The Use of Fluorescence Resonance Energy Transfer to Study the Disintegration Kinetics of Liposomes Containing Lysolecithin and Oleic Acid in Rat Plasma

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Purpose. To validate Fluorescence Resonance Energy Transfer (RET) as method to monitor disintegration of fluorescently labeled liposomes varying in lysolecithin/oleic acid (equimolar) content, lysolecithin fatty acid composition and vesicle size in rat blood plasma and buffer.

Methods. NBD-PE and Rho-PE were used for RET. The measurements were performed on a Perkin Elmer LS-50 spectrofluorimeter. Liposomes were prepared by the extrusion method.

Results. Analysis of the RET data was optimised using a fitting procedure to correct for fluorescence interference by plasma. The disintegration patterns of liposomes could be described by a bi-exponential decay model. Disintegration rate increased at increasing lysolecithin/oleic acid content and decreasing size. In contrast, all liposomes showed no disintegration in buffer.

Conclusions. RET is a suitable method to monitor liposome disintegration in non-diluted plasma. Rate and extent of liposome disintegration increases at decreasing liposome size and increasing lysolecithin/oleic acid content.

KEY WORDS: Fluorescence Resonance Energy Transfer (RET); liposomes; lysolecithin; blood plasma

INTRODUCTION

Liposomes are vesicles whose aqueous volume is entirely enclosed by one or more membranes composed of a bilayer of phospholipids. Liposomes are able to entrap hydrophilic drugs within the aqueous compartment and amphiphilic and lipophilic drugs within the membrane.

The stability of liposomes in biological fluids is usually monitored by release of an entrapped hydrophilic dye like 6(5)-carboxyfluorescein (CF). The fluorescence of CF is quenched at high concentrations and enables measurements of the continuous release of the dye from the liposomes containing the self-quenching CF concentration without the need of separation of the liposomes from the incubation medium (1–3). However, even though the permeability of the liposomal membrane for CF is measured, no information is obtained on the integrity of the liposomal structure. The observed release of the dye could have been caused by serious packing defects without simultaneous loss of liposomal integrity (3). To get more information on the integrity of the membrane, we investigated therefore liposome stability in rat plasma using liposomes labeled with lipophilic fluorescent markers and the Fluorescence Resonance Energy Transfer method (RET). This technique has been used earlier to monitor e.g. membrane fusion of vesicles and drug exchange between liposomes in non biological media (4–6).

RET (4–5) is based on the transfer of the excited state energy from a fluorescence donor (FD) to an fluorescent acceptor (FA), provided that the emission spectrum of the FD partially overlaps with the excitation spectrum of the FA. RET can be observed by a decrease of the fluorescence of FD and an increase of the fluorescence intensity of FA, if the distance between FD and FA and is less than about 5 nm.

A widely used RET couple in liposome characterisation studies consists of NBD-PE (L-α-Phosphatidylethanolamine-N-(4-nitrobenzo-2-1,3-diazole) as fluorescence donor and Rho-PE (L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) as fluorescence acceptor. Just like with the carboxyfluorescein assay, RET measurements on a liposomal suspension can be performed without the need to isolate the liposomes from the incubation medium.

It is known that size, charge and lipid composition influence liposome stability in blood (7–9). Incorporation of phospholipids that are in the gel state at 37°C and/or cholesterol increase liposome stability in blood.

To validate the RET assay in plasma stable liposomes containing cholesterol were compared to labile liposomes containing lysolecithin and oleic acid. As will be explained below, these liposomes should be less stable in plasma. Lysolecithin is a naturally occurring impurity of phospholipids and its influence on membrane properties has been investigated by various groups (10–13). Although lysolecithin alone increases the permeability of the membrane for aqueous markers, it forms, codispersed with fatty acids, a lamellar bilayer vesicle. This is the result of an association of the fatty acid with the lysolecithin molecule, thus forming a complex with structural similarity to an intact phospholipid molecule. However, when they are dispersed in an aqueous solution together with serum albumin, the fatty acids are bound to the albumin, and the bilayer structure collapses, eventually forming lysolecithin micelles.

In this work, the capability of RET to monitor liposome disintegration in rat plasma and buffer was investigated in detail. Using this assay, the disintegration characteristics of the lysolecithin/oleic acid liposomes of different sizes were studied.

MATERIALS AND METHODS

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholin (POPC), purity 98%, and 1,2-dioleoyl-sn-glycero-3-phospho-

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ABBREVIATIONS: CF, 4(5)-Carboxyfluorescein; Chol, Cholesterol; FA, Fluorescence Acceptor; FD, Fluorescence Donor; Lyso-OPC, 1-Oleoyl-2-hydroxy-sn-glycero-3-phosphocholine; Lyso-PPC, 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine; Lyso-Soy-PC, Lysolecithin from soy bean; MPS, Mononuclear phagocytotic system; NBD-PE, L-α-Phosphatidylethanolamine-N-(4-nitrobenzo-2-1,3-diazole); OA, Oleic Acid; OOPS, 1,2-Dioleoyl-sn-glycero-3-phospho-L-serine; POPC, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; RET, Resonance Energy Transfer; Rho-PE, L-α-Phosphatidyl-ethanolamine-N-(lissamine rhodamine B sulfonyl)

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L-serin (OOPS), purity 97%, were synthesised at Novartis Pharma AG, Basel, Switzerland, using patented procedures .

Lyso-soy PC (Lysolecithin from soy bean), purity ≥ 96% was from Lipoid KG, Ludwigshafen, Germany. 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso-OPC), purity > 99%, was from Avanti Polar-Lipids, Inc. Birmingham, Alabama. 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso-PPC), purity > 95%, was synthesised at Novartis Pharma AG, Basel, Switzerland, using patented procedures. Cholesterol, purity > 99%, was from Merck KGaA, Darmstadt, Germany. Oleic acid, purity > 97%, and Triton® X-100 were from Fluka Chemie AG, Buchs, Switzerland. L-α-Phosphatidylethanolamine-N-(4-nitrobenzo-2-1,3-diazole) (Egg) NBD-PE, purity > 99%, and L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Egg) Rho-PE, purity > 99%, were from Avanti Polar-Lipids, Inc. Birmingham, Alabama. (Fluorescent markers attached to the head group). 4(5)-Carboxyfluorescein, purity > 99%, was from Fluka Chemie AG, Buchs, Switzerland.

Rat plasma was prepared from blood from animals in fasting state and collected in tubes containing EDTA. After centrifugation at 3000 *g* for 30 min. at 6 °C, plasma was used fresh or kept frozen at –70°C.

Preparation of Liposomes

Liposomes of defined sizes were prepared by extrusion technique (14). Lipids and markers were dissolved in chloroform, mixed, and dried under vacuum. The lipid film was hydrated with an isotonic 36 mM phosphate buffer (with 100 mM sodium chloride (pH 7.4), containing 0.001% NaN_3 as antimicrobial preservative) yielding 6.7 μmol lipid per ml buffer. In preliminary experiments it was observed, that a marker ratio of 1:1 in a concentration of 0.25 mole % each, leads to a RET of about 70–80%. In this range, RET degree is very sensible to minor changes in distances between donor and acceptor and therefore suitable to detect already small changes in membrane integrity. Therefore, fluorophore concentration in the lipid membrane was 0.25 mole %, and NBD-PE/Rho-PE molar ratio was (1:1). For the preparation of CF liposomes, the lipid film was hydrated with 100 mmol CF (36.2 mg/ml) in phosphate buffer.

The resulting multilamellar vesicles were extruded up to 15 times through polycarbonate filters with either 50, 100, or 200 nm pore size (Nucleopore, Pleasanton, CA) mounted in the mini-extruder (Avestin, Inc., Ottawa, Canada). Average size and size distribution of liposomes after extrusion was examined using photon correlation spectroscopy on a Malvern Lo-C Autosizer.

The CF liposomes were dialysed against phosphate buffer to remove free CF. Liposomes containing (Chol/POPC/Lyso-OPC) 1:1:1 were analysed for chemical stability of the phospholipids by HPLC (as described in reference 15) directly after preparation and after storage at 4°C for 7 weeks. During this period Cholesterol and POPC content decreased (2%, and 5% respectively), whereas lysolecithin content increased (4%). The decrease of content is within acceptable limits. The increase of lysolecithin is a consequence of the degradation of POPC. Liposomes were therefore stored at 4°C and generally used within 2 weeks after preparation.

Fluorescence Resonance Energy Transfer Measurements

Fluorescence measurements were performed on an Perkin Elmer LS 50 Spectrofluorimeter. To monitor liposome disintegration in rat plasma, samples were excited at 470 nm where NBD-PE shows a characteristic absorption peak. The emission maximum of Rho-PE (590 nm) was the observation wavelength for time scans. Fluorescence was recorded every 2 s. Fluorescence emission spectra were recorded from 480 nm to 650 nm.

Six hundred microliters of plasma were preincubated in a 114 F-QS Cuvette (Hellma S.A., Basel, Switzerland) at 37°C for 10 minutes. Thirty microliters liposomal suspension (6.7 μmol lipid per ml, marker concentration 0.25 mole%) were added to the plasma and immediately mixed using a pipette. In order to disintegrate the fraction of liposomes still intact after the incubation period and to obtain the minimal value of RET, 30 μl of 20% Triton® X-100 solution was added after the final recording, resulting in 1 % Triton® X-100 concentration in the sample. Emission values obtained after addition of Triton® X-100 were corrected for sample dilution and the interference of Triton® X-100 on the quantum yield of NBD-PE and Rho-PE.

Carboxyfluorescein Leakage Assay

The continuous measurement of carboxyfluorescein leakage from liposomes containing carboxyfluorescein at self-quenching concentrations in plasma was performed as described in detail in (1).

Statistical Analysis

Statistical comparison of the results was done by analysis of variance (ANOVA) and simple linear regression. Statistical hypotheses were tested at the 0.05 significance level. Comparison of means was performed using the Bonferroni t-test of differences between means for all main effect means. Bound for mean comparisons were 95%. All statistical calculations were performed using *SAS/LAB*°® Version 6 (80). Data are expressed as mean ± S.D.

RESULTS

In order to assess the degree of RET of the fluorescently labeled liposomes in plasma a new data analysis approach was used and compared to the one used in the original paper of Struck and Hoekstra (4). In principle, in media where fluorescence interference (e.g. aqueous buffers) is not be expected and concentration and quantum yields of the fluorescent probes stay constant, the RET degree can be calculated from the changes of either donor fluorescence intensity or acceptor fluorescence intensity. Based on this underlying principle the RET degree was measured using the following approach.

Emission spectra of liposomes containing 0.25 mol% NBD-PE alone, and of liposomes containing 0.25 mol% NBD-PE and 0.25 mol% Rho-PE were recorded in buffer (excitation wavelength was 470 nm). Fluorescence emission spectra of stable cholesterol and labile lysolecithin / oleic acid liposomes were recorded in buffer and after addition of Triton® X-100.

RET for liposomes containing both fluorescence markers can be calculated from the fluorescence intensity at 530nm (I) where the NBD-PE emission is at its maximum and where the Rho-PE emission is almost zero (4). As a reference point, the emission intensity (I_0) where no RET can be observed (i.e. liposomes containing only NBD-PE) has to be known.

$$RET = 1 - I/I_0. \quad (1)$$

Equation (1) uses only spectral information at one wavelength and is only valid if concentrations and quantum yields are the same when I and I_0 are measured. In order to correct for fluorescence interference due to plasma components resulting in variable I and I_0 values, Eq. (1) can be rewritten for the whole spectral range of fluorescence emission yielding Eq. (2).

$$I_\lambda = C \cdot \{(1 - RET) \cdot I_{NBD,\lambda} + RET \cdot I_{Rho,\lambda}\} \quad (2)$$

I_λ is the fluorescence intensity of the sample at wavelength λ after excitation at 470 nm, $I_{NBD,\lambda}$ is the fluorescence intensity of NBD-PE liposomes at wavelength λ after excitation at 470 nm, $I_{Rho,\lambda}$ is the intensity of Rho-PE at λ for complete energy transfer after excitation at 470 nm, RET is the degree of energy transfer, and C is a constant factor proportional to fluorophore concentration and quantum yield. If concentrations and quantum yields are constant when I_λ and $I_{NBD,\lambda}$ are measured, C is equal to 1. $I_{Rho,\lambda}$ can be calculated from measured fluorescence spectra of a Rho/NBD-liposome solution and a NBD-liposome solution with precisely equal NBD and phospholipid concentrations where RET is calculated using Eq. (1).

Since now entire spectras are compared with each other instead of emissions at a fixed wavelength, it is not necessary any more that the absolute concentrations and quantum yields stay constant. The only assumption to use this method is that the **ratio** of Rho-PE and NBD-PE concentrations and quantum yields will stay constant during the measurement.

The measured fluorescence spectra of liposomes in rat plasma were then fitted with Eq. (2) to calculate the two unknowns C and RET, using a least-squares method with C and RET as adjustable parameters. The experimental data showed that C changed in some cases up to 20% during incubation in rat plasma. This is most likely due to the fact that the environment and hence the quantum yield of the fluorescent markers changes during the decay of the liposomes. This observation proves the necessity of using Eq. (2) and not Eq. (1) to calculate RET in plasma.

In order to validate the RET assay in plasma, four liposome compositions differing in plasma stability were prepared giving Chol/POPC/OOPS (molar ratio 3:1:6), POPC/OOPS (molar ratio 9:1), Lyso-OPC/OA/POPC (molar ratio 1:1:1), and Lyso-OPC/OA (molar ratio 1:1). Liposomes were fluorescently labeled as described in methods section. All formulations were extruded through 50nm nucleopore filters. The mean diameter and standard deviations were 86nm ± 2nm for the Chol/POPC/OOPS and the POPC/OOPS liposomes, and 76nm ± 2nm for the lysolecithin containing liposomes. Polydispersity (range from 0-1) was below 0.1 for all liposomes, indicating a monomodal size distribution.

In Figure 1, fitted time scan curves (RET calculated using Eq. (2)) representing the disintegration of the four different liposome formulations in plasma at 37 °C are shown. The various liposome formulations exhibit clear differences in decay. After 1h of incubation only a minor fraction of the cholesterol containing liposomes disintegrates before addition of Triton® X-100. The POPC/OOPS liposomes disintegrate to a larger extent of about 50% in 1h. Incorporation of lysolecithin clearly increases the disintegration rate. After 1h of incubation 83% of the Lyso-soy PC/OA/POPC liposomes are disintegrated. Lyso-PC/OA liposomes are almost completely disintegrated after a few minutes of incubation (Figure 1). In general, the disintegration of the liposomes could be approximated by a bi-exponentional fluorescence decay pattern using Eq. (3) with a first, fast and a second, slow phase.

$$RET = RET_\infty + A_1 \cdot e^{-k_1(t-t_0)} + A_2 \cdot e^{-k_2(t-t_0)} \quad (3)$$

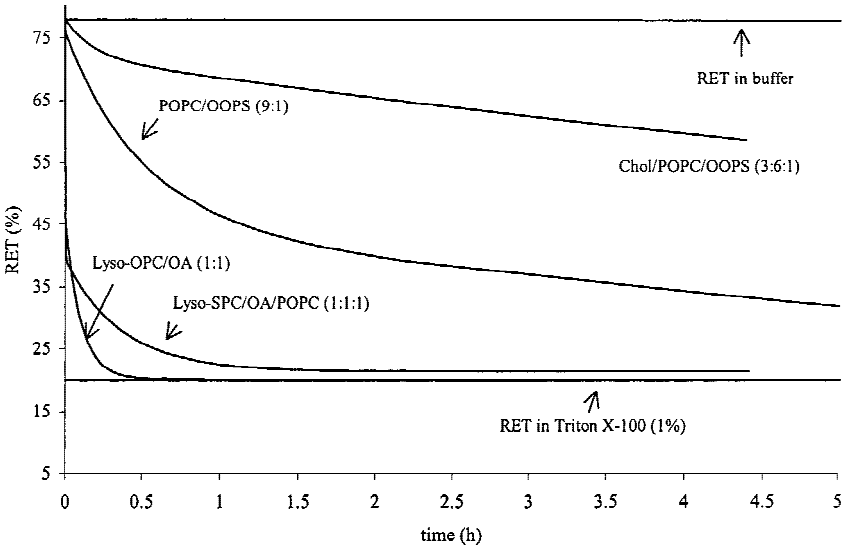

Fig. 1. Time scan curves, fitted using Eq. (3). (RET calculated using Eq. (2)) representing the disintegration of the four different liposome formulations in plasma at 37 °C are shown, demonstrating the effect of lysolecithin/OA and Cholesterol content on disintegration rate of liposomes.

RET_∞ is the RET of the totally disintegrated liposomes at $t = \infty$ (= RET after addition of Triton® X-100) , A_1 is the amplitude of the fast phase, A_2 is the amplitude of the slow phase. k_1 is the rate constant of the fast phase, and k_2 the rate constant of the slow phase. Time t is the actual incubation time, and t_0 the start time. Half life $t_{1/2}$ was calculated from k values using ($t_{1/2} = \ln 2 / k$).

The corresponding kinetic parameters of the various disintegration patterns of the liposomal formulations after incubation in rat plasma are calculated using Eq. (3) (Table I). Strong statistical evidence of differences between A_1, t_1 and t_2 was found ($p<0.001$). For A_1 the Bonferroni test gave 3 groups with statistically different means: Group 1 consisting of Lyso-OPC/OA, group 2 consisting of Lyso-OPC/OA/POPC and POPC/OOPS, and group 3 consisting of Chol/POPC/OOPS liposomes. For t_1 all liposomes show statistically different means. For t_2 the Bonferroni test yields 3 groups: Group 1 consisting of Lyso-OPC/OA, group 2 consisting of Lyso-OPC/OA/POPC, and group 3 consisting of POPC/OOPS and Chol/POPC/OOPS liposomes.

The half life t_1 of the Lyso-PC/OA (1:1) liposomes in plasma is an estimated value because it was too short to allow accurate calculation. The half life t_1 in plasma of the liposomes increased in the following order Lyso-PC/OA (1:1) < Lyso-PC/OA/POPC (1:1:1) < Chol/POPC/OOPS (3:6:1) <POPC/OOPS (9:1). The fraction of the liposomes which decays in a fast process (A_1) is 80% for Lyso-PC/OA (1:1), about 50% for Lyso-PC/OA/POPC (1:1:1) and POPC/OOPS (9:1), respectively. In case of Chol/POPC/OOPS (3:6:1) liposomes, only 10% of the liposomes disintegrate during the first phase. The half life t_2 of the liposomes increases in the following order Lyso-PC/OA (1:1) < Lyso-PC/OA/POPC (1:1:1) < POPC/OOPS (9:1) < Chol/POPC/OOPS (3:6:1).

Lyso-PC/OA/POPC (1:1:1) liposomes were also investigated using the CF leakage assay. It was found that CF release in rat plasma at 37°C from the lysolecithin containing liposomes was too fast to be detected. Under these conditions all CF was immediately released, whereas in buffer no CF leakage from the liposomes could be observed (detailed results not shown).

Starting from the liposomes composed of lysolecithin/oleic acid/POPC (molar ratio 1:1:1), three lysolecithins differing in fatty acid composition were compared with respect to disintegration in rat plasma: Lyso-oleoyl-PC (lyso-OPC), lyso-palmitoyl-PC (lyso-PPC) and lysolecithin from soy bean (lyso-soy-PC. The liposomes were extruded through 50nm nucleopore filters. The mean diameter and standard deviation of the liposomes was 76nm ± 2nm. The corresponding kinetic parameters of the various liposomal formulations after incubation in rat plasma are summarised in Table II.

Only the A_1 values of these different lysolecithin types containing liposomes show significantly different values ($p<0.035$). A_1 decreases in the following order: Lyso-soy-PC > Lyso-PPC > Lyso-OPC . According to the Bonferroni test 2 groups with statistically different means exist: Group 1 consisting of Lyso-soy-PC and Lyso-PPC, and group 2 consisting of Lyso-PPC and Lyso-OPC. No significant differences were found between t_1 and t_2 parameters of the formulations.

In order to study the influence of the size of the liposomes on disintegration kinetics in plasma, Lyso-OPC/OA/POPC (molar ratio 1:1:1) liposomes were prepared and sized by extrusion either through 50, 100 or 200 nm pore size nucleopore filters. The mean and standard deviation of liposome diameters were 76nm ± 2nm, 117nm ± 2nm, and 176nm ± 4nm, respectively. The corresponding kinetic parameters of these liposomes after incubation in rat plasma are summarised in Table III. It can be seen that an increase in size is associated with an increase in half lives t_1 and t_2, and a decrease in A_1. This demonstrates, that smaller liposomes disintegrate faster and to a larger extent during the first phase. Values for amount of disintegration, A_1 and A_2, of liposomes extruded through 50 nm pore size filters were significantly higher compared to A_1 and A_2 values of liposomes extruded through a 100 nm and 200 nm pore size filter, respectively. Half life t_1 of liposomes extruded through a 50 and 100 nm pore size filter was significantly shorter compared to t_1 of liposomes extruded through a 200nm pore size filter. From simple linear regression analysis of the logarithmic values of t_1 and t_2 it can be concluded, that an increase in size of the liposomes results in a slower disintegration.

DISCUSSION

Calculation of RET

In contrast to the original paper of Struck *et al.* (4), RET was calculated in this work using the whole spectral information of the emission scans instead of using the emission at one wavelength. The advantage of the calculation method of RET in this paper is that the results are independent on sample concentration and not influenced by interference caused by plasma environment. RET is calculated from the ratio of NBD-PE and Rho-PE emission.

The continuous scanning in time of fluorescence intensity of fluorescently labeled liposomes in rat plasma yielded bi-exponential decay curves. By means of kinetic modelling half

Table I. Influence of Lipid Composition on Kinetic Parameters of Liposome Disintegration in Rat Plasma at 37°C[a]

Group	Lipid composition	A_1 (%)	A_2 (%)	t_1	t_2
1	Lyso-OPC/OA (1:1)	82 ± 2	19 ± 2	4 ± 3 s	0.12 ± 0.07 h
2	Lyso-OPC/OA/POPC (1:1:1)	44 ± 8	56 ± 8	9 ± 3 s	0.33 ± 0.11 h
3	POPC/OOPS (9:1)	60 ± 10	40 ± 10	0.5 ± 0.17 h	3.61 ± 0.56 h
4	Chol/POPC/OOPS (3:6:1)	11 ± 4	89 ± 4	0.07 ± 0.05 h	9.2 ± 1.9 h
	Sets of means (Bonferroni)[b]	(1) (2, 3) (4)	(1) (2, 3) (4)	all means compare different	(1) (2) (3, 4)

[a] Values calculated from Eq. (3). Data are expressed as mean ± SD.

[b] Numbers in parenthesis are compared equal by Bonferroni t-test of differences between means for all main effect means. Bound for mean comparisons is 95%.

Table II. Influence of Fatty Acid Composition of Lysolecithin on Kinetic Parameters of Liposome Disintegration in Rat Plasma at 37°C[a]

Group	Lipid composition	A_1 (%)	A_2 (%)	t_1 (s)	t_2 (h)
1	Lyso-soy-PC/OA/POPC (1:1:1)	60 ± 5	40 ± 5	7 ± 1	0.31 ± 0.17
2	Lyso-PPC/OA/POPC (1:1:1)	50 ± 10	50 ± 10	9 ± 3	0.39 ± 0.18
3	Lyso-OPC/OA/POPC (1:1:1)	44 ± 8	56 ± 8	9 ± 3	0.33 ± 0.11
	sets of means (Bonferroni)[b]	(1, 2) (2, 3)	(1, 2) (2, 3)	all means compare equal	all means compare equal

[a] Values calculated from Eq. (3). Data are expressed as mean ± SD.
[b] Numbers in parenthesis are compared equal by Bonferroni t-test of differences between means for all main effect means. Bound for mean comparisons is 95%.

lives (t_1 and t_2) and extent of liposomal degradation (A_1 and A_2) can be calculated and enable comparison of the different liposome formulations. In Table I t_1 of Chol/POPC/OOPS liposomes is smaller than t_1 of POPC/OOPS liposomes. Although this result may suggest that liposomes containing cholesterol disintegrate faster than liposomes without cholesterol, it should be realised that during this first, fast process, about 60% of the non cholesterol containing liposomes are disintegrated and in case of the cholesterol containing liposomes only 10%.

With respect to the interpretation of the extent of disintegration in the slow phase, it should be realised that the slow phase is not relevant regarding the use of standard liposomes (without extended circulation time) as drug carriers for intravenous administration. In this period the liposomes have already mostly been taken up by the MPS (16) and interaction with plasma is irrelevant. However for stealth liposomes it could be of importance.

The carboxyfluorescein assay was not suitable to characterise the disintegration kinetics of lysolecithin containing liposomes under the experimental conditions used in this study. CF release after incubation in rat plasma appeared to be too fast and was not detectable. This fast release was only observed in plasma, not in buffer, indicating that indeed vesicles which were not permeable for CF were present in buffer. It can be concluded that the CF leakage assay is suitable for the characterisation of more stable liposomes in plasma or in media that do not promote vesicle disintegration.

In contrast, it was possible to study and compare the disintegration behaviour of these lysolecithin-containing liposomes with the RET method. RET efficiency is mainly influenced by concentration of the fluorophores in the membrane. RET can therefore still be used to monitor structural disintegration in spite of (partial) membrane disrupture. The sensitivity of the RET assay and correctness of the interpretation procedure was further underscored by the observations that liposomes which vary only slightly in size and composition showed distinctly different behaviours with respect to their fluorescence and RET patterns when incubated in rat plasma (Table III). It was possible to characterise liposomes consisting of Lyso-OPC/OA (molar ratio 1:1) with respect to their stability in buffer and rat plasma. In buffer these liposomes were completely stable, while only a few seconds after incubation in rat plasma they were almost completely disintegrated.

Liposome size, lysolecithin/OA ratio and cholesterol content influenced the *in-vitro* disintegration kinetics of liposomes significantly in rat plasma. The fatty acid composition of lysolecithin investigated in this study influenced the extent but not the half lives of liposome degradation. Liposomes larger than 100nm showed a much smaller tendency to disintegrate compared to liposomes smaller than 100nm. This size effect may be explained by the larger curvature of the smaller liposomes, making the surface less stable. Consequently, vesicle stability may not only be manipulated by lipid composition but also by choosing the appropriate size. (Table III). The mechanisms of disintegration may not be the same for the investigated liposome compositions. The lysolecithin containing vesicles will mainly disintegrate by extraction of the oleic acid out of the membrane by albumin, whereas the vesicles without lysolecithin become unstable by interactions of phospholipids with lipoproteins (lipid exchange) (7).

Beside the use as reference liposomes to validate the RET assay, these lysolecithin and oleic acid containing liposomes can also be of pharmaceutical interest for water insoluble drugs which can be formulated in liposomes and which are intended for treatment of disease targets outside the mononuclear phagocytic system (MPS) compartment. The fast disintegration of the liposomes should lead to complete transfer of the lipophilic drug to the blood components. A concomitant loss of the drug by uptake of the liposomes by the MPS can thus be avoided.

The properties of these fast disintegrating liposomes, containing a lipophilic model drug, and the influence of lipo-

Table III. Influence of Liposome Size on Kinetic Parameters of Lyso-OPC/OA/POPC (1:1:1) Liposomes Disintegration in Rat Plasma at 37°C[a]

Group	Extrusion pore size (nm)	Size (nm)	A_1 (%)	A_2 (%)	t_1 (s)	t_2 (h)
1	50	76 ± 2	44 ± 8	56 ± 8	9 ± 3	0.33 ± 0.11
2	100	117 ± 2	22 ± 8	78 ± 8	18 ± 8	1.78 ± 0.75
3	200	176 ± 4	23 ± 5	77 ± 5	80 ± 30	24.2 ± 0.67
	sets of means (Bonferroni)[b]	/	(1) (2, 3)	(1) (2, 3)	(1, 2) (3)	(1) (2, 3)

[a] Values calculated from Eq. (3). Data are expressed as mean ± S.D.
[b] Numbers in parenthesis are compared equal by Bonferroni t-test of differences between means for all main effect means. Bound for mean comparisons is 95%.

some formulation on the extent of drug release in human plasma and on the distribution of the drug among human plasma proteins, will be reported in a following publication.

CONCLUSIONS

By applying a fitting procedure to correct for fluorescence interference in plasma the RET method could be considerably improved and adapted for measurement of *in-vitro* liposome stability in rat plasma. Liposome size, Lyso-OPC/OA and cholesterol content influenced significantly the disintegration rate in rat plasma.

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