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Long-term stability of liposomes containing both tissue-type Plasminogen Activator and glu-plasminogen

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Abstract

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 2 h). 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- 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.

Keywords: Freeze-drying; Drug-stability; Drug-delivery system; Liposomes; t-PA; Plg

1. Introduction

For treatment of acute myocardial infarction aggressive thrombolytic therapy is used in Western societies (Gaffney, 1992). However, during thrombolytic treatment systemic effects can occur, such as bleeding episodes, which can be life-

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threatening when located intracranially (Collen, 1988). Moreover, thrombolytic treatment is associated with increased thrombin generation (Owen et al., 1988; Seitz et al., 1988; Seitz et al., 1993; Andreotti et al., 1993; Gram et al., 1993), 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. Crommelin and Schreier, 1994). 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 (Heeremans et al., 1995). 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 (Heeremans et al., 1992).

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 (Talsma and Crommelin, 1993; Grit et al., 1993a,b; Crowe et al., 1987). 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) into 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 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 and Crowe, 1993). 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.

2. Materials and methods

2.1. Materials

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, Germany).

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 and Papghadjopoulos (1982).

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

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

2.2. Vesicle preparation

2.2.1. 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 (Heeremans et al., 1994). Subsequently, the procedure as previously described (Heeremans et al., 1992) 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 et al., 1983, 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., 1965) 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 (3 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 min. After stopping the reaction with *N*-ethylmaleimide, the liposomes were separated from free protein by two ultracentrifugation steps (Beckman Instruments Inc., CA, USA) for 45 min, at 80 000 × *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.

2.2.2. 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' (Bangham et al., 1965). The lipid film was redispersed in 10 mM Hepes buffer[2], pH 7.5, after which t-PA was added. A t-PA incubation concentration of 2.0×10^5 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 (Hope et al., 1986; Chapman et al., 1990). Non-entrapped t-PA was removed by ultracentrifugation for 45 min, at 150 000 × *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 ultracentrifugation/t-PA before ultracentrifugation} × 100%.

2.2.3. Preparation of Plg- t-PA- 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 prevent 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.

[2] 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.

For comparison reasons 1 mg/ml of cysteine was also used during the first ultracentrifugation step for the Plg-liposomes (see Section 2.2.1.). The obtained pellet of (Plg-)liposomes was subsequently resuspended in 10 mM Hepes buffer[2], with a PL concentration of about 5 μmol/ml. Then the t-PA entrapment procedure by freeze-thawing was performed, as described before.

2.3. Freeze-drying experiments

For the freeze-drying experiments, samples of 0.25 ml of liposomes (either t-PA-containing, Plg-containing or Plg- plus 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 × 15 mm) in liquid nitrogen for 10 min. 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 min, at 150 000 × *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 × 100% (= [total entrapped + leaked t-PA after freeze-drying]/[total entrapped t-PA before freeze-drying] × 100%). *Retention* was calculated by c/b × 100% (= [entrapped t-PA after freeze-drying]/[entrapped t-PA before freeze-drying] × 100%).

2.3.1. Choosing the proper control conditions: 1 ultracentrifugation step instead of 2 (cf. Table 3)

As described in the Results section, ultracentrifugation of t-PA- containing liposomes (= both t-PA-liposomes and t-PA- Plg-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 × *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 the liposomes). 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 × 100% (= [entrapped t-PA after freezing]/[total entrapped plus non-entrapped t-PA after freezing] × 100%). The recovery of t-PA upon freeze-drying was calculated by c/a × 100% (= [total entrapped plus non-entrapped plus leaked t-PA after freeze-drying]/[total entrapped plus non-entrapped t-PA after freezing] × 100%). The t-PA retention after freeze-drying was calculated by d/b × 100% (= [entrapped t-PA after freeze-drying]/[entrapped t-PA after freezing] × 100%).

2.4. 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.

2.5. 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. (1978). 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.2HCl, which was measured by monitoring the absorbance of the reaction-product pNA (*para*-nitro-aniline) at 405 nm.

2.6. 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 Verheijen et al. (1982). 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.

2.7. Assessment of (loss of) Plg upon preparation of Plg-t-PA-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-liposomes, samples of (free) Plg and t-PA, with a concentration ratio as used during preparation of Plg-t-PA-liposomes, were incubated for 2 h. PPACK (D-Phe-Pro-Arg-Chloromethylketone), in a final concentration of 10 μM, was added immediately after taking a sample from the Plg- and t-PA- containing incubation mixture. PPACK is a small peptide which inhibits t-PA in an irreversible way (Mohler et al., 1986), 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.

2.8. Statistics

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

3. Results

3.1. Vesicle preparation

3.1.1. 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/μmol PL. These values are in agreement with those found in our previous study (Heeremans et al., 1992). However, upon freeze-thawing Plg-liposomes in the presence of cryoprotectant (prior to the freeze-drying procedure) the coupling ratio decreased by 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.

3.1.2. 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 (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.

Table 1
Liposomal t-PA entrapment as a function of the buffer medium used

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)

t-PA incubation concentration 2.0×10^5 IU/ml, PL incubation concentration 30 μmol/ml. Average values ± standard deviation are shown.

3.1.2.1. 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-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. 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.

3.1.3. Plg-t-PA-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

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

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)

Free Plg and t-PA are incubated for 2 h at pH 7.5, at room temperature, at a concentration ratio as used during preparation of Plg and 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.

take place, requiring special precautionary measures. Measurements of the Plg concentration upon a 2 h 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).

3.2. Freeze-drying experiments

3.2.1. 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).

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 was 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

Table 3
Recovery and retention of liposomal t-PA upon freeze-drying in the presence of variable buffer compositions

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)

Freeze-dried concentrations: PL 30 μmol/ml, t-PA between 0.9 and 1.6 × 10^5 IU/ml. Average values ± standard deviations are shown.

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 and freeze-drying procedure including two ultracentrifugation steps may therefore be ascribed to the damaging effect of the second ultracentrifugation step.

3.2.2. *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 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 conditions used 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 (Heeremans et al., 1992). 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).

3.2.3. *Freeze-drying of Plg-t-PA-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-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-

Table 4
Recovery and retention of liposomal t-PA and glu-plasminogen (Plg) upon freeze-drying t-PA-Plg-liposomes

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

Freeze-dried concentrations: PL 4 μmol/ml, t-PA 2 × 10^5 IU/ml, Plg between 1.5 and 3.0 × 10^2 μg/ml. Buffer medium: 10 mM Hepes buffer, 7.5% (w/v) lactose, pH 7.5. Average values ± standard deviations are shown.

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- 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.

3.3. 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 $2.8 \pm 0.4\%$ (w/w) was measured irrespective of the freeze-dried PL concentration.

4. 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 (Nieuwenhuizen, 1988). However, also several denatured proteins can be active in that respect (Radcliffe, 1983). Under our experimental conditions the reaction between t-PA and Plg was found to be very slow: only upon addition of fibrin fragments (Nieuwenhuizen et al., 1983) 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 (Heeremans et al., 1992), t-PA entrapment is best performed at 100 μmol/ml PL. 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-liposomes. Lyophilization (freeze-drying) in the presence of cryoprotectants is considered a promising means of extending the shelf-life of liposomes (e.g. Crowe et al., 1987; Crowe and Crowe, 1993; Özer et al., 1988; Talsma et al., 1991). 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. Isele et al., 1994). 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 (Nguyen and Ward, 1993). 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-liposomes), causing t-PA leakage, 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 and 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-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-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.

5. 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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