed t-PA

(Table 3) to the overall t-PA entrapment percentage (Table 1). The t-PA entrapment percentage showed a drop after each ultracentrifugation sequence (Figures 2a and 2b), far beyond the adsorption percentages found in Table 3. Moreover, t-PA loss upon repeated ultracentrifugation was not influenced by increasing the dilution-factor used: upon diluting the samples 6-fold more (200 instead of 33 times) prior to ultracentrifugation, no increase in t-PA loss was observed. Therefore, simple loss of adsorbed t-PA due to dilution was no longer a plausible explanation. Instead, we considered the occurrence of damage to the liposomal bilayer during ultracentrifugation (and/or during redipersion of the obtained liposome-pellet) as a major cause for leakage. Part of this effect may be the result of an enhanced lipid-permeability due to the presence of Tween 80 and / or interaction of t-PA with the bilayer. In the literature the liposome destabilizing effect of BSA due to penetration into the bilayer was described before [3, 7, 21]. Furthermore, the lipid composition is known to influence the interaction of liposomes with (serum-)proteins. Cholesterol and PC containing liposomes are considered relatively inert with respect to their protein interacting properties [22]. It was also concluded by Cevc et al. [23] that the efficiency of non-specific protein adsorption to lipid bilayers is a function of the number of defects in the bilayer. Therefore, changing the liposome bilayer composition can be expected to introduce different t-PA - liposome interactions and possibly also a different behaviour of t-PA containing liposomes upon repeated ultracentrifugation.
In conclusion: ultracentrifugation of the t-PA containing liposomes used in this study should be avoided since it damages the liposomes, leading to t-PA loss. As a consequence, the liposomal t-PA entrapment percentages measured in this study (all obtained following an ultracentrifugation step as a means of separating liposomally entrapped t-PA from free t-PA) are in fact underestimations of the true encapsulation percentages. As an alternative way of separating liposomal t-PA from non-entrapped t-PA the use of Sephadex gel filtration (PD-10 column) and Dowex cation exchange resin (as a column) was examined. However, substantial loss of about 70% of phospholipid to these columns occurred (data not shown). The tendency of t-PA to adsorb to various surfaces was probably responsible for this loss. Therefore, dialysis and ultrafiltration are probably no acceptable alternative. The use of Tween 80 can prevent adsorption of t-PA. However, as the presence of Tween 80 influences the liposome bilayer stability as well (see below), this could not solve our problem either. In the absence of any acceptable alternatives for the separation of liposome-entrapped t-PA from free t-PA, we decided to use the ultracentrifugation method in this study and consider the obtained data on t-PA entrapment as underestimates.
Tween 80, 0.05%, was generally included in the buffer solutions to prevent non-specific adsorption of t-PA and to assure a 100% yield of t-PA in the supernatant plus pellet. We suspected that its presence in the buffer solutions used for preparation and ultracentrifugation of t-PA-liposomes might contribute to the damaging ultracentrifugation-effect. Therefore, repeated sequential ultracentrifugation was also performed with t-PA liposomes which were

prepared and ultracentrifuged in the absence of Tween 80. A substantial, although reduced, damaging effect by ultracentrifugation was still observed (cf. Figures 2a and 2b).
We conclude from this study that the described standard-method, using unsized liposomes, PL concentration 100 μmol/ml, freeze-thawed in standard Hepes buffer, pH 7.5 results in substantial t-PA entrapment. However, a more quantitative entrapment percentage (90%) is obtained in Hepes buffer devoid of arginine, with low ionic strength, pH 7.5. It is important to avoid ultracentrifugation steps for removal of non-entrapped t-PA, since it damages the liposomes, leading to t-PA loss beyond stripping the outer bilayer from t-PA. In the absence of acceptable alternative separation techniques, ultracentrifugation was used during this study. Therefore, the encapsulation-percentage values shown in this study in fact underestimate the original entrapment of t-PA.
When developing a pharmaceutical t-PA-liposome formulation, one can decide to simply not remove the remaining 10% in the case of 90% t-PA liposome association. Furthermore, for optimal stability, Tween 80 should be excluded from the buffers, since it leads to enhanced t-PA leakage upon ultracentrifugation and does not improve entrapment efficiency under the chosen conditions. Finally, as aqueous t-PA-dispersions lack the required long term stability, freeze-drying procedures for the t-PA-liposomes are currently under investigation in our laboratory, to create a product with a pharmaceutical acceptable shelf life.

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Chapter 5

Long term stability of liposomes containing both enzyme and its substrate: the challenge to formulate stable liposomes containing both tissue-type Plasminogen Activator & glu-plasminogen

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Summary

In this study a procedure is described for preparation and storage of liposomes containing glu-plasminogen (Plg) -as a homing device- coupled to the outside of the liposome bilayer and physically entrapped tissue-type Plasminogen Activator (t-PA). Since in this concept both enzyme and substrate are introduced into one formulation, establishing preparation and storage conditions which prohibit plasmin-formation, loss of enzymatic activity and t-PA-leakage for these complex liposomes are of utmost importance.

During preparation of these liposomes no interaction between Plg and t-PA was observed under the conditions used (up to two hours). Plg-activation occurred only in the presence of fibrin fragments. Therefore, no special stability precautions had to be taken during the preparation process. Suitable preparation and freeze-drying conditions were examined for t-PA-liposomes first and subsequently applied to Plg-liposomes and Plg & t-PA containing liposomes. Freeze-drying of the liposomes in a Hepes buffer pH 7.5, containing 7.5% (w/v) lactose, resulted in maximal recovery of enzyme activity of both Plg and t-PA. Plg was also shown to retain its fibrin binding capacity upon freeze-drying. Furthermore, no loss of t-PA retention was induced by the freeze-drying procedure under the conditions used, provided that freeze-dried liposomes were not exposed to an extra ultracentrifugation step compared to the non-freeze-dried control liposomes. In conclusion, the described preparation and freeze-drying procedure is considered appropriate.

Introduction

For treatment of acute myocardial infarction aggressive thrombolytic therapy is used in Western societies [1]. However, during thrombolytic treatment systemic effects can occur, such as bleeding episodes, which can be life-threatening when located intracranially [2]. Moreover, thrombolytic treatment is associated with increased thrombin generation [3-7], which may favour reocclusion.

Liposomes have been shown to serve as carriers for drugs, thereby improving their efficacy and reducing their systemic toxicity [e.g. 8]. In a previous study we described the improvement of the therapeutic index obtained in thrombolytic treatment with tissue-type Plasminogen Activator (t-PA) upon entrapment of t-PA into liposomes (Chapter 6; [9]). In order to achieve site specific targeting, a homing device with affinity for the target-tissue can be coupled to the outside of the liposome. In our study, where we seek specific delivery of a thrombolytic agent (t-PA) at the site of a thrombus, we provide the liposomal external surface with glu-plasminogen (referred to as Plg), which has affinity for fibrin-containing thrombi (Chapter 2a; [10]).

Protection of liposomes and liposomal proteins from general chemical and physical degradation upon storage over prolonged periods of time is a prerequisite for acceptance of

pharmaceutical formulations. This issue has been addressed by several groups before [11-14]. The concept described above, combining liposomal entrapment of t-PA with coupling of the targeting device glu-plasminogen to the outside of the liposomes, creates the extra problem of introducing enzyme (t-PA) and substrate (Plg) in one formulation. As a consequence, plasmin-formation may take place in the liposome-formulation, during storage or possibly already during preparation, which might cause systemic side-effects upon administration in vivo. Therefore, providing stable conditions for both the preparation and storage of these complex liposomes was the challenge we dealt with in the present study.

First, conditions for preparation of the Plg & t-PA containing liposomes were selected. Then we looked for a suitable freeze-drying procedure for preservation of the liposomes. Lyophilization (freeze-drying) is considered a promising means of extending the shelf-life of liposomes, especially in the presence of cryoprotectants (e.g. Crowe et al. [15]). We first investigated conditions needed for suitable freeze-drying of t-PA-liposomes, which were then tested for the Plg-liposomes and ultimately for the liposome formulation containing both glu-plasminogen and t-PA. We aimed at complete recovery of the enzymatic activity of both t-PA and Plg, as well as maximal retention of t-PA within the liposomes upon freeze-drying.

Materials and methods

Glu-Plasminogen (Plg) was obtained from Pharmacia (Stockholm, Sweden, courtesy of Prof. L.O. Andersson). Recombinant t-PA (Actilyse) was obtained as a freeze-dried product from Boehringer Ingelheim GmbH (Frankfurt, West Germany).

Egg-phosphatidylcholine (egg-PC) was a gift from Lipoid KG (Ludwigshafen, Germany), cholesterol was obtained from Sigma (St. Louis, MO, USA); egg-phosphatidylglycerol (egg-PG) was a gift from Nattermann Phospholipid GmbH (Cologne, FRG).

Maleimido-4-(p-phenylbutyrate)-phosphatidylethanolamine (MPB-PE) was synthesized from Succinimidyl-4-(p-maleimidophenyl)butyrate (SMPB) (Pierce Chemical Co., Rockford, USA) and egg-phosphatidylethanolamine (egg-PE) (Lipid Products, Nutfield, UK) as described by Martin et al. [16].

Streptokinase (Kabikinase) was obtained from Kabi Pharmacia (Woerden, The Netherlands); the chromogenic plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide.2 HCl from Chromogenix (Mölndal, Sweden).

All other chemicals were of analytical quality and were used without additional purification.

Vesicle preparation

1) Preparation of Plg-liposomes

Before derivatizing, glu-plasminogen was extensively purified over a Sephadex G-25 column, in order to remove (small quantities of) EACA (Chapter 2b; [17]). Subsequently, the

procedure as previously described (Chapter 2a; [10]) was used. The coupling-reaction is based on the reaction between thiol groups, introduced into the protein, and maleimide groups of liposomes and is briefly outlined below.

Glu-plasminogen was derivatized by the method of Duncan and co-workers [18], using SATA (N-succinimidyl S-acetylthioacetate) for the introduction of thiol groups. The obtained acetylthioacetyl-glu-plasminogen was deacetylated with hydroxylamine-HCl, generating free thiol groups, available for coupling to liposomes.

Liposomes, consisting of egg-phosphatidylcholine, egg-phosphatidylglycerol, cholesterol and MPB-PE, molar ratio 39.5 : 4 : 16 : 0.5, were prepared by the "film-method" (first described by Bangham et al. [19]) in a 10 mM Hepes buffer pH 7.5 with 135 mM NaCl and 1 mM EDTA. Maleimido-4-(p-phenylbutyrate)-phosphatidylethanol-amine (MPB-PE), a maleimide-containing anchor molecule, was introduced in order to be able to couple the homing device to the outside of the liposomes. The liposomes were extruded through polycarbonate membrane filters with pores of 0.6 μm (once) and 0.2 μm (three times). After extrusion their average diameter was determined by dynamic light scattering with a Malvern 4700 system, Malvern Ltd, UK. For the liposomes in this study, the actual coupling was performed by incubating 0.5 - 1.5 mg/ml of deacetylated protein with liposomes, phospholipid concentration around 7 μmol/ml, at room temperature for 75 minutes. After stopping the reaction with N-ethylmaleimide, the liposomes were separated from free protein by two ultracentrifugation steps* (Beckman Instr. Inc., California, USA) for 45 minutes, at 80,000 x g at 4°C.

When Plg-liposomes as such (without t-PA entrapment) were to be freeze-dried, they were freeze-thawed in advance in the presence of cryoprotectant (lactose), lactose-lipid ratio about 25 (w/w), to obtain cryoprotection both inside and outside these liposomes.

2) Preparation of t-PA-liposomes

Small unilamellar vesicles consisting of egg-phosphatidylcholine, egg-phosphatidylglycerol and cholesterol in a molar ratio of 10 : 1 : 4 were prepared by the "film-method" [19]. The lipid film was redispersed in 10 mM Hepes buffer**, pH 7.5, after which t-PA was added. A t-PA incubation concentration of 2.0 x 10^5 IU/ml was used, corresponding with about 300 μg/ml; the phospholipid (PL) incubation concentration was 30 μmol/ml. The liposomes were subsequently freeze-thawed 5 times to increase the encapsulation capacity of the liposomes [20, 21]. Non-entrapped t-PA was removed by ultracentrifugation for 45 minutes, at 150,000 x g and 4°C. For ultracentrifugation liposome dispersions were diluted with a buffer consisting of 10 mM Hepes, 0.135 M NaCl, 1 mM EDTA, 0.05% Tween 80. By comparing the amount of t-PA after ultracentrifugation (= liposome-associated t-PA) with the amount of t-PA just after freeze-thawing, before ultracentrifugation (= total amount of incubated t-PA), the entrapped percentage of t-PA could be calculated: {t-PA after ultracentr. / t-PA before ultracentr.} x 100%.

** In order to establish suitable freeze-drying conditions, the buffer-composition was varied with respect to the concentration of lactose (for cryoprotection), arginine (for stabilization of t-PA) and Tween 80 (to prevent t-PA from adsorbing to surfaces). The osmolarity of the used buffer composition was in all cases about 300 mOsm/kg.

3) Preparation of Plg & t-PA containing liposomes
For the preparation of liposomes containing both glu-plasminogen and t-PA, first the Plg-coupling procedure as described above was performed. In order to mask unreacted anchor molecules and thereby preventing possible reaction of t-PA with anchor molecules upon incubation, 1 mg/ml cysteine was added to the buffer during the first ultracentrifugation step. Excess cysteine was removed in the second ultracentrifugation step. (*For comparison reasons 1 mg/ml of cysteine was also used during the first ultracentrifugation step for the Plg-liposomes). The obtained pellet of (Plg-)liposomes was subsequently resuspended in 10 mM Hepes buffer**, with a PL concentration of about 5 μmol/ml. Then the t-PA entrapment procedure by freeze-thawing was performed, as described before.

Freeze-drying experiments
For the freeze-drying experiments, samples of 0.25 ml of liposomes (either t-PA containing, Plg containing or Plg & t-PA containing) in a concentration of 30 μmol/ml PL for t-PA-liposomes and 5 μmol/ml PL for Plg-liposomes and Plg & t-PA liposomes, were frozen in vials (25 x 15 mm) in liquid nitrogen for 10 minutes. The vials were transferred to a precooled freeze-dryer (Leybold GT4 pilot production). Samples were dried under reduced pressure (p = 11-13 Pa) for 40 h with a plate temperature of -40°C and a condenser temperature of -60°C, after which they were stored at -20°C until use. Then each sample was rehydrated with distilled water adding the same quantity as lost during freeze-drying. Leaked protein was removed by ultracentrifugation for 45 minutes, at 150,000 x g and 4°C.
For measurement of the original amount of protein a sample was taken before starting the freeze-drying process (a). Further samples were taken after freeze-drying, before ultracentrifugation (b) and after freeze-drying plus ultracentrifugation (c). Recovery was calculated by $^{b}/_{a}$ x 100% (= [total entrapped + leaked t-PA after freeze-drying] / [total entrapped t-PA before freeze-drying] x 100%). Retention was calculated by $^{c}/_{b}$ x 100% (= [entrapped t-PA after freeze-drying] / [entrapped t-PA before freeze-drying] x 100%).
Choosing the proper control conditions: 1 ultracentrifugation step instead of 2 (cf. Table 3)
As described in Chapter 4 and in the Results section, ultracentrifugation of t-PA containing liposomes (= both t-PA-liposomes and t-PA & Plg containing liposomes) damages the liposomes, causing t-PA leakage. Ultracentrifugation steps are performed both after t-PA entrapment and after freeze-drying of t-PA containing liposomes. Therefore, the difference between freeze-dried and non-freeze-dried (control) t-PA containing liposomes would not

only be the presence or absence of the freeze-drying procedure, but also the presence of one extra ultracentrifugation step for the freeze-dried liposomes, causing extra liposomal damage plus t-PA leakage. In order to correct for this difference, the procedure for preparation plus freeze-drying of t-PA containing liposomes was also performed using only one instead of two ultracentrifugation steps (under buffer conditions that stood out favourably in the preceding freeze-drying experiments). To this end t-PA was entrapped into liposomes by freeze-thawing as usual, but without removing non-entrapped t-PA by ultracentrifugation. Part of these t-PA containing liposomes was frozen in liquid nitrogen and stored at -196°C (control), the other part was freeze-dried. After 3 days, the control liposomes were thawed. A sample was taken (a = total amount of t-PA). Then a regular ultracentrifugation step (45 minutes at 150,000 x g and 4°C) was performed to remove non-entrapped t-PA after which a second sample was taken (b = entrapped amount of t-PA upon freezing of the liposomes before ultracentrifugation). The freeze-dried liposomes were treated simultaneously: after 3 days they were rehydrated and a sample was taken (c = total amount of t-PA after freeze-drying). The freeze-dried liposomes were ultracentrifuged to remove non-entrapped plus leaked t-PA. Afterwards sample d was taken (d = amount of t-PA remaining after freeze-drying). The entrapment percentage was calculated by $^{b}/_{a}$ x 100% (= [entrapped t-PA after freezing] / [total entrapped plus non-entrapped t-PA after freezing] x 100%). The recovery of t-PA upon freeze-drying was calculated by $^{c}/_{a}$ x 100% (= [total entrapped plus non-entrapped plus leaked t-PA after freeze-drying] / [total entrapped plus non-entrapped t-PA after freezing] x 100%). The t-PA retention after freeze-drying was calculated by $^{d}/_{b}$ x 100% (= [entrapped t-PA after freeze-drying] / [entrapped t-PA after freezing] x 100%).

Measurement of the residual water content

The residual water content was determined with the Karl-Fisher method. A Mitsubishi moisturemeter model CA-05 (Tokyo, Japan) was used. After weighing of the empty vial plus cap, the sample was freeze-dried. Subsequently, air was allowed into the chamber and the vial with the freeze-dried cake inside was closed and weighed again. The cake was solubilized by injection af an adequate amount of titration solution through the rubber cap into the closed vial. An aliquot of this solution was taken and directly injected into the reaction cell. The water content was expressed as the measured mass percentage of water of the freeze-dried cake.

Measurement of glu-plasminogen activity

The activity of bound and unbound glu-plasminogen (free or liposomal) was determined by activation with streptokinase according to Friberger et al. [22]. The glu-plasminogen samples were incubated with a molar excess of streptokinase and the activity of the streptokinase-plasminogen complex was determined by the conversion of the synthetic substrate H-D-Val-

Leu-Lys-pNA.2 HCl, which was measured by monitoring the absorbance of the reaction-product pNA (para-nitro-aniline) at 405 nm.

Measurement of t-PA activity

The t-PA-activity in supernatant samples was determined in the presence of 0.05% w/v Tween 80 (reducing adsorption of t-PA to surfaces); when measuring liposomal t-PA, 1% w/v triton X-100 was used for destruction of the liposomes.

The activity of free (supernatant) or liposomal (pellet) t-PA was determined by assessing the amount of plasmin formed, following incubation with a molar excess of plasminogen, using the reaction as described by Verheyen et al. [23]. In short: the plasmin concentration is determined by measuring its ability to convert the synthetic substrate H-D-Val-Leu-Lys-pNA.2HCl. The absorbance of the reaction-product para-nitro-aniline at 405 nm is proportional to the concentration of t-PA in the sample.

Assessment of (loss of) Plg upon preparation of Plg & t-PA containing liposomes

During t-PA entrapment into Plg-containing liposomes, incubation of the enzyme t-PA with its substrate glu-plasminogen occurs, with as a possible consequence plasmin-formation in the liposome-formulation. To determine possible loss of Plg during the preparation of Plg & t-PA containing liposomes, samples of (free) Plg and t-PA, with a concentration ratio as used during preparation of Plg & t-PA containing liposomes, were incubated for 2 hours. PPACK (D-Phe-Pro-Arg-Chloromethylketone), in a final concentration of 10 μM, was added immediately after taking a sample from the Plg & t-PA containing incubation mixture. PPACK is a small peptide which inhibits t-PA in an irreversible way [24], thereby prohibiting continuation of the conversion of Plg to plasmin by t-PA. Moreover, PPACK also inhibits any activity of plasmin already present before sample-taking. Therefore, during the Plg determination, performed after hydrolysis of PPACK, only non-t-PA-converted Plg is measured.

Statistics

Statistics for more than two groups were evaluated by one-way ANOVA. P values < 0.05 were considered significant.

Results

Vesicle preparation

1) Plg-liposomes

After the coupling-procedure, the particle size of the Plg-liposomes was between 0.22 and 0.25 μm. The Plg-liposomes used for freeze-drying in this study had a Plg-coupling ratio

varying between 25 and 70 µg Plg per µmol PL. These values are in agreement with those found in our previous study (Chapter 2a; [10]). However, upon freeze-thawing Plg-liposomes in the presence of cryoprotectant (prior to the freeze-drying procedure) the coupling ratio decreased with about 20%. In control experiments it was shown that this effect could not be ascribed to a reduction in enzymatic activity of Plg: after freeze-thawing of either non-liposomal Plg, non-liposomal derivatized Plg (= acetylthioacetyl-glu-plasminogen) or liposomal Plg in the presence of 1% w/v triton X-100 (known to destroy the liposomal structure), the enzymatic activity of the protein was not reduced (data not shown). Therefore, we hypothesize that during freeze-thawing of the Plg-liposomes glu-plasminogen, originally coupled to the outside of the outer liposome-bilayer, is subjected to a relocation process. The liposome bilayer is probably disrupted upon freezing and thawing, causing part of the Plg to move away from the outer leaflet of the liposome. Since Plg measurement is performed with intact liposomes, Plg at that position (not present at the liposome outside) will not be detected.

2) t-PA-liposomes

The mean diameter of the freeze-thawed, t-PA containing liposomes was about 0.53 µm. Liposomal t-PA-entrapment was shown to be dependent upon the buffer-composition used (Table 1). The buffer-composition was (non-systematically) varied with respect to

Table 1 Liposomal t-PA entrapment as a function of the buffer medium used. t-PA incubation concentration 2.0×10^5 IU/ml, PL incubation concentration 30 µmol/ml. Average values ± standard deviations are shown.

Hepes buffer 10 mM, pH 7.5, 300 mOsm/kg, with:	% of t-PA entrapped into the liposomes
a) 0.21 M arginine, 0.05% (w/v) Tween 80	45 ± 4 (n=4)
b) 7.5% (w/v) lactose, 0.05% (w/v) Tween 80	64 ± 6 (n=9)
c) 7.5% (w/v) lactose	78 ± 15 (n=6)

1) the concentration of cryoprotectant, 2) the presence of arginine and 3) the presence of Tween 80, in order to find favourable freeze-drying conditions. Buffer a) contained 10 mM Hepes, 0.21 M arginine, 0.05% Tween 80, buffers b) and c) 10 mM Hepes, 7.5% (w/v) lactose, with or without 0.05% Tween 80, respectively. The osmolarity of all three buffers was about 300 mOsm/kg, their pH 7.5. t-PA entrapment in buffer a) was significantly lower than in buffers b) and c) ($p < 0.01$). The difference in t-PA-entrapment between buffers b) and c) was only just significant ($p < 0.05$ but > 0.01). In earlier independent experiments this apparent difference was shown not to be significant (cf. Chapter 4).

Note with respect to the use of ultracentrifugation for t-PA containing liposomes

For t-PA containing liposomes (= both t-PA-liposomes and Plg & t-PA containing liposomes), two ultracentrifugation steps were used: a) after t-PA-entrapment and b) after the freeze-drying procedure. The first was included to remove non-entrapped t-PA after preparation and the second to remove leaked t-PA after freeze-drying of the liposomes. However, in separate experiments ultracentrifugation steps were proven to damage t-PA containing liposomes, inducing t-PA leakage (cf. Chapter 4). Since alternative approaches to separate free t-PA from liposomal t-PA (gel filtration, dialysis, ion-exchange) caused substantial loss of lipid and protein, ultracentrifugation was used in this study. As a consequence, the values for t-PA-entrapment (after preparation of t-PA containing liposomes) and for t-PA retention (after freeze-drying of t-PA containing liposomes) obtained after one and two ultracentrifugation steps, respectively, in this study in fact underestimate the true values.

3) Plg & t-PA containing liposomes

Plg-liposomes as described above were used for t-PA entrapment. Upon freeze-thawing, the 20% decrease in Plg coupling ratio was observed (see above).

During t-PA entrapment into Plg-containing liposomes, interaction of the enzyme t-PA with its substrate glu-plasminogen occurs, with as a possible consequence plasmin-formation in the liposome-formulation. Freeze-drying is assumed to be an effective means of preventing this t-PA - Plg interaction on long term storage. But, during preparation of these liposomes, prior to freeze-drying, significant plasmin formation may already take place, requiring special precautionary measures. Measurements of the Plg concentration upon a 2 hour incubation of t-PA and Plg under similar conditions as used during liposome-formation did not display any Plg-activation whatsoever. It was shown that only in the presence of fibrin fragments (corresponding with a fibrinogen concentration of 1.2×10^{-9} M), significant amounts of Plg were converted into plasmin (Table 2).

Table 2 Decrease in glu-plasminogen (Plg) concentration in the presence of t-PA (due to Plg-activation by t-PA), in the absence and presence of fibrin fragments.
Free Plg and t-PA are incubated for 2 hours at pH 7.5, at room temperature, at a concentration ratio as used during preparation of Plg & t-PA containing liposomes (molar ratio of Plg : t-PA = 1 : 3). The concentration of fibrin fragments corresponds with a fibrinogen concentration of 1.2×10^{-9} M. Average values ± standard deviations are shown.

Plg - t-PA incubation time	Percentage of Plg left in the absence of fibrin	Percentage of Plg left in the presence of fibrin
0	100	100
120	99 ± 5 (n=12)	30 ± 4 (n=4)

Freeze-drying experiments

1) freeze-drying of t-PA-liposomes

t-PA-liposomes were freeze-dried in the presence of a Hepes buffer, pH 7.5, varying with respect to the concentration of cryoprotectant, arginine and Tween 80. Buffer a) contained 10 mM Hepes, 0.21 M arginine, 0.05% Tween 80, buffers b) and c) 10 mM Hepes, 7.5% (w/v) lactose, respectively with or without 0.05% Tween 80. The osmolarity of all three buffers was about 300 mOsm/kg. As shown in Table 3, an acceptable t-PA recovery was obtained after freeze-drying in all three buffers. Since we made use of an enzymatic activity-measurement, this implies that no t-PA activity was lost upon freeze-drying under the conditions used. It should be noted, however, that inter-experimental differences were substantial for buffers b) and c). In buffer c), devoid of Tween 80, the absolute values for the t-PA concentration were not reduced compared to buffers a) and b), indicating that the absence of Tween 80 in this buffer did not cause substantial loss of t-PA due to adsorption.
Not all t-PA remained liposome-associated during freeze-drying. The retention (percentage of t-PA still liposome-associated after freeze-drying) depended on the buffer composition used. The highest retention was obtained in buffer c), although large inter-experimental differences were measured ($p < 0.01$) (Table 3).

Table 3 Recovery and retention of liposomal t-PA upon freeze-drying in the presence of variable buffer compositions.
Freeze-dried concentrations: PL 30 µmol/ml, t-PA between 0.9 and 1.6 x 10^5 IU/ml.
Average values ± standard deviations are shown.

Hepes buffer 10 mM, pH 7.5, 300 mOsm/kg, with:	Recovery (%) upon freeze-drying	Retention (%) upon freeze-drying
a) 0.21 M arginine, 0.05% (w/v) Tween 80	98 ± 4	28 ± 5 (n=4)
b) 7.5% (w/v) lactose, 0.05% (w/v) Tween 80	102 ± 22	42 ± 3 (n=9)
c) 7.5% (w/v) lactose	93 ± 20	64 ± 13 (n=6)
c) using 1 instead of 2 ultracentrifugation steps	106 ± 7	96 ± 11 (n=4)

As described before (see materials and methods) the preparation plus freeze-drying procedure of t-PA containing liposomes in buffer c) was also performed using one instead of two ultracentrifugation steps. Therefore, control-t-PA-liposomes (non-freeze-dried) and freeze-dried t-PA-liposomes no longer differed with respect to the number of ultracentrifugation steps that were performed. Under those conditions the t-PA entrapment percentage was 78 ± 5 % (n=4), similar to the value found in Table 1. This implies that freezing of the t-PA-liposomes before ultracentrifugation does not influence the obtained entrapment-value. The recovery and retention of t-PA after freeze-drying and one ultracentrifugation step are shown

in Table 3 on the last line. The high recovery supports our earlier finding that freeze-drying does not impair the enzymatic activity of t-PA. The t-PA retention after freeze-drying, using this preparation and freeze-drying procedure with only one ultracentrifugation step, was 96 ± 11 %, significantly higher than those obtained in buffers a), b) and c) performed with two ultracentrifugation steps ($p < 0.01$). In other words: the t-PA entrapment for freeze-dried t-PA-liposomes using one ultracentrifugation step was similar to the entrapment-value that was obtained for control (non-freeze-dried) t-PA-liposomes using one ultracentrifugation step (75 ± 7% compared to 78 ± 5%, respectively). This implies that no t-PA-leakage occurred upon freeze-drying of t-PA containing liposomes under the chosen conditions. The low t-PA retention of about 63% (Table 3) upon freeze-drying of t-PA-liposomes in buffer c) using the preparation & freeze-drying procedure including two ultracentrifugation steps may therefore be ascribed to the damaging effect of the second ultracentrifugation step.

2) freeze-drying of Plg-liposomes

Considering the promising results obtained with t-PA liposomes in buffer c), Plg-liposomes were also freeze-dried in the presence of buffer c), containing 10 mM Hepes, 7.5% w/v lactose and no arginine or Tween 80. Since the liposomes were freeze-thawed with this buffer prior to freeze-drying (see Materials and methods), lactose was present both at the in- and outside the liposomes. The recovery of Plg-activity after freeze-drying under these circumstances was 98 ± 3 % (n=8) (compared to freeze-thawed and frozen, but not freeze-dried Plg-liposomes), implying that the freeze-drying procedure under the used conditions was not harmful to the enzymatic activity of liposomal glu-plasminogen. The retention of Plg after freeze-drying was 94 ± 8 % (n=4). In other words: no significant Plg-leakage occurred, which was expected since Plg is not liposome-entrapped like t-PA, but instead, covalently bound to the liposome bilayer. For the same reason, the introduction of a second ultracentrifugation step after freeze-drying was no problem with respect to the Plg-liposomes. The fibrin binding capacity of freeze-dried liposomal Plg was measured in vitro, using a fibrin monolayer (Chapter 2a; [10]). It was shown that the binding behaviour of liposomal Plg (which was improved over that of non-liposomal Plg) was not impaired by freeze-drying: the binding curve of freshly made Plg-liposomes was closely similar to that of identical liposomes which went through a freeze-drying procedure (data not shown).

3) freeze-drying of Plg & t-PA containing liposomes

Liposomes containing both Plg and t-PA were prepared and freeze-dried in buffer c), containing 7.5% w/v lactose and no arginine or Tween 80, using only one ultracentrifugation step (as described in Materials and methods). Therefore, control-Plg-t-PA-liposomes (non-freeze-dried) and freeze-dried Plg-t-PA-liposomes were comparable with respect to the fact

that they both underwent one ultracentrifugation step. The recovery and retention of t-PA and Plg after freeze-drying Plg & t-PA containing liposomes are shown in Table 4.
Apparently, the presence of both t-PA and Plg in one liposome formulation did not influence the behaviour of the liposomal proteins upon freeze-drying: after freeze-drying of t-PA-Plg liposomes the recovery and retention values were similar to those obtained after freeze-drying of liposomes with only t-PA (cf. Tables 3 and 4) or only Plg. However, the value for t-PA retention as shown in Table 4 should be interpreted with caution. Since Plg & t-PA containing liposomes showed a low t-PA entrapment (low PL concentration), most of the t-PA was non-entrapped when the freeze-drying process was initiated.

Table 4 Recovery and retention of liposomal t-PA and glu-plasminogen (Plg) upon freeze-drying t-PA-Plg-liposomes.
Freeze-dried concentrations: PL 4 μmol/ml, t-PA 2 x 10^5 IU/ml, Plg between 1.5 and 3.0 x 10^2 μg/ml. Buffer medium: 10 mM Hepes buffer, 7.5% (w/v) lactose, pH 7.5. Average values ± standard deviations are shown.

	Recovery (%)	Retention (%)
t-PA	97 ± 5 (n = 6)	102 ± 10 (n=6)
Plg	99 ± 5 (n=12)	96 ± 11 (n=9)

Residual water content upon freeze-drying
Upon freeze-drying the liposomes in Hepes buffer c) as described above, the obtained cakes had a porous, light appearance and were easy to rehydrate. An average residual water content of 2.8 ± 0.4 % (w/w) was measured irrespective of the freeze-dried PL concentration.

Discussion

The concept of combining liposomal entrapment of the fibrinolytic t-PA with covalent coupling of the targeting device glu-plasminogen to the outside of the liposomes generates the problem of introducing both enzyme and substrate in one formulation. Therefore, plasmin-formation may take place in the liposome-formulation, possibly already during preparation. In this study we first tried to stabilize all constituents during preparation (short term stability) of these liposomes. It is known from literature that the presence of fibrin as a third component in the ternary complex of t-PA, Plg and fibrin is needed for optimal activation of Plg [25]. However, also several denatured proteins can be active in that respect [26]. Under our experimental conditions the reaction between t-PA and Plg was found to be very slow: only upon addition of fibrin fragments [27] significant Plg-conversion was measured (Table 2). Therefore, no specific (inhibiting-)precautions had to be taken.

For the preparation of liposomes containing both Plg and t-PA we were confronted with the conflicting 'optimal' conditions for Plg-coupling and t-PA entrapment. Whereas PL concentrations < 10 μmol/ml are used for obtaining high liposomal Plg surface-densities (Chapter 2a), t-PA entrapment is best performed at 100 μmol/ml PL (Chapter 4). In this exploratory study only Plg-t-PA-liposomes with high Plg surface-densities and low t-PA entrapment percentages were used. In future experiments liposomes with both a high Plg surface-density and a high t-PA entrapment will be prepared (and freeze-dried) as well.
Our second aim in this study was to provide stable, long term storage-conditions for the Plg & t-PA containing liposomes. Lyophilization (freeze-drying) in the presence of cryoprotectants is considered a promising means of extending the shelf-life of liposomes (e.g. [14, 15, 28, 29]). We first investigated conditions needed for suitable freeze-drying of t-PA-liposomes, which were then tested for the Plg-liposomes and ultimately for the liposome formulation containing both glu-plasminogen and t-PA. Only a number of freeze-drying conditions were explored, since systematic variation of all variables (including buffer conditions) would be too laborious. Buffer-conditions were varied with respect to the presence of lactose, arginine and Tween 80. In this study, lactose was selected from a number of possible cryoprotectants, because of its pharmaceutical acceptability and favourable protective properties (e.g. [30]). Arginine was used for its known contribution to stabilize t-PA on storage: it increases t-PA solubility and prevents self-association, heat denaturation and cleavage [31]. The rationale for including Tween 80 was its ability to prevent loss of t-PA by adsorption. Different buffer compositions resulted in differences in the entrapped percentage of t-PA (Table 1). Furthermore, the retention of t-PA after freeze-drying was also dependent on the buffer composition used (Table 3). For maximal t-PA entrapment plus retention, buffer c), composed of 10 mM Hepes, 7.5% lactose, without Tween 80, was shown to be superior over buffers a) and b). Under the chosen circumstances, the lactose-lipid ratio was 3 (on a weight to weight basis). However, the obtained retention of about 63% in buffer c) still indicated considerable loss of t-PA upon freeze-drying. Since ultracentrifugation is known to damage t-PA containing liposomes (= both t-PA-liposomes and Plg & t-PA containing liposomes), causing t-PA leakage (Chapter 4), it was interesting to compare the preceding results with a freeze-drying procedure performed in the absence of the extra ultracentrifugation step (see Materials and methods section). We were able to show that in buffer c) the t-PA entrapment percentage for freeze-dried t-PA-liposomes was similar to the entrapment-value that was obtained for control (non-freeze-dried) t-PA-liposomes upon using just one ultracentrifugation step: t-PA retention upon freeze-drying was about 100%. The low t-PA retention of about 63% (Table 3) upon freeze-drying of t-PA-liposomes in buffer c) using the preparation & freeze-drying procedure including two ultracentrifugation steps may therefore be ascribed to the damaging effect of the second ultracentrifugation step. Alternatively, the presence of non-entrapped t-PA might reduce the leakage tendency.

Freeze-drying of Plg-liposomes in buffer c) showed that the enzymatic activity of Plg and its liposomal retention were not reduced by the freeze-drying procedure. The covalent Plg - liposome association was not impaired by a (second) ultracentrifugation step, in contrast to t-PA. Liposomal Plg was shown to have intact fibrin binding properties upon freeze-drying, as shown in an in vitro fibrin monolayer model. Therefore, freeze-drying was not harmful to the targeting capacity of the homing device of this liposome formulation. It should be noted that the lactose-lipid ratio for Plg-liposomes and also for Plg & t-PA containing liposomes was higher than for t-PA-liposomes, namely about 25 (weight to weight basis). This difference was due to the fact that Plg-liposomes were prepared at lower PL concentrations than t-PA-liposomes, while the same lactose-containing buffer was used for all liposomes.

Freeze-drying of Plg & t-PA containing liposomes was also performed in buffer c). Since these t-PA containing liposomes are sensitive to ultracentrifugation damage, the freeze-drying procedure with only one ultracentrifugation step for both freeze-dried and control liposomes was used. Under these conditions maximal recovery and retention values were obtained for both t-PA and Plg (Table 4). Therefore, the presence of both proteins in one formulation clearly did not interfere with the freeze-drying results obtained with liposomes containing only Plg or only t-PA.

Stable (non-collapsing) cakes were obtained with the described freeze-drying procedure, containing about 3% of residual water.

In conclusion: the described preparation and freeze-drying procedure provides stable conditions for complex liposomes containing both t-PA and Plg in one formulation. No plasmin-formation takes place, complete enzymatic recovery of both proteins is obtained, liposomal Plg retains its fibrin binding capacity, and no leakage of t-PA is induced, provided that no extra ultracentrifugation step is introduced after freeze-drying.

Final remarks

The freeze-drying procedure as described in the materials and methods section was used throughout this study: no efforts to optimize the chosen freeze-drying procedure were undertaken. A secondary drying phase will reduce the residual water content below the value of 3% that was obtained in this study. It may improve the long-term stability of the cake (e.g. avoidance of collapse) of the liposomal formulation, but removal of strongly protein-bound water may on the other hand cause conformational changes in t-PA and / or Plg. Although no extensive optimization steps were undertaken, the promising results in this study form an interesting starting-point for further improvement of the liposomal product, e.g. aimed at quantitative liposomal entrapment of therapeutically relevant doses of t-PA. The present explorative study did not report on the 'real life' of the freeze-dried liposomal product. These long-term stability experiments will be performed in the near future as well.

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Chapter 6

Thrombolytic treatment with tissue-type Plasminogen Activator (t-PA) containing liposomes in rabbits: a comparison with free t-PA

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Summary

In this study, we aimed at improving the therapeutic index of tissue-type Plasminogen Activator (t-PA) as thrombolytic agent in the treatment of myocardial infarction. Liposome-encapsulated t-PA was tested in a rabbit jugular vein thrombosis model: administration of free t-PA (t-PA) as a bolus injection in the ear vein was compared to a similar administration of liposomal t-PA (t-PA-lip), liposomal t-PA in glu-plasminogen-coated liposomes (Plg-t-PA-lip), a mixture of free t-PA and empty liposomes (t-PA+ empty lip) and a saline-blank (blank) in terms of thrombolytic activity and side-effects.

Liposomal t-PA (t-PA-lip / Plg-t-PA-lip) showed a significantly better thrombolysis efficiency than equimolar doses of free t-PA (t-PA / t-PA+ empty lip): about 0.24 mg/kg of liposomal t-PA practically equalled the lysis-activity of a dose of free t-PA of 1.0 mg/kg (t-PA$^{1mg/kg}$). On the other hand, liposome encapsulation did not affect the systemic activation of alpha-2-antiplasmin and plasminogen by t-PA.

We conclude that for this model an improvement in thrombolytic efficacy of t-PA is achieved by liposome encapsulation of t-PA. As t-PA-lip and Plg-t-PA-lip-treatment induced similar results, targeting of liposomal t-PA by coupled glu-plasminogen remains a topic to be optimized in future studies.

Introduction

Thrombolytic therapy is generally used for treatment of acute myocardial infarction in Western cultures [1]. An important drawback of thrombolytic treatment is the occurrence of systemic effects, which can either lead to bleeding episodes [2], or to increased thrombin generation in blood, with reocclusion as a possible consequence [3-7].

Tissue-type Plasminogen Activator (t-PA) is known as a fibrin-specific thrombolytic agent under physiological circumstances. However, for thrombolytic treatment of an acute myocardial infarction high dosages of t-PA are required and the occurrence of systemic side-effects cannot be excluded [1, 8]. Furthermore, t-PA has a short plasma half life of 3-5 minutes in humans, which may favour reocclusion [6, 9]. To circumvent these problems, literature reports on administration of t-PA over an extended period of time [9] and on recombinant plasminogen activators with prolonged half lives (e.g. [10]).

In this study a new concept is tested, aimed to improve the therapeutic index of t-PA. The concept comprises liposomal encapsulation of t-PA combined with coupling of a targeting device to the outside of the liposomes [11]. Liposomal encapsulation of t-PA separates t-PA from the blood, which is expected to reduce systemic activity and to prevent inactivation of t-PA by circulating inhibitors. The homing device at the outside of the t-PA-containing liposomes will guide the liposomes to fibrin deposits, which increases the amount of t-PA actually reaching the thrombus compared to non-targeted delivery.

In order to create these complex liposomes various technologies need to be worked out and

eventually combined. We separately developed technologies for coupling a homing device to the external surface of liposomes and for encapsulation of t-PA into the liposomes.
Concerning the first technology, we developed a covalent binding procedure for glu-plasminogen (referred to as Plg) to the surface of liposomes (Chapter 2a; [12]). It was possible to introduce various densities of glu-plasminogen on the liposomes and the in vitro fibrin binding of liposomal Plg was found to be significantly increased compared to free Plg ([11], Chapter 2a; [12]).
The technology of encapsulation of t-PA was successful for liposomes without coupled glu-plasminogen and resulted in stable liposomes on storage with encapsulation efficiencies up until 90% (Chapter 4). The encapsulation of t-PA into glu-plasminogen-bearing liposomes was less efficient, and at the time of performing the experiments described in this chapter only low surface-densities of coupled glu-plasminogen were achieved when combining the two technologies. Further development is required to solve this problem (cf. Chapter 5).
In the present study we looked for an answer to the following questions:
a) What is the effect of encapsulation of t-PA into liposomes on its clearance rate in animals: can liposomal encapsulation of t-PA prolong its circulation time?
b) Do these t-PA containing liposome formulations still exert a lytic effect in vivo?
c) Does the encapsulation of t-PA into liposomes change the therapeutic index of lysis versus systemic effects compared to free t-PA?
In order to obtain a first indicative answer to these basic questions we tested a number of liposome types available at that point of time (non-optimized). These included t-PA containing liposomes without targeting device (t-PA-lip) and liposomes with a low surface density of targeting device (Plg-t-PA-lip). As a control also free t-PA mixed with empty liposomes was tested. All liposomes were made with a composition of lipids that was expected not to be highly stable under in vivo conditions: leakage of the contents during circulation in the blood is likely.
The thrombolytic efficacy and the systemic side-effects of the thrombolytic mixtures were tested and compared to the effects observed after administration of free t-PA in rabbits, utilizing the jugular-vein-thrombosis model as described by Collen et al. [13] employing bolus injection through an ear vein.

Materials and methods

Recombinant t-PA (Actilyse) was obtained as a freeze-dried product from Boehringer Ingelheim GmbH (Frankfurt, West Germany). Glu-Plasminogen (Plg) was obtained from Pharmacia (Stockholm, Sweden, courtesy of Prof. L.O. Andersson).
Egg-phosphatidylcholine (egg-PC) was a gift from Lipoid KG (Ludwigshafen, Germany), cholesterol was obtained from Sigma (St. Louis, MO, USA) ; egg-phosphatidylglycerol

(egg-PG) was a gift from Nattermann Phospholipid GmbH (Cologne, FRG). [1α, 2α (n)-^{3}H]-Cholesteryl oleoyl ether was obtained from Amersham (Buckinghamshire, UK).
Maleimido-4-(p-phenylbutyrate)-phosphatidylethanolamine (MPB-PE) was synthesized from succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) (Pierce Chemical Co., Rockford, USA) and egg-phosphatidylethanolamine (egg-PE) (Lipid Products, Nutfield, UK) as described by Martin et al. [14].
Bovine thrombin was obtained from Leo Pharmaceuticals (Ballerup, Denmark); streptokinase (Kabikinase) from Kabi Pharmacia (Woerden, The Netherlands); the chromogenic plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide.2 HCl from Chromogenix (Mölndal, Sweden).
^{125}I-Fibrinogen (human) was purchased from Amersham (Houten, The Netherlands); D-phenyl-alanyl-prolyl-arginyl-chloromethylketone (PPACK) from Bachem, (Bubendorf, Switzerland). Nembutal: sodium pentobarbital 60 mg/ml, and Vetranquil: acepromazine maleate 10 mg/ml, were obtained from Sanofi (Paris, France); Nimatek: ketamine.HCl 100 mg/ml from A.U.V. (Cuyk, The Netherlands).
All other chemicals were of analytical quality and were used without prior purification.

Preparation of t-PA-containing liposomes with or without homing device
1) Preparation of (Plg-)liposomes
Small unilamellar vesicles consisting of egg-phosphatidylcholine, egg-phosphatidyl-glycerol, cholesterol and MPB-PE in a molar ratio of 39.5 : 4 : 16 : 0.5 were prepared. Maleimido-4-(p-phenylbutyrate)-phosphatidylethanolamine (MPB-PE), a maleimide-containing anchor molecule, was introduced in order to be able to couple the homing device to the outside of the liposomes (Chapter 2a; [12]).
For an optimal comparison of the effect of Plg-attachment to liposomes on t-PA-activity, liposomes with and without homing device (groups Plg-t-PA-lip and t-PA-lip) both contained the anchor-molecule in the bilayer. Full details of the coupling procedure and analytical assays are described in our previous publication (Chapter 2a; [12]). Basically, the "film-method" (first performed by Bangham et al. [15]) was used. Twenty ml of a liposomes dispersion, phospholipid (PL) concentration 60 μmol/ml, was prepared in 10 mM Hepes buffer pH 7.5, containing 135 mM NaCl and 1 mM EDTA. The liposomes were extruded through polycarbonate membrane filters with pores of 0.6 μm (once) and 0.2 μm (three times); after extrusion their average diameter -as determined by dynamic light scattering with a Malvern 4700 system, Malvern Ltd, UK- ranged between 0.23 and 0.25 μm. The liposomes were then incubated with (or without) derivatized glu-plasminogen in Hepes buffer (incubation concentration about 0.4 mg Plg/ml), after which they were pelleted by ultracentrifugation (Beckman Instr. Inc., California, USA) for 60 minutes, at 200,000 g and 4°C (for removal of unbound homing device) as described (Chapter 2a; [12]).*

2) Encapsulation of t-PA

The obtained pellet of (Plg-)liposomes was subsequently resuspended in a buffer containing 0.033 M citric acid, 0.135 M NaCl, 0.05% Tween 80, pH 4. By means of this ultracentrifugation step the liposomes were concentrated to about 140 μmol/ml phospholipid (volume ± 8 ml). Varying amounts, up to 2 ml, of a t-PA-solution consisting of 1.95 x 10^6 IU/ml (± 2.9 mg/ml) t-PA in citrate-buffer (as described above), were added. The liposomes were subsequently freeze-thawed 5 times to encapsulate t-PA as efficiently as possible [e.g. 16, 17]. In separate experiments we found that the execution of more than 5 freeze-thawing steps did not improve the encapsulation (data not shown). Non-encapsulated t-PA was removed by ultracentrifugation, again for 60 minutes at 200,000 g and at 4°C. As free t-PA (in contrast to liposomal t-PA) has the propensity to adsorb to surfaces, 0.05% Tween 80 was included in the buffer during ultracentrifugation to prevent loss of non-encapsulated t-PA from the supernatant. The mean diameter of the liposomes after freeze-thawing was about 0.53 μm.

The liposomes were then resuspended in about 4 ml of Hepes buffer. The pH was adjusted to 7.4 prior to injection into the animal. In order to keep t-PA in solution during this pH increase, arginine was added (in advance) up to a total concentration of 0.2 M. To minimize the amount of free t-PA in the t-PA-liposome formulation, t-PA-encapsulation (plus ultracentrifugation step) was performed just before the injection. Therefore, the t-PA encapsulation efficiency (and consequently the exact administered dose) could only be measured retrospectively.

*For the t-PA+ empty lip-group, liposomes were redispersed in Hepes buffer (+ 0.2M arginine) immediately after the first ultracentrifugation step, leaving out the t-PA-encapsulation step (freeze-thawing under citric acid conditions). Free t-PA was added and mixed with the empty liposomes 30 min prior to injection.

Kinetics of t-PA-containing liposomes in healthy rabbits

The disappearance kinetics of t-PA-containing liposomes from the plasma were studied in healthy rabbits: without an artificial thrombus. t-PA-containing liposomes (t-PA-lip) were prepared as described above, except that ^{125}I trace-labeled t-PA (Iodogen-method) was encapsulated this time. In a second experiment we also labeled the lipid bilayer: with ^{3}H-labeled cholesteryl oleoyl ether (representing a negligible fraction on a lipid-basis). Labeled t-PA-containing liposomes, the volume and t-PA / PL concentrations as described above, were administered as a bolus injection. Blood samples were obtained from the femoral artery cannula, the first one taken at t=1', then samples were taken every 5 minutes up until 30', every 15 minutes up until 2h, and every 30 minutes up until 3.5h. Plasma t-PA concentrations were determined by radio-activity-measurements. The kinetic data were analyzed using a two-compartment mammalian model with peripheral elimination. The t-

PA distribution between a central plasma compartment and a peripheral liver compartment was estimated according to Gibaldi and Perrier [18]. This model was chosen since the predominant role of the liver in the clearance of t-PA from the blood was demonstrated in animals [19, 20].

Phospholipid-determination

Phospholipid concentrations were assessed (after perchloric acid destruction) by a phosphate-assay according to Fiske-Subbarow [21].

Measurement of liposomal t-PA

t-PA concentrations were assessed by measurement of the clot-lysis-time [22]. When measuring liposomal t-PA, 4% w/v Tween 80 was used for destruction of the liposomes.
Briefly: the sample to be measured was incubated with 4% Tween 80, then human fibrinogen, plasminogen and thrombin were added. After mixing, the sample was placed in a 37°C waterbath and the time until complete lysis of the clot was measured. Calibration-curves of free t-PA (showing a linear relationship between log t-PA conc. and log lysis time) were used to calculate the concentration of t-PA in the sample from the measured clot-lysis-time. Control-experiments showed the absence of a significant influence by 4% w/v Tween 80 on the clot-lysis-time of free t-PA in the calibration-curves (data not shown).

Measurement of liposomal glu-plasminogen

The enzymatic activity of liposomal glu-plasminogen was determined by activation with streptokinase according to Friberger et al. [23]. The Plg samples were incubated with a molar excess of streptokinase and the activity of the streptokinase-Plg complex was determined by conversion of the synthetic substrate H-D-Val-Leu-Lys-pNA.2 HCl, which was measured by monitoring the absorbance of the reaction-product pNA (para-nitro-aniline) at 405 nm.

Thrombosis model

The thrombosis procedure as described by Collen et al. [13] was used with some minor adjustments [24].
New Zealand White rabbits, 5 to 6 months old (2-3 kg body weight), were sedated with 0.9 ml Vetalar plus 0.1 ml Vetranquil; injected i.m., followed by induction of anaesthesia with slow i.v. administration of Nembutal, 1:1 diluted in sterile saline. Anaesthesia was maintained by i.v. Nembutal. A femoral artery cannula was introduced for blood sampling [24].
A thrombus was formed in the external jugular vein as described by Collen et al. [13]. In short: a labeled fibrin-clot was made in an isolated part of the jugular vein by introducing a

woollen thread, thrombin and a ^{125}I-labeled fibrinogen-blood-mixture (incubation for 30 min). The blood-flow through the isolated part was restored and 30 min later the thrombolytic agent was injected as a bolus into the contralateral ear-vein. Blood-samples were taken every 30 minutes. After 120 min the animal was sacrificed by an overdose of the narcotic and the (remaining) thrombus was removed [24].
The extent of thrombolysis and isotope recovery were calculated as described by Collen et al. [13]. Systemic side-effects of t-PA were monitored by following the changes in concentration of plasminogen and alpha$_2$-antiplasmin in the blood samples.

Thrombolytic treatment
A bolus injection into the ear vein of 0.20 mg/kg or 1.0 mg/kg of free t-PA was compared to about 0.25 mg/kg t-PA encapsulated in liposomes (t-PA-lip) and 0.22 mg/kg t-PA (average amounts) encapsulated in Plg-bearing liposomes (t-PA-Plg-lip). Inter-experimental differences in encapsulation-efficiency caused some variation in the injected amount of t-PA in these groups. As controls also saline (blank) and 0.20 mg/kg free t-PA mixed with empty liposomes (t-PA + empty lip) were tested.
Free t-PA was administered in saline + 0.2 M arginine, the liposomal formulations in Hepes-buffer + 0.2 M arginine. In each experiment a total volume of about 4 ml solution or dispersion was injected.

Plasma assays
For the determination of systemic effects blood samples (± 0.5 ml) were taken every 30 min. They were anticoagulated with sodium citrate (final concentration: 13 mM). Then PPACK [25] was added to a final concentration of 2 μM and the mixture was kept on ice for 30 min. Platelet-poor plasma was prepared (2000 x g, 10 min, 4°C) and stored at -30°C.
Plasminogen and α_2-antiplasmin concentrations were determined spectrophotometrically according to procedures described before [26, 27].

Statistics
Clot-lysis results: the statistical significance of differences between the lysis-behaviour of the six thrombolytic groups was evaluated by one-way ANOVA. P values < 0.05 were considered significant. Two 'a priori' hypotheses were examined by t-tests; differences were considered significant in case P values < 0.025 were obtained.
Systemic effects: repeated measurements-ANOVA was performed to evaluate the differences between the systemic effects of the six thrombolytic groups. P values < 0.05 were considered to be significant. Two 'a priori' hypotheses were examined by t-tests. Differences were considered significant in case P values < 0.025 were obtained.

Results

Coupling efficiency of glu-plasminogen

In the Plg-t-PA-lip-group (liposomal t-PA in Plg-bearing liposomes), the coupled amount of Plg was within the range of 1.5 to 2.2 μg per μmol PL, corresponding with an average coupling efficiency of about 16% (data not shown).

When comparing these results with our previous experiences concerning the coupling of Plg to liposomes (Chapter 2a; [12]), (reporting coupled amounts of 20-94 μg Plg per μmol PL) it is important to notice that the applied incubation ratio s of Plg : PL are significantly different, causing corresponding differences in the coupled amount of Plg per PL. Since the in vivo experiments require relatively high concentrations of phospholipid as a carrier to administer appropriate doses of t-PA, we were -for the time being- obliged to use a (± tenfold) lower incubation ratio of Plg to PL.

Encapsulation efficiency of t-PA

The freeze and thaw t-PA-encapsulation procedure for groups t-PA-lip and Plg-t-PA-lip as described above lead to an average encapsulation efficiency of about 17%, corresponding with about 9 x 10^2 IU t-PA per μmol PL. However, it has become clear from later in vitro optimization studies that an encapsulation efficiency of about 90% can be obtained when selecting optimal encapsulation conditions (Chapter 4).

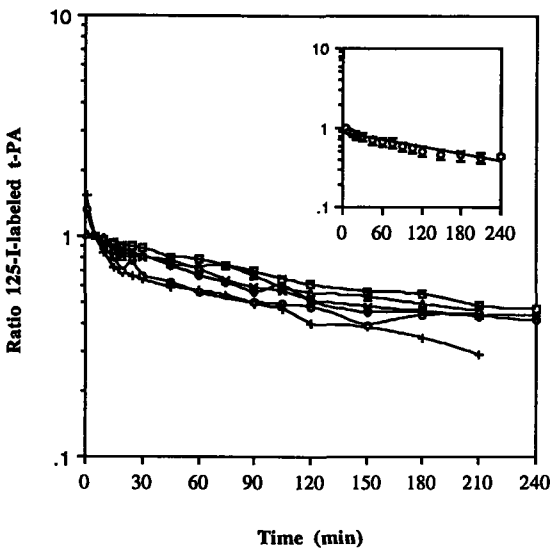
Kinetics of t-PA-containing liposomes in healthy rabbits (t-PA-lip)

The disappearance rate of liposomal t-PA from plasma was studied by injecting liposomes with ^{125}I-labeled t-PA as a bolus in the ear vein of healthy rabbits (n=3). In a second experiment a double-labeling technique was used: apart from using the ^{125}I-labeled t-PA, we labeled the liposome-bilayer with ^{3}H-cholesteryl oleoyl ether (n=3). Radio-activity of blood samples was observed at regular intervals.

For the double-labeled liposomes we also measured the ratio of ^{125}I-labeled t-PA over ^{3}H-labeled lipid. A significant reduction in this ratio was found initially after in vivo administration: the ratio was 1.1 in the injected liposome-mixture, compared to about 0.5 at t=1' in the first blood sample (data not shown). This observation suggests a rapid clearance of a considerable part of the liposome-associated t-PA upon in vivo administration. Throughout the experiment the ratio remained almost unchanged: a small decrease to about 0.43 at t=210' was seen. Assuming the recovery of ^{3}H-labeled lipid at t=1' to be 80 - 100% (lipid vesicles will not extravasate nor be substantially cleared within one minute after injection), the corresponding recovery at t=1' of ^{125}I-labeled t-PA is about 45% (n=3). Even if the assumption of the high lipid recovery at t=1' would prove to be incorrect, the more pronounced fast clearance of t-PA over that of lipid indicates loss of t-PA from the liposomes upon injection.

Liposomal ^{125}I-labeled t-PA showed a biphasic pattern in this model with a very short half-life for the alpha-phase (< 4 min) and a beta-phase half-life of about 160 min (n=6) (Figure 1). Figure 2 shows the very similar behaviour of the ^{3}H-label of the liposome-bilayer: the obtained alpha-and beta half life are < 4 min and about 180 min, respectively.

1



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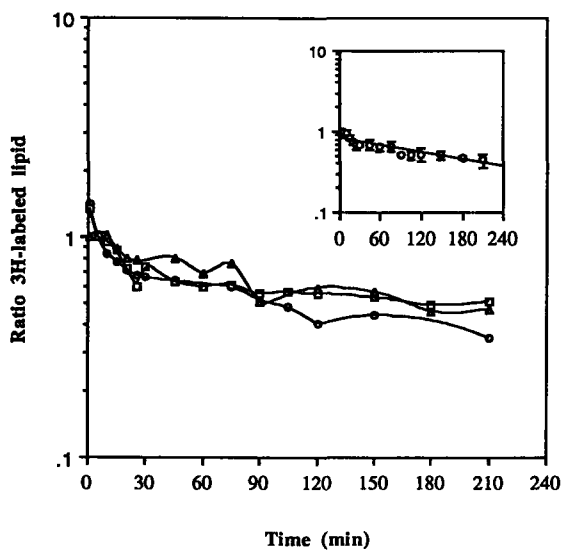


Figure 1, 2 Kinetics of i.v. administered t-PA-liposomes in 6 healthy rabbits.
The decrease of (1) ^{125}I-labeled t-PA, plotted as the ratio of ^{125}I-label concentration at t=x to that at t=5', and (2) ^{3}H-labeled lipid, plotted as the ratio of ^{3}H-label concentration at t=x to that at t=5', as a function of time. The inserted figures represent the calculated curves for the concerned ratios, based on linear regression analysis of the time-averaged data points (shown with standard deviations).

Free t-PA is known to show a single phase log-linear decline with an apparent half-life in this rabbit model of approximately 2 min [19]. Apparently, in our liposomal t-PA formulation there is an instantaneous loss of over 50% of liposome-associated t-PA. However, the clearance behaviour of the remaining 45% of t-PA is very similar to the lipid bilayer, indicating that this part remains liposome-associated in vivo, and probably adopts the clearance behaviour of the liposomes used [28].

Lysis

The lysis behaviour of different delivery protocols of t-PA was studied in a rabbit-jugular-vein-thrombosis model. The effects of free t-PA (t-PA) were compared with the effects of liposomal t-PA (t-PA-lip), liposomal t-PA in Plg-bearing liposomes (Plg-t-PA-lip), a mixture of free t-PA and empty liposomes (t-PA+ empty lip) and a saline-blank (blank). A standard dose of t-PA of around 0.2 mg/kg was used, except for the high dose-group of free t-PA, therefore marked by the superscript $^{1mg/kg}$.

Lysis-efficiencies for all six thrombolytic groups are shown in Table 1. One-way ANOVA proved the presence of significant differences between groups. Two a priori hypotheses were tested.

In a first t-test we looked for the effect of liposomal encapsulation by comparing groups t-PA plus t-PA+ empty lip (both referring to free t-PA, 0.20 mg/kg) with groups t-PA-lip plus Plg-t-PA-lip (both referring to liposomal t-PA, about 0.24 mg/kg). A significant difference ($P < 0.025$) was demonstrated. A second t-test investigated the difference between groups t-PA-lip and Plg-t-PA-lip (as an indication of the effect of coupled Plg). The difference was shown not to be significant ($P >> 0.025$).

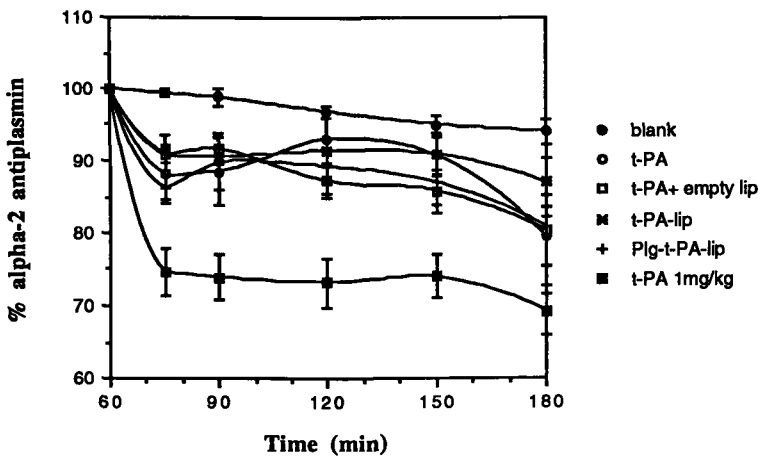
Table 1 Mean thrombolysis induced by different (thrombolytic) administrations. Administered were bolus injections of: saline-blank (blank), free t-PA (t-PA) [two doses], free t-PA + empty liposomes (t-PA+ empty lip), liposomal t-PA (t-PA-lip) and liposomal t-PA in Plg-bearing liposomes (Plg-t-PA-lip). Isotope recovery was >90% for all rabbits.

Thrombolytic category	Number of rabbits	Dosage (mg/kg t-PA)	Thrombolysis (%) (mean ± SEM)
blank	(n=8)	-	14.1 ± 1.9
t-PA+ empty lip	(n=5)	0.20	20.8 ± 3.1
t-PA	(n=6)	0.20	26.3 ± 3.8
Plg-t-PA-lip	(n=5)	0.22	34.2 ± 3.8
t-PA-lip	(n=6)	0.25	34.8 ± 3.6
t-PA$^{1mg/kg}$	(n=7)	1.0	37.7 ± 3.6

Systemic effects

The systemic activation in the six thrombolytic groups was compared by measuring the decrease in plasma concentration of alpha-2-antiplasmin and plasminogen after thrombolytic treatment. Figures 3 and 4 show the relative decrease in plasma concentration of alpha-2-antiplasmin and plasminogen in time. Values are expressed as a percentage of the concentration at 60 minutes (= prior to thrombolytic treatment).

3



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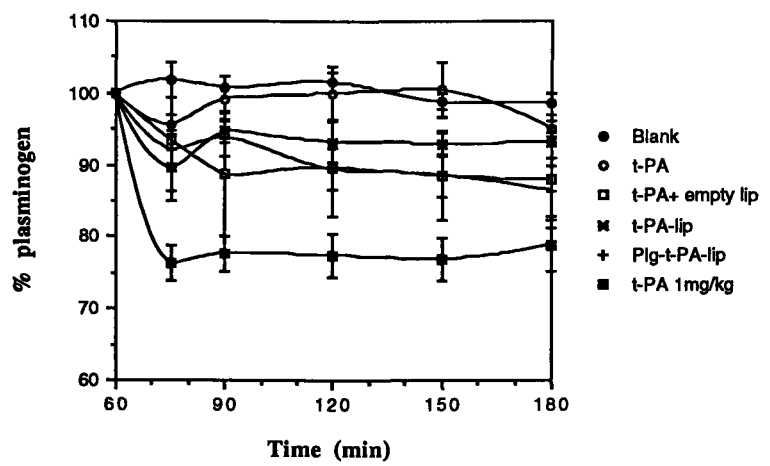

Figure 3, 4 Mean decrease in (3) alpha-2-antiplasmin and in (4) plasminogen [both ± SEM] induced by different (thrombolytic) administrations.
Administered were bolus injections of: saline-blank (blank), free t-PA (t-PA) [two doses], free t-PA + empty liposomes (t-PA+ empty lip), liposomal t-PA (t-PA-lip) and liposomal t-PA in Plg-bearing liposomes (Plg-t-PA-lip). Values shown are obtained by expressing plasma concentrations of (3) alpha-2-antiplasmin and (4) plasminogen after several time-points as a percentage of the concentration at 60' (= prior to thrombolytic treatment).

Regarding all groups, a significant difference in the average percentages of both alpha-2-antiplasmin and plasminogen (in time) was found between groups ($P < 0.05$ in repeated measurements ANOVA). This effect was mainly due to the difference between the t-PA$^{1mg/kg}$-group and the mean of the other groups (t-test, $P < 0.025$). In addition, no difference was found between groups t-PA plus t-PA+ empty lip (free t-PA) and t-PA-lip plus Plg-t-PA-lip (liposomal t-PA) (t-test, $P >> 0.025$).
Furthermore, there was no difference in curve shape between groups ($P >> 0.05$ in repeated measurements ANOVA).

Discussion

Kinetics of t-PA-containing liposomes in healthy rabbits

After in vivo administration, more than 50% of the administered t-PA is rapidly removed from the circulation. This probably concerns the part of liposomal t-PA that is exposed at the outside of the liposome or is present in free form in the external phase (cf. Chapter 4). The remaining part of liposomal t-PA shows a prolonged circulation time after intravenous administration compared to the free drug: the estimated half-life is increased up to about 160 minutes. From the double-labeling experiments with the t-PA-liposomes we could derive that the half-life of 160 minutes is probably a consequence of the clearance behaviour of the liposomes (as shown by the clearance behaviour of the lipid-associated ^{3}H-label). This remaining part of t-PA is tightly attached to or encapsulated into the liposomes in vivo.

The effect of encapsulation of t-PA

It is concluded that there is a significant difference in thrombolysis efficiency between equimolar (around 0.20 mg/kg) doses of free t-PA (t-PA, t-PA+ empty lip) and liposomal t-PA (t-PA-lip, Plg-t-PA-lip). The thrombolytic activity of about 0.24 mg/kg liposomal t-PA approximates the lysis-behaviour of the high dose of free t-PA; t-PA$^{1mg/kg}$ (34.5 <-> 37.7 % lysis, respectively). On the other hand, with respect to systemic activation, liposomal t-PA (t-PA-lip, Plg-t-PA-lip) behaves like equimolar doses (0.20 mg/kg) of free t-PA (t-PA, t-PA+ empty lip), while the systemic influence of the t-PA$^{1mg/kg}$-group is significantly larger. Liposome t-PA encapsulation efficiency in this stage of the project was too low to allow liposomal administration of 1 mg/kg for direct comparison at that dose level. More efficient loading techniques (cf. Chapter 4) should solve this problem.
In conclusion: when using the rabbit jugular vein thrombosis model, liposomal t-PA is more potent than free t-PA while inducing similar systemic activation for the doses studied: an improvement of the therapeutic index in thrombolytic treatment is achieved by the encapsulation of t-PA into liposomes.

In the present liposome formulation about 50% of the liposomal t-PA is already present in free form in the external phase (cf. Chapter 4) or is stripped rapidly upon contact with blood. The rest is released much more slowly. This biphasic behaviour does not provide a direct insight into the mechanism for therapeutic index enhancement. In subsequent experiments we will 'pre-strip' t-PA-liposomes before administration and follow fibrinolytic activity and systemic activation under those conditions.
Furthermore, it would be interesting to monitor our liposomal t-PA in this model for a longer period of time. Whereas it is unlikely that free t-PA with its short half-life of 2 minutes would introduce any additional fibrinolysis after 3 hours, liposomal t-PA with its much prolonged half life might. After 3 hours there is still a significant amount of circulating liposomal t-PA, which could provide an effective protection against possible reocclusions, a feature often seen after thrombolytic treatment [3-7]. Besides, since the jugular vein thrombosis model used does not usually lead to complete vessel occlusion, it should be emphasized that for the clinical situation with complete coronary obstructions different findings may occur.

The effect of the homing device glu-plasminogen
As was shown in Table 1, the percentages of thrombolysis obtained in groups t-PA-lip and Plg-t-PA-lip are similar, indicating no detectable contribution of the targeting capacity of coupled glu-plasminogen.
Inappropriate densities of glu-plasminogen at the surface of the t-PA-containing liposomes could be the reason for this lack of improvement of thrombolytic efficiency. As was already mentioned, the glu-plasminogen density was much lower than in previous in vitro experiments (Chapter 2a; [12]). Apparently, the favourable binding behaviour of the Plg-liposomes that we found in vitro can not be obtained with the tenfold lower Plg density in the in vivo experiments. Alternatively, the presence of only t-PA (partly) sticking out of the liposomes may already result in a targeting behaviour similar to that of Plg in the presently used low density.

Concluding remarks
This study showed that an improvement in therapeutic index of t-PA can be achieved by encapsulation of the thrombolytic agent into liposomes. However, since the results described in this publication are obtained with 'non-optimized liposomes', an even larger improvement might be achieved by:
1) Enhancement of the Plg-density at the outside of the liposomes.
The t-PA-containing liposomes with and without Plg coupled to the outside showed a similar behaviour with respect to lysis and systemic effects. Thus, although Plg did not introduce an improvement, the applied procedure for the Plg-coupling apparently did not

introduce deleterious effects either. An increase of the Plg-density may lead to a (higher) targeting-efficiency which improves the therapeutic index even more.

2) Optimizing the liposome characteristics.

The liposome-composition used is a relatively unstable one. Release kinetics for t-PA are not established. Optimization of this release pattern is definitely possible by using liposomal membranes with different permeability and / or longer circulation times. Several different approaches are possible. For instance, a prolonged vascular circulation time and prolonged action can be achieved by attaching polyethyleneglycol to the membrane (e.g. [29-31]).

3) Increasing the t-PA encapsulation efficiency and stripping the outside of t-PA containing liposomes prior to in vivo administration.

The administration of few targeted liposomes loaded with a high payload of t-PA is expected to be more effective (by a more pronounced local concentration-effect) than many, low-payload t-PA liposomes. Furthermore, the absence of t-PA at the outside of the liposomes would rule out the possibility of instantaneous release of t-PA into the circulation upon administration, which would most likely reduce systemic effects of the t-PA containing liposome formulations. On the other hand, the hypothesis that surface exposed t-PA helps in the targeting process deserves attention.

In view of these possibilities to be explored, we conclude that this concept holds a promise for further improvement of the therapeutic index in thrombolytic therapy.

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Chapter 7

Final considerations and prospects / results of ongoing research

Introduction

This thesis reports on the concept where liposomal encapsulation of the thrombolytic agent tissue-type Plasminogen Activator (t-PA) is combined with coupling of glu-plasminogen (Plg), as an endogenous homing device, to the outside of the liposome. The rationale of this concept is that targeted delivery of the thrombolytic agent at the site of action (the thrombus) is accomplished, thereby minimizing systemic side-effects elsewhere in the body. Moreover, physical separation of t-PA from the blood will prevent its degradation or inhibition by plasma-components before reaching the target tissue.

The described concept is multifaceted and complicated for the following reasons. (a) Both enzyme (t-PA) and substrate (Plg) are present in one formulation. (b) t-PA and Plg are both complex and fragile proteins. (c) Little was known about the actual 'homing activity' of glu-plasminogen in vivo and (d) about the required release kinetics of t-PA from the liposomes. In this study, different elements of the concept were studied separately, with a "target-oriented" research approach. Therefore, this thesis reports on an evolutionary learning-process and should not be considered as an exhaustive study of all details involved. The chapters in this thesis all refer to independent parts of research, not all presented in chronological order. This may confront the reader with discrepancies between the chosen experimental conditions per chapter. In spite of these limitations, the results obtained in this study (as described below) clearly point out the interesting possibilities that the chosen concept offers with respect to target oriented thrombolytic treatment.

In vitro studies

Plg-liposomes

Preparation, in vitro fibrin binding

A suitable method was developed for coupling the homing device glu-plasminogen to liposomes *(Chapter 2a)*. Thiol groups are introduced into the Plg-molecule and they are subsequently covalently coupled to liposomal anchor-molecules. With the obtained method, liposomes with a wide range of surface Plg-densities can be prepared.

The purity of glu-plasminogen turned out to be a critical parameter for the coupling method. The presence of -seemingly harmless- small quantities of EACA (ε-amino-caproic acid) in commercially available glu-plasminogen can interfere with the thiol introduction into the protein *(Chapter 2b)*. Therefore, highly purified glu-plasminogen should be used.

Liposomal glu-plasminogen exhibits promising targeting properties in vitro *(Chapter 3)*. Binding of liposomal Plg to a fibrin monolayer is much enhanced over that of non-liposomal Plg: even a 100-fold excess of free Plg could not successfully compete with the

fibrin binding of liposomal Plg. This phenomenon is ascribed to the multivalent nature of the interaction of liposomal Plg with fibrin: Plg-liposomes can interact with the fibrin surface through more Plg-molecules than 'monovalent' non-liposomal Plg. Rearrangement of Plg on the surface of the liposome may lead to relatively high Plg concentrations at the liposome - fibrin interaction site, a process ('contact capping') which possibly intensifies the multivalent character.
From these in vitro results it can be concluded that Plg-liposomes can be prepared, which are able to bind (target) to fibrin efficiently.

t-PA-liposomes

Preparation, ultracentrifugation damage

t-PA-liposomes are prepared by freeze-thawing liposomes in the presence of t-PA. The obtained t-PA entrapment percentage (for liposomes without coupled Plg) depends strongly on the experimental conditions *(Chapter 4)*. Whereas in acidic medium (citric acid, NaCl, pH 4) about 35% t-PA was entrapped, almost quantitative (90%) entrapment could be obtained in a pH 7.5 Hepes-buffer with lactose, low ionic strength (using similar t-PA and phospholipid incubation concentrations). This high entrapment efficiency is attributed to strong t-PA - bilayer interactions. During this study, the pronounced stability-interfering effect of ultracentrifugation (especially in the presence of 0.05% Tween 80) on t-PA containing liposomes was encountered. As repeated ultracentrifugation caused cumulative t-PA loss from the liposomes, alternative approaches to separate free t-PA from liposomal t-PA were considered, with little success. Consequences of this unexpected finding (ultracentrifugation damage) for other protein-liposome systems remain to be seen. At the present, ultracentrifugation is considered a harmless and widely accepted method (e.g. [2, 3]).

Combined Plg-t-PA-liposomes

Preparation, stability

Our studies demonstrate that for the preparation of liposomes containing both t-PA and Plg, e.g. both enzyme and its substrate, no specific precautions need to be taken to prohibit plasmin-formation when the dispersion is stored in freeze-dried state or kept under aqueous conditions for at most a few hours *(Chapter 5)*. This is in accordance with the very low plasminogen conversion rate in the absence of fibrin [4].
Freeze-drying of the liposomes in a Hepes buffer pH 7.5, containing 7.5% (w/v) lactose, results in complete recovery of enzyme activity of both Plg and t-PA. Plg was shown to retain its fibrin binding capacity upon freeze-drying as well. Furthermore, no leakage of t-PA was induced by the freeze-drying procedure under the conditions used, provided that

freeze-dried liposomes were not exposed to an extra ultracentrifugation step compared to the non-freeze-dried control liposomes.

However, further development is required to solve the problem of combining the conflicting 'optimal' conditions for Plg-coupling and t-PA entrapment. Whereas Plg-coupling is most efficient at a high Plg to PL ratio (Ch. 2a), t-PA entrapment is best performed at 100 µmol/ml PL (Ch. 4). In other words, a low phospholipid concentration promotes high liposomal Plg surface-densities, but it reduces t-PA entrapment. This phenomenon leads to a reduction of the t-PA entrapment to 70% at 30 µmol/ml in Ch. 5, compared to 90% t-PA entrapment at 100 µmol/ml PL in Ch. 4, at otherwise similar conditions. Conversely, at high PL concentrations more t-PA can be entrapped at the cost of the liposomal Plg surface-densities (Ch. 6). A number of approaches can be proposed to formulate liposomes with both a high Plg density at their surface and a high t-PA entrapment efficiency. E.g., the Plg binding process can be performed at low PL concentration, which is then increased by ultracentrifugation or ultrafiltration steps to ensure efficient quantitative t-PA entrapment. These Plg-t-PA-liposomes containing both a high Plg surface-density and a high concentration of t-PA will be freeze-dried in the near future. Additionally, long-term stability experiments on the freeze-dried product will be performed.

Alternatively, the option of loading t-PA directly during the rehydration process of the freeze-dried Plg-liposomes ('at the bedside') should be investigated. This would be a highly interesting protocol as it is technologically relatively simple. The idea for 'at the bed side loading' of the liposomes with t-PA is based on the high entrapment value for t-PA under conditions selected in Ch. 4 (low ionic strength, lactose, pH 7.5).

In conclusion: combining the methods described in Chapters 2, 4 and 5, we will be able to prepare liposomes containing both t-PA and Plg, which meet the requirements of short term and long term stability. Future efforts will be mainly focused on optimization of the combination of t-PA entrapment and Plg-coupling in this formulation.

In vivo studies

Thrombolytic behaviour of t-PA-liposomes

Liposomal t-PA was shown to display an improved therapeutic index over free t-PA for thrombolytic treatment in the rabbit jugular vein thrombosis model *(Chapter 6)*. It should be noted that non-optimized t-PA-liposomes (prepared in pH 4 citric acid + NaCl, with an ultracentrifugation step to remove non-entrapped t-PA) were used for these in vivo experiments. However, in spite of this, a dose of only about 0.24 mg/kg of liposomal t-PA practically equalled the lysis activity of 1.0 mg/kg of free t-PA, whereas the systemic

side-effects by t-PA were not affected by liposomal entrapment. Therefore, we feel that these results hold a promise for further improvement of the therapeutic index in thrombolytic therapy. More specifically, measurement of the in vivo thrombolytic activity of t-PA liposomes which are prepared under conditions where 90% entrapment is obtained (Ch. 4: low ionic strength, lactose, pH 7.5) would be an obvious next step for further research, which may lead to even further improvement of the therapeutic index beyond that of the t-PA liposomes tested in Ch. 6.

Targeting of Plg-liposomes in vivo

The attachment of Plg as a homing device at the outside of t-PA containing liposomes did not affect their thrombolytic performance in vivo *(Chapter 6)*. Apparently, there was no detectable contribution of the targeting capacity of liposome coupled Plg. This may seem to contradict with the promising in vitro fibrin binding characteristics of Plg-liposomes as described in Ch. 3. However, as was mentioned before, the in vivo tested liposomes in Ch. 6 were prepared at relatively high PL concentrations (in order to entrap as much t-PA as possible). As a consequence, relatively low liposomal Plg surface-densities were obtained: only 2 μg Plg per μmol PL, whereas in Ch. 3 Plg-densities between 8 and 60 μg Plg per μmol PL were used. Therefore, it can not be concluded from Ch. 6 that the Plg targeting concept fails in vivo: liposomes which contain substantial amounts of t-PA in combination with high densities of Plg (as described previously) need to be prepared and tested. In vivo thrombolysis tests with these optimized liposomes may show a further increase of the therapeutic index of Plg-t-PA-liposomes over that of t-PA-liposomes, due to (improved) targeting-efficiency through liposome coupled Plg.

Preliminary additional data from currently performed studies

Targeting of Plg-liposomes in a flow-through system

The static fibrin binding system in vitro as used in Chapter 3 demonstrated strong and specific binding of Plg-liposomes to fibrin. Here we present preliminary data on a flow-through system in vitro. In other words, we currently study the binding of Plg-liposomes under dynamic flow conditions. The experimental scheme is presented in Figure 1.

Materials and methods

A flow-through system was used, consisting of silicone tubes (diameter 0.9 mm), connected with a 2120 Varioperpes II pump (LKB, Woerden, The Netherlands). Total volume of the system: 600 μl of either buffer (10 mM Hepes, 135 mM NaCl, 1 mM EDTA, 0.01% Tween 20) or CTAD-plasma (containing citrate, theophylline, adenosine and dipyrimidol), flow-rate 2 ml/min. A clot was introduced into a segment of the tubing

system. Temperature: 37°C. Either a fibrin clot (50 µl of 4 mg/ml plasminogen-free fibrinogen, coagulated with Ca^{2+}, thrombin and a woollen thread), or a whole blood clot (50 µl of citrated blood coagulated with Ca^{2+}, thrombin and a woollen thread) was used. ^{125}I-radio-labeled Plg (Iodogen method, see Ch. 6) was coupled to ^{3}H-labeled liposomes ([1α, 2α (n)-^{3}H]-Cholesteryl oleoyl ether, see Ch. 6). These double-labeled liposomes were introduced into the closed loop system after which the accumulation of radio-label on the entire clot was measured with a NaI (Tl) probe, model 5XM-5/1-5LP-x (Bicron Corp. Newbury, USA). A data processing program from the Canberra Nuclear Products group was used.

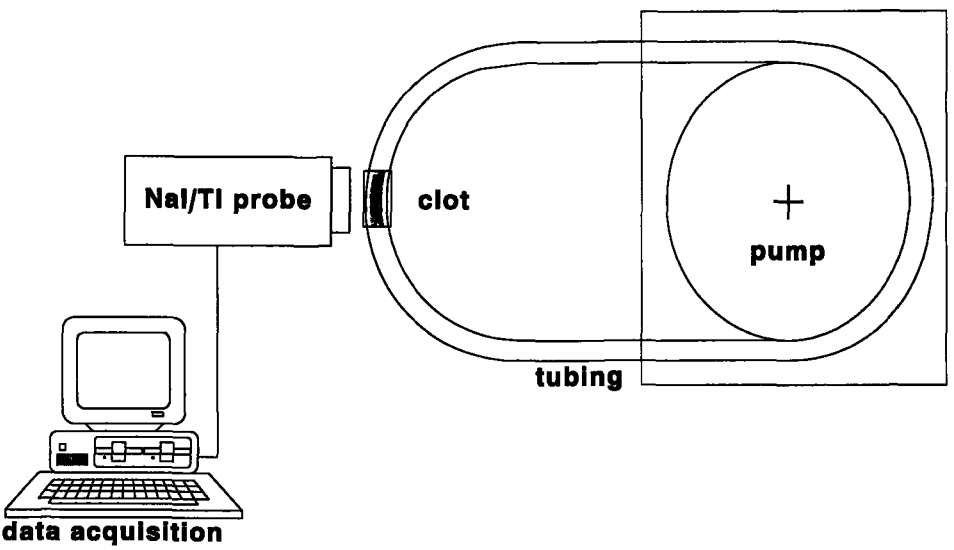

Figure 1 Schematic representation of the flow-through system.
The flow-through model consists of a closed system of silicone tubes (diameter 0.9 mm), connected with a pump. Total volume of the system: 600 µl, flow-rate 2 ml/min, temperature: 37°C. A clot was introduced into a segment of the tubing system. Either radio-labeled Plg or Plg-liposomes were injected into the closed loop system after which the accumulation of Plg on the entire clot was measured with a NaI (Tl) probe. A data processing program was used. (For further details or explanation see text.)

Variables under investigation

The clot binding behaviour of liposomal Plg by itself or in the presence of an excess of free Plg was studied under different experimental conditions: **(I)** first in a buffer system using a fibrin clot, **(II)** in plasma using a fibrin clot and eventually **(III)** in a plasma system with a whole blood clot.

Results / discussion

(I) In Figure 2 the fibrin clot binding of 2 µg/ml liposomal Plg (surface-density 60 µg Plg / µmol PL) is compared to that of 2 µg/ml radio-labeled free Plg in a buffer system. It is clear that, under the chosen conditions, liposomal Plg shows improved fibrin binding characteristics over free Plg (60% compared to 20% binding, respectively). Moreover, washing of the clot by introducing fresh buffer into the system (at t = 25') did not reduce the amount of fibrin bound liposomal Plg, whereas fibrin bound free Plg could be washed away almost quantitatively. The open triangles in Figure 2 represent the fibrin binding behaviour (duplo) of 2 µg/ml liposomal Plg upon introduction into a system which was already pre-equilibrated for 20 min with an excess of 80 µg/ml of free (unlabeled) Plg. It is obvious that liposomal Plg is capable to compete successfully with this excess of non-liposomal Plg for clot binding. Several control-experiments were performed: firstly, introduction of Plg-free liposomes into the system showed hardly any clot accumulation (< 0.5% upon washing). Secondly, in the absence of a clot, negligible binding of free Plg to the tubing system was seen. Finally, the constant ratio of ^{125}I(-Plg) to ^{3}H(-liposomes) (data not shown) indicated that the observed binding concerns liposomal Plg, not free Plg.

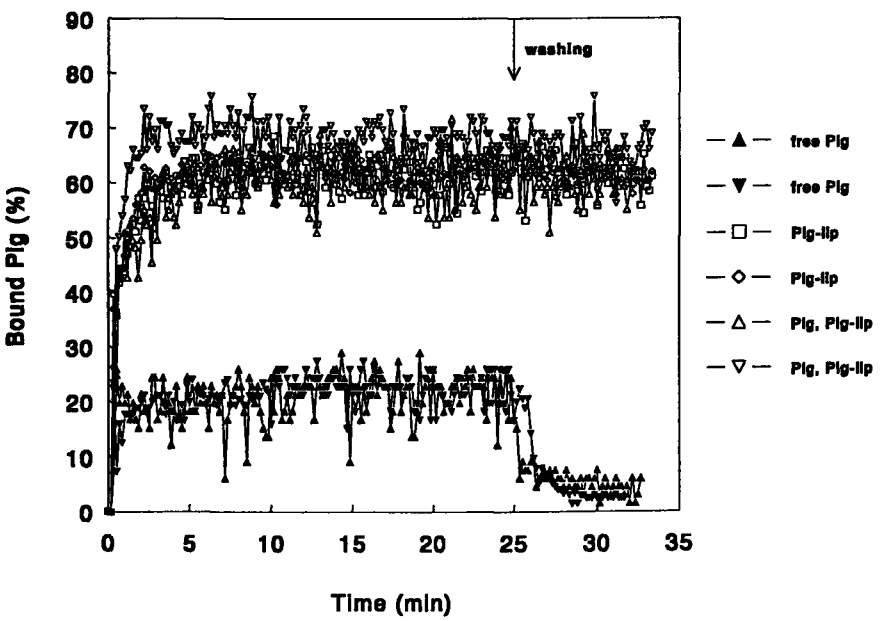

Figure 2 Binding of free Plg and liposomal Plg to a fibrin clot in a flow-through buffer system. 2 µg/ml free or liposomal ^{125}I-labeled Plg is incubated with a fibrin clot in buffer (10 mM Hepes, 135 mM NaCl, 1 mM EDTA, 0.01% Tween 20). Liposomal surface-density used: 60 µg Plg / µmol PL. The open triangles represent the fibrin binding behaviour of 2 µg/ml liposomal Plg upon introduction into a system which was already pre-equilibrated for 20 min with an excess of 80 µg/ml of free (unlabeled) Plg. After 25 min the clot is washed with fresh buffer solution. Plg clot accumulation is detected with a NaI probe. Duplicate experiments are shown.

(II) In Figure 3 the fibrin clot binding of 2 μg/ml liposomal Plg (surface-density 40 μg Plg / μmol PL) is compared to that of 2 μg/ml radio-labeled free Plg in plasma instead of buffer. Measurement in plasma implies the presence of an excess of about 200 μg/ml of endogenous free, unlabeled Plg throughout the experiment. Labeled free Plg shows a clot 'binding' of about 10%, which corresponds more or less to the expected level based on dilution of the original solution (50 μl clot volume in a 600 μl total volume), i.e.: no clot binding is observed. Liposomal Plg shows a slow increase in binding up to ± 30%, about half the value observed in buffer (cf. Figure 2). Upon addition of t-PA to the system (at t = 15', final concentration 90 IU/ml), no clot accumulation is detected for free Plg, whereas for liposomal Plg binding is at first slightly enhanced, followed by a strong decrease. The initial increase was foreseen, since partly degraded fibrin is known to bind Plg more strongly, because of the appearance of extra binding sites on the partly degraded fibrin surface compared to 'intact' fibrin [5]. The subsequent strong decline is ascribed to t-PA induced clot-lysis.

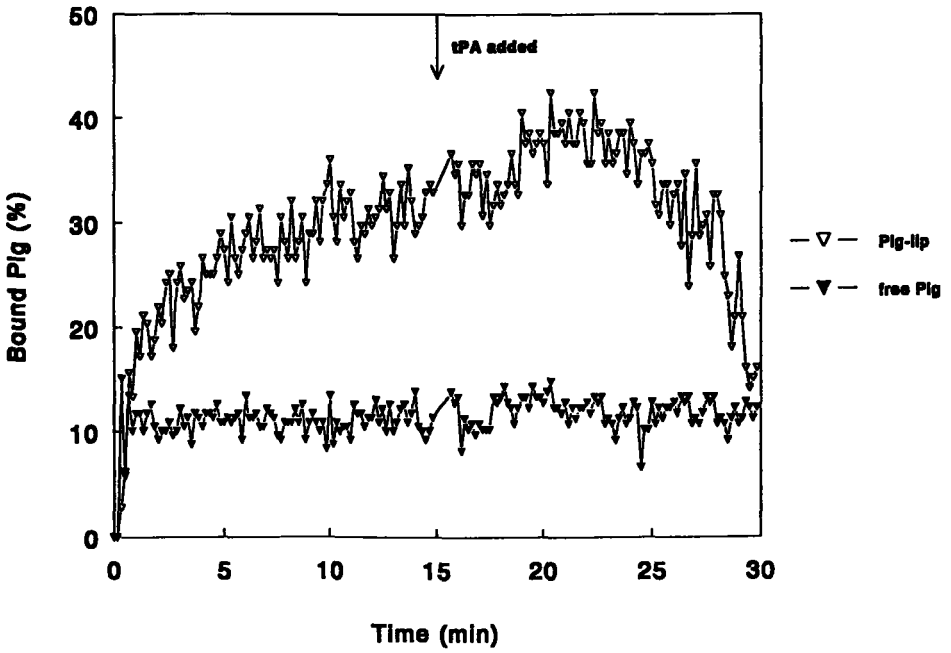

Figure 3 Binding of free Plg and liposomal Plg to a fibrin clot in a flow-trough plasma system. 2 μg/ml free or liposomal ^{125}I-labeled Plg is incubated with a fibrin clot in CTAD-plasma (containing citrate, theophylline, adenosine and dipyrimidol). Liposomal surface-density used: 40 μg Plg / μmol PL. After 15 min t-PA is added to the closed system, final concentration 90 IU/ml. Plg clot accumulation is detected with a NaI probe.

In Figure 4 the binding of liposomal Plg as seen in Figure 3 is compared to its fibrin binding behaviour after incubation with a reduced t-PA concentration (10 IU/ml instead of 90 IU/ml). The initial increase is clearly more pronounced for the 10 IU/ml incubation, whereas the strong decline is not observed. Apparently, the equilibrium between increase in binding due to the appearance of extra binding sites and decrease in binding due to clot-lysis is shifted in favour of the first process.

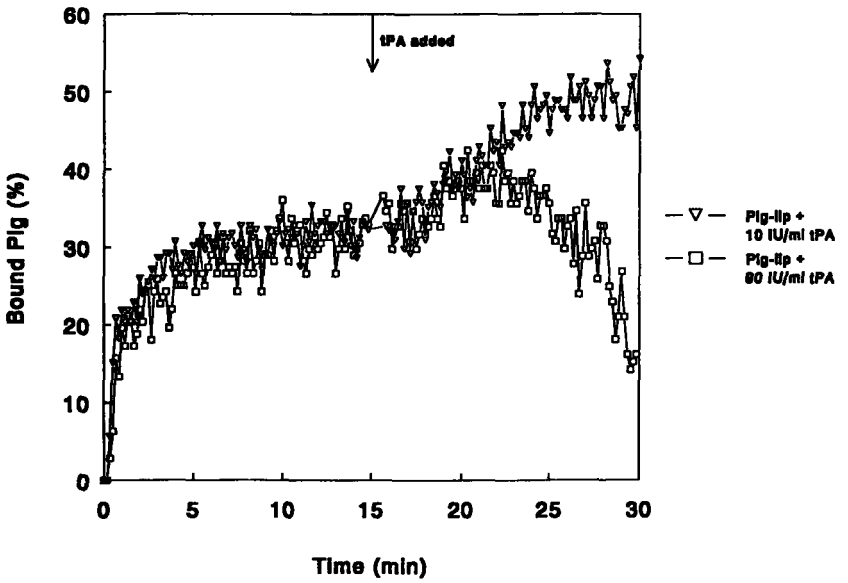

Figure 4 Binding of free Plg to a fibrin clot in a flow-through plasma system.
2 µg/ml free or liposomal ^{125}I-labeled Plg is incubated with a fibrin clot in CTAD-plasma (containing citrate, theophylline, adenosine and dipyrimidol). Liposomal surface-density used: 40 µg Plg / µmol PL. A comparison is made between the effect of addition (after 15 min) of 90 IU/ml and 10 IU/ml t-PA. Plg clot accumulation is detected with a NaI probe.

(III) Figure 5 shows binding of free and liposomal Plg (surface-density 40 µg Plg / µmol PL) in plasma to a whole blood clot, followed by incubation with 90 IU/ml t-PA. Figures 3 and 5 are quite similar. This implies that we could not discern a difference between binding behaviour of (liposomal) Plg to a fibrin clot and a whole blood clot.

In conclusion: Plg-liposomes show improved binding over free Plg in this flow-through system. This binding phenomenon was observed both with a fibrin clot and a whole blood clot, both in buffer and in plasma. It confirms our previous findings with a static system (Ch. 3) qualitatively. Therefore, new in vivo experiments with Plg-liposomes containing similar Plg surface-densities as used in this flow through system will be performed in the future to see whether the therapeutic index of t-PA can indeed be further improved by efficient targeting of liposomal Plg.

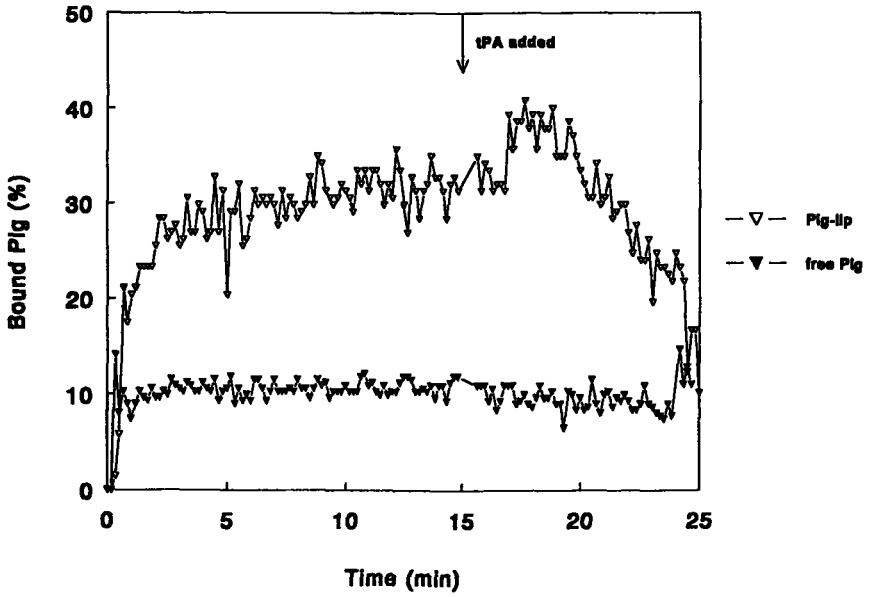

Figure 5 Binding of free Plg and liposomal Plg to a whole blood clot in a flow-through plasma system.
2 µg/ml free or liposomal ^{125}I-labeled Plg is incubated with a fibrin clot in CTAD-plasma (containing citrate, theophylline, adenosine and dipyrimidol). Liposomal surface-density used: 40 µg Plg / µmol PL. After 15 min t-PA is added to the closed system: final concentration 90 IU/ml. Plg clot accumulation is detected with a NaI probe.

Conclusions, plans for further research

The 'target-oriented' approach that was used throughout this study leaves us with unanswered questions: many aspects still need to be investigated and variation of the experimental conditions leaves ample room for optimization. Future plans were already indicated throughout this last chapter. Efforts to combine high t-PA entrapment with high liposomal Plg surface-densities into one formulation have a high priority rating. In the context of optimization of the pharmaceutical characteristics of the formulation a better method than the existing ultracentrifugation procedure should be found for separation of free t-PA from liposome-associated t-PA, both for preparatory and analytical purposes.
Conditions have to be chosen to ensure sufficient long-term stability of the freeze-dried form of these eventually optimized Plg-t-PA-liposomes. Large quantities of liposomes can then be prepared and stored in freeze-dried form: they can be redispersed when needed. Further in vivo work in relevant animal models with these "high Plg-density / high t-PA payload" liposomes will be done and attention will be paid to in vivo thrombus degradation and side effects. These data will be correlated with in vivo release kinetics of t-PA from the

liposomes.

If the in vivo targeting capacities of liposomal Plg may, after all, appear to be insufficient, replacement of the homing device glu-plasminogen by higher-affinity substances can be considered. Monoclonal antibodies against fibrin may be used (e.g. [6]), as well as antimyosin antibodies [7].

Once optimal targeting towards thrombi in vivo is accomplished, these targeted liposomes could also be used for diagnostic purposes to provide images of fibrin-rich tissue or for delivery of other drugs besides t-PA, e.g. anticoagulants.

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Appendices

Summary

Liposomes, vesicular structures consisting of hydrated lipid bilayers, have drawn much interest in their role of drug carriers, in particular of bioactive molecules such as peptides and proteins. This thesis reports on the concept where liposomal encapsulation of a thrombolytic (thrombus dissolving) protein: tissue-type Plasminogen Activator (t-PA), is combined with coupling of an endogenous homing device (tissue-selective agent): glu-plasminogen (Plg) to the outside of the liposome. These liposomes are designed to be used for specific delivery of the thrombolytic agent at the site of a thrombus, as with myocardial infarction. A schematic representation of the concept is presented as Figure 1 in Chapter 1. Whereas liposomal encapsulation of thrombolytic agents was described before, combination with endogenous glu-plasminogen as a homing device for these thrombolytic liposomes created a novel approach towards thrombolytic therapy. This concept is expected to improve the therapeutic index of thrombolytic treatment relative to free t-PA for two main reasons: a) a reduction of unwanted systemic activity by t-PA is obtained by its encapsulation and, consequently, its physical separation from the blood flow, b): liposomes are targeted to fibrin deposits by glu-plasminogen, which increases the fraction of t-PA actually reaching the thrombus compared to non-targeted delivery.
In order to prepare and test these technologically complex liposomes, a stepwise approach was followed to build up the different parts of the delivery system. These findings are described in separate chapters. First, Plg-liposomes were prepared and tested for their in vitro targeting capacity (Chapters 2a, 2b, 3). Then, t-PA-liposomes were prepared (Chapter 4) after which both in vitro preparation technologies were combined to produce Plg-t-PA-liposomes (Chapter 5). Finally, preliminary data on the in vivo thrombolysis activity and side effects in an animal model are discussed in Chapter 6.

In *Chapter 1* a brief introduction to the subject and an outline of this thesis is presented.

Chapter 2a describes a suitable way of coupling the homing-device glu-plasminogen to the outside of liposomes. The procedure is based on the reaction of thiol groups introduced into the protein with thiol-reactive groups anchored in the liposome bilayer. Glu-plasminogen was thiolated with the reagent N-succinimidyl S-acetylthioacetate (SATA), after which the protein was incubated with liposomes containing the thiol-reactive anchor maleimido-4-(p-phenylbutyrate)-phosphatidyl-ethanolamine (MPB-PE). A wide range of surface Plg-densities could be obtained. Although SATA-derivatization of glu-plasminogen induced some loss of its enzymatic activity, in a preliminary experiment it was shown that liposomal glu-plasminogen could efficiently bind liposomes to their target, fibrin. Therefore, the described

coupling procedure was considered an effective way to bind the homing-device glu-plasminogen to liposomes without critically interfering with its fibrin binding / homing capacity.
The presence of small quantities of EACA (ε-amino-caproic acid) in commercially available glu-plasminogen can interfere with this SATA-thiol introduction *(Chapter 2b)*. It was shown that only 0 to 1.8 mol thiol groups could be introduced per mol of commercially available glu-plasminogen, whereas up to 9 thiol groups could be introduced per glu-plasminogen molecule of a batch previously subjected to an additional purification step on a large Sephadex G-25 column. Therefore, only highly purified glu-plasminogen should be used.

In *Chapter 3*, the fibrin binding properties of liposomal Plg are compared to those of free Plg in an in vitro model system, using fibrin monolayer coated 96-wells plates. The results suggest that on the average only 40% of the total amount of liposomal Plg was involved in the fibrin binding. Liposomal Plg showed a binding rate which was at least one order of magnitude higher than free Plg. Moreover, liposomal Plg could successfully compete for fibrin binding sites with a 100 fold higher concentration of free Plg. The multivalent character of liposomal Plg was probably responsible for the improved binding characteristics. Fibrin binding of liposomal Plg was thought to be limited by geometrical restrictions, dictated by the available space per well and by the dimensions of the liposomes.
These in vitro findings indicate that liposomes with attached glu-plasminogen (preferably at a high density) are promising vehicles for in vivo targeting of encapsulated drugs to fibrin based thrombi.

Chapter 4 reports on the development of a method for efficient entrapment of active tissue-type Plasminogen Activator into liposomes. Liposomal t-PA entrapment strongly depended on experimental conditions like buffer composition, the incubation concentration of phospholipid (PL) and liposome-size. Almost quantitative (90%) entrapment (for definition see Ch. 4), was obtained in Hepes buffer pH 7.5, devoid of arginine, with low ionic strength, at 100 μmol/ml PL. Furthermore, ultracentrifugation, which was used to separate liposomal t-PA from free t-PA, was shown to have a damaging effect on the liposomes (especially in the presence of 0.05% Tween 80), leading to t-PA loss. Therefore, the encapsulation-percentage values shown in this chapter are in fact underestimates for the true entrapment of t-PA.
In conclusion: almost quantitative t-PA entrapment in liposomes can be achieved by selecting the proper milieu and inducing a strong interaction between t-PA and bilayer.

Preparation and storage of liposomes containing both Plg, covalently coupled to the outside of the liposome bilayer, and physically entrapped t-PA is the topic dealt with in *Chapter 5*. As both enzyme (t-PA) and its substrate (Plg) are introduced into one liposome formulation, it

was important to find preparation and storage conditions which prohibit (1) plasmin-formation, (2) loss of enzymatic activity and (3) t-PA-leakage for these complex liposomes. Since t-PA induced Plg-activation only occurred in the presence of fibrin fragments, no special stability precautions had to be taken during the preparation of Plg-t-PA-liposomes. Stable storage conditions were obtained by freeze-drying of the liposomes in a Hepes buffer pH 7.5, containing 7.5% (w/v) lactose. Quantitative recovery of enzyme activity of both Plg and t-PA was obtained upon rehydration. Plg was also shown to retain its fibrin binding capacity after a freeze-drying rehydration cycle. Furthermore, no loss of t-PA retention was induced by the freeze-drying procedure if the conditions were properly chosen.

In *Chapter 6,* t-PA-containing liposomes were tested for their thrombolytic activity and systemic side effects in a rabbit jugular vein thrombosis model. Liposome encapsulated t-PA showed a significantly better thrombolysis efficiency than equimolar doses of free t-PA: about 0.24 mg/kg of liposomal t-PA practically equalled the lysis-activity of a dose of free t-PA of 1.0 mg/kg. Moreover, liposome encapsulation was shown not to affect the undesired systemic activation of alpha-2-antiplasmin and plasminogen compared to free t-PA. We conclude from these in vivo experiments that an improvement in thrombolytic efficacy of t-PA can be achieved by liposome encapsulation of t-PA. Attachment of Plg to t-PA-liposomes provided similar results as those obtained with t-PA-liposomes without a homing device. This indicates that the low Plg surface-densities used in this in vivo study (about 10-fold lower than in both Chapters 3 and 7) were not adequate for efficient targeting of liposomal t-PA.

An evaluation of the most important results is presented in *Chapter 7* and plans for future research in this field are outlined. Furthermore, in this chapter preliminary results of targeting experiments with Plg-liposomes (densities comparable to Ch. 3) in a clot containing flow-through system are shown. Plg-liposomes showed increased binding over free Plg both to fibrin clots and whole blood clots, both in buffer and in plasma (implying the presence of a 100-fold excess of endogenous free Plg). Washing of the clot with fresh medium did not reduce the amount of clot associated liposomal Plg, indicating increased affinity and prolonged residence time. In contrast, fibrin bound free Plg could be washed away almost quantitatively. By addition of free t-PA the dynamics of the lysis process were mimicked. With a low concentration of free t-PA increased clot binding of liposomal Plg was observed, in accordance with the formation of additional Plg binding sites in a clot during its degradation. Upon addition of a higher concentration of t-PA liposomal Plg remained bound, showing the expected decline in quantity when the clot dissolved. Based on these promising results, we conclude that further improvement of the therapeutic index of t-PA (cf. Ch. 6) may be attainable by efficient targeting of t-PA laden liposomes with coupled Plg. To achieve this goal, encapsulation of sufficient amounts of t-PA into the liposome should be combined

with generation of adequate densities of Plg to the outside of the liposome. An option to be further developed is, for example, freeze-drying of the Plg-liposomes followed by 'at the bedside' loading of these liposomes with t-PA.
In conclusion, this thesis describes the 'state of the art' of the ongoing efforts in our group to improve the therapeutic index of t-PA by targeted delivery using 'homed' liposomes.

Samenvatting

Liposomen zijn bolvormige structuren, opgebouwd uit lipide bilagen die een waterig compartiment omsluiten. Een veelbelovende toepassing voor het gebruik van liposomen ligt op het terrein van de toediening van geneesmiddelen, met name van peptiden en eiwitten met een geneeskrachtige werking.
Dit proefschrift beschrijft de inbouw in liposomen van een thrombolyticum (een stolsel oplossend agens): tissue-type Plasminogen Activator, kortweg t-PA. Dit inbouwen wordt gecombineerd met het koppelen aan de buitenzijde van het liposoom van een 'homing device' (een weefsel-specifiek eiwit): glu-plasminogen ofwel Plg. Het is de bedoeling dat intraveneus gebruik van deze liposomen bij een acuut hartinfarct leidt tot specifieke afgifte van het stolsel oplossende middel t-PA op de plaats van werking: bij het stolsel in het hart. Figuur 1 in Hfdst. 1 geeft een schematische weergave van het concept waarop dit onderzoek is gebaseerd. Inbouw van thrombolytica in liposomen is al eerder beschreven, maar de combinatie met het gebruik van het lichaamseigen eiwit glu-plasminogeen maakt dit tot een nieuw concept binnen de thrombolytische therapie. Met deze benadering wordt om twee redenen een verhoging van de therapeutische index van t-PA verwacht boven die van vrij t-PA: a) door t-PA in te bouwen en af te schermen van de bloedcirculatie kan het minder systemische bijwerkingen veroorzaken, b) de liposomen worden door glu-plasminogeen bij het stolsel vastgehouden ('getarget'), hetgeen de fractie aan ter plaatse afgeleverd t-PA verhoogt ten opzichte van niet getargete systemen.
Om deze complexe liposomen te maken en uit te testen, zijn de praktische werkzaamheden verdeeld in separate stukken, elk met een eigen doelstelling, die beschreven worden in de verschillende hoofdstukken. Allereerst zijn Plg-bevattende liposomen gemaakt en is hun in vitro 'targetings'-capaciteit gemeten (Hoofdstuk 2a, 2b en 3). Vervolgens zijn t-PA-bevattende liposomen gemaakt (Hoofdstuk 4), waarna beide technologieën zijn gecombineerd voor de productie van Plg-t-PA-liposomen (Hoofdstuk 5). De thrombolytische activiteit en de systemische bijwerkingen van een dergelijk t-PA-Plg product zijn in vivo gemeten in een diermodel, hetgeen is beschreven in Hfdst. 6.

In *Hoofdstuk 1* wordt een korte introductie en de indeling van dit proefschrift gegeven.

Hoofdstuk 2a beschrijft een geschikte manier om het homing-device glu-plasminogeen te koppelen aan de buitenzijde van de liposomen. De methode is gebaseerd op een reactie tussen thiolgroepen die geïntroduceerd worden in het eiwit, met thiol-reactieve groepen die verankerd zijn in de liposomale bilaag. Glu-plasminogeen werd gethioleerd met het reagens N-succinimidyl S-acetylthioacetaat (SATA), waarna het eiwit werd geïncubeerd met liposomen die het thiol-reactieve anker maleimido-4-(p-phenylbutyraat)-phosphatidyl-

ethanolamine (MPB-PE) bevatten. Een grote verscheidenheid aan oppervlakte-dichtheden van Plg op het liposoom kon worden verkregen. Hoewel het SATA-gederivatiseerde glu-plasminogeen wel wat van zijn enzymatische activiteit verloor, toonde een eerste experiment in vitro aan dat liposomaal Plg zeer wel in staat was om liposomen aan het doel-weefsel, fibrine, te binden. Daarom wordt de hier beschreven methode beschouwd als een effectieve manier om het homing-device aan het liposoom te koppelen zonder dat het sturend vermogen van het Plg teveel wordt aangetast.
De aanwezigheid van kleine hoeveelheden EACA (ε-amino-caproic acid) in algemeen verkrijgbaar glu-plasminogeen kan de SATA-thiol introductie verstoren *(Hoofdstuk 2b)*. Er konden maar tussen 0 en 1,8 mol thiolgroupen worden geïntroduceerd per mol commerciëel verkrijgbaar glu-plasminogeen, terwijl tot 9 thiolgroepen konden worden geïntroduceerd per Plg molekuul in fracties die tevoren gezuiverd waren over een grote Sephadex G-25 kolom. Daarom kan het beste met sterk gezuiverd glu-plasminogeen worden gewerkt.

In *Hoofdstuk 3* worden de fibrine-bindingseigenschappen van liposomaal Plg vergeleken met die van vrij Plg in een in vitro model system waarbij gebruik wordt gemaakt van een 96-wells-plaat bedekt met een monolaag fibrine. Er bleek gemiddeld slechts ongeveer 40% van de totale liposomale hoeveelheid Plg te binden. Liposomaal Plg bond minstens 10x sneller dan vrij Plg en kon bovendien een 100-voudige overmaat aan vrij Plg verdringen. De binding aan fibrine door liposomaal Plg werd geacht gelimiteerd te worden door geometrische factoren: het 'well'-oppervlak en de afmetingen van de liposomen. De binding van liposomaal Plg werd sterker naarmate de dichtheid op het liposoom toenam als gevolg van het fenomeen van multivalente binding.
Deze in vitro resultaten geven aan dat liposomen met gekoppeld glu-plasminogeen (liefst hoge dichtheden) goed bruikbaar lijken te zijn voor de targeting van farmaca in vivo naar fibrine-bevattende stolsels.

Hoofdstuk 4 beschrijft de ontwikkeling van een methode om actief tissue-type Plasminogen Activator op efficiënte wijze in te bouwen in liposomen. Liposomale inbouw van t-PA bleek sterk afhankelijk te zijn van de experimentele omstandigheden zoals buffersamenstelling, incubatie-concentratie fosfolipid (PL) en liposoom-grootte. Bijna kwantitatieve (90%) inbouw (zie voor definitie Hfdst. 4) werd verkregen in een Hepes buffer met pH 7,5, zonder arginine, met lage ionsterkte, bij een concentratie van 100 μmol/ml PL. Verder werd aangetoond dat ultracentrifugatie, een methode die gebruikt wordt om liposomaal van vrij t-PA te scheiden, schadelijk is voor de liposomen, hetgeen leidt tot t-PA verlies (in het bijzonder in de aanwezigheid van 0.05% Tween 80). Daarom zijn de inbouw-percentages die in dit hoofdstuk worden vermeld eigenlijk een onderschatting van de werkelijke t-PA inbouw. Concluderend: bijna volledige t-PA inbouw in liposomen kan worden bereikt door

de juiste omstandigheden te selecteren en een sterke interactie tussen t-PA en de bilaag te bewerkstelligen.

Het bereiden en bewaren van liposomen die zowel Plg -gekoppeld aan de buitenkant van de liposoom-bilaag- als fysisch ingebouwd t-PA bevatten is het onderwerp van *Hoofdstuk 5*. Omdat zowel enzym (t-PA) als substraat (Plg) in één liposoom-preparaat worden geïntroduceerd, was het belangrijk om bereidings- en bewaringscondities te vinden voor deze complexe liposomen waarbij zowel plasmine-vorming, verlies van enzymatische activiteit als ook t-PA lekkage voorkomen worden. t-PA geïnduceerde Plg-activering trad slechts op in aanwezigheid van fibrine fragmenten. Daarom hoefden geen speciale stabiliteits-maatregelen getroffen te worden tijdens de bereiding van Plg-t-PA-liposomen. Stabiele bewaringscondities werden verkregen door de liposomen te vriesdrogen in een Hepes buffer met een pH van 7,5, met 7,5% (w/v) lactose. Een volledige recovery van de enzymatische activiteit van zowel Plg als t-PA werd gevonden. Plg bleek ook zijn fibrine-bindende vermogen te behouden na een vriesdroog-rehydratatie cyclus. Bovendien ontstond er geen t-PA lekkage, mits de omstandigheden gunstig gekozen werden.

In *Hoofdstuk 6* worden t-PA bevattende liposomen getest op hun thrombolytische activiteit en hun systemische bijwerkingen in een konijne-thrombose model. Liposomaal ingebouwd t-PA bleek thrombolytisch efficiënter te werken dan equimolaire doses vrij t-PA: 0,24 mg/kg liposomaal t-PA had een lysis-activiteit die vrijwel gelijk was aan die van een dosis van 1,0 mg/kg vrij t-PA. Liposomale inbouw bleek geen effect te hebben op de bijwerkingen van t-PA, zoals systemische activering van alpha-2-antiplasmine en plasminogeen. Uit deze in vivo experimenten concluderen wij dat een verbetering van de thrombolytische efficiëntie optreedt door t-PA in te bouwen in liposomen. Behandeling met Plg-t-PA-liposomen gaf soortgelijke resultaten als t-PA-liposomen zonder homing device. Dit kan betekenen dat de lage Plg oppervlakte-dichtheden die in dit hoofdstuk zijn gebruikt (ongeveer 10 maal lager dan in Hfdst. 3 en 7) niet voldoende waren voor efficiënte targeting van liposomaal t-PA.

Een evaluatie van de belangrijkste resultaten wordt gepresenteerd in *Hoofdstuk 7*.
Verder geeft dit hoofdstuk voorlopige resultaten van targetings-experimenten met Plg-liposomen (dichtheden vergelijkbaar met Hfdst. 3) in een stolsel-bevattend doorstroom systeem. Plg-liposomen bleken beter aan een fibrine stolsel en aan een bloedstolsel te binden dan vrij Plg. Dit gold zowel in buffer als in plasma (wat betekent dat een 100-voudige overmaat aan vrij, endogeen Plg aanwezig is). Wassen van het stolsel gaf geen vermindering te zien van de hoeveelheid gebonden liposomaal Plg, hetgeen wijst op een verhoogde affiniteit en langdurigere binding. Gebonden niet-liposomaal Plg kon daarentegen vrijwel geheel worden weggewassen. Door middel van toevoeging van vrij t-PA werden dynamische

lysis-omstandigheden nagebootst. Bij lage t-PA concentraties werd een versterkte stolsel-binding van liposomaal Plg waargenomen in overeenstemming met het ontstaan van nieuwe Plg-bindingsplaatsen in een stolsel tijdens thrombolyse. Bij hogere t-PA concentraties bleef liposomaal Plg gebonden, waarbij de gebonden hoeveelheid afnam bij afbraak van het stolsel. Op grond van deze veelbelovende resultaten concluderen wij dat verdere verbetering van de therapeutische index van t-PA mogelijk is door t-PA beladen liposomen te targeten door middel van gekoppeld Plg. Voor dit doeleinde moet liposomale inbouw van adequate hoeveelheden t-PA gecombineerd worden met koppeling van Plg in voldoende hoge dichtheden aan de buitenzijde van het liposoom. Dit kan mogelijk worden bewerkstelligd door Plg-liposomen te vriesdrogen, waarna vlak voor gebruik t-PA wordt ingebouwd tijdens het rehydratatie-proces.

Concluderend: dit proefschrift beschrijft de huidige stand van zaken in een project dat gericht is op het verbeteren van de therapeutische index van t-PA door gebruik te maken van getargete liposomen.

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Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 21 januari 1966 te Heemstede. In 1984 haalde zij haar VWO-B diploma aan het Atheneum Hageveld te Heemstede, waarna werd begonnen aan de studie Farmacie aan de Universiteit Utrecht. In 1985 werd de propedeuse behaald. Tijdens de bijvakperiode is onderzoek verricht bij de vakgroep Veterinaire Farmacie, Farmacologie en Toxicologie, begeleid door Dr. Niek Snoey.
Na het behalen van het doctoraalexamen Farmacie in 1989 werd in januari 1990 begonnen met het promotie (AIO)-onderzoek bij de vakgroep Klinische Farmacie van de Universiteit Utrecht, in samenwerking met het Gaubius Laboratorium, TNO-PG, te Leiden. In 1990 werkte zij gedurende 3 maanden aan de University of Tennessee, onder begeleiding van Prof. Dr. Leaf Huang. Het onderzoek heeft geleid tot het verschijnen van dit proefschrift.
In april 1994 werd de 4-jarige onderzoeksperiode verlengd met 2 jaar; na de promotie in juni 1995 zal het huidige onderzoek in de functie van post-doc worden voortgezet.

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