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CHARACTERIZATION OF PLASMA-STABILIZED LIPOSOMES COMPOSED OF DIOLEOYL-PHOSPHATIDYLETHANOLAMINE AND OLEIC ACID

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Summary: We have previously reported that small unilamellar liposomes (d≤200 nm) composed of dioleoylphosphatidylethanolamine and oleic acid can be stabilized by incubating with normal human plasma (Liu and Huang, Biochemistry 1989, in press). The stabilized liposomes were very stable even under relatively harsh conditions such as extreme pH, high salt and trypsin treatment. Fluorescence depolarization of diphenylhexatriene showed that the stabilized liposome had a high microviscosity in the lipid core, which did not decrease even after the majority of proteins were removed by trypsin. These data suggest that plasma proteins inserted into the lipid bilayer are probably responsible for the stabilization activity. After i.v. injection into mouse, stabilized liposomes showed a relatively low affinity to liver and spleen as compared to a conventional liposome composition.

Liposomes composed of unsaturated phosphatidylcholine (PC) are not stable in the serum or plasma, unless high concentrations of cholesterol are included in the membrane (for review see 1). Blood proteins such as the high density lipoprotein interact with the liposome membrane causing a rapid liposome leakage (1). We have recently reported that small (d≤200 nm), but not large, unilamellar liposomes composed of dioleoylphosphatidylethanolamine and oleic acid show unusual stability characteristics (2). These liposomes are not stable at 37°C in a simple buffered solution, but become stabilized when incubated with normal human plasma or serum. This behavior is just opposite to what is commonly found for the cholesterol-free, PC liposomes. We have also reported that plasma proteins and lipids are found in the stabilized liposomes and that

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Abbreviations: DOPE, dioleoylphosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; DOC, deoxycholate; DPH, 1,6-diphenyl-1.3.5-hexatriene; OA, oleic acid; PBS, phosphate buffered saline.

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oleic acid is extracted from liposomes by some plasma component (probably albumin). We now report a few additional observations about the plasma-stabilized liposomes which include the stability of the stabilized liposomes under extreme conditions, the microviscosity of the lipid core, and the *in vivo* biodistribution of the stabilized liposomes.

MATERIALS AND METHODS

Dioleoyl PE, egg PC, PS were purchased from Avanti Polar Lipids, Inc. Oleic acid, cholesterol, inulin, calcein and trypsin were obtained from Sigma Chemical Co. Freshly collected human plasma was obtained from Fort Sanders Regional Medical Center, Knoxville, TN. Tyraminyl inulin was synthesized according to Sommerman et al. (3) followed by standard radioiodination with ^{125}I performed by the chloramine T methods.

Small unilamellar vesicles containing either calcein (50 mM in PBS which was isotonic to plasma) or [^{125}I]tyraminyl inulin were prepared by sonication according to Liu and Huang (2). A trace amount of hexadecyl [^{3}H]cholestanyl ether was included in the lipid to monitor the lipid.

Calcein containing liposomes (20 mM) were incubated with an equal volume of human plasma for 1 hour at 37°C followed by gel chromatography using a Bio-Gel A 1.5m column to separate free plasma components from liposomes. To test the stability of liposomes, 200 µl of liposomes containing 0.4 µmole of lipids were mixed with either PBS, KCl (1M in PBS) or trypsin containing buffer in a final volume of 400 µl. The ones mixed with PBS were adjusted to a desired pH by NaOH or HCl and left at room temperature for 1 hour. The fluorescence intensity was measured with a LS-5 fluorescence spectrophotometer. Release of the entrapped calcein from the liposomes was calculated according to Liu and Huang (2).

Liposomes for protein association measurement were prepared in a simple PBS buffer and mixed with a Ficoll solution to a final Ficoll concentration of 20% after incubation with plasma. The mixture was placed in a 15 ml COREX centrifuge tube. 6 ml of 15% Ficoll were overlayed, followed by 0.5 ml PBS. The mixture was centrifuged in a Sorvall HB4 swing bucket rotor for 1 hour at 5000 rpm at room temperature. The liposomes floated to the PBS/15% Ficoll interface were collected and treated as described above. The liposomes were floated one more time after treatments. Protein content was determined by Lowry assay.

Fluorescence depolarization of DPH in liposome was measured by a method similar to that described by Shinitzky and Barenholz (4) by including 0.2% (mole/mole) of DPH in lipid composition. The anisotropy was calculated using the following equation:

$$\gamma = \frac{I_{\parallel} - I_{\perp} \times G}{I_{\parallel} + 2 I_{\perp} \times G}$$

where $I_{\parallel}$ is the fluorescence intensity parallel to and $I_{\perp}$ is the intensity perpendicular to the plane of polarization of the excitation beam. G is the correction factor obtained using horizontally polarized light ($G = I_{\perp} / I_{\parallel}$). Fluorescence was measured with excitation at 360 nm and emission at 430 nm.

150 µl [^{125}I]tyraminyl inulin containing liposome (1 µmole lipid) was administered intravenously by tail vein injection into Balb/c mice (6-8 weeks old, male). After 5 hours, the mice were anaesthetized with diethyl ether and bled by eye puncture. Blood collected was weighed for each mouse. The mice were then killed by cervical dislocation and all organs were collected. The individual organ was counted for ^{125}I-counts in a gamma counter. The total

radioactivity in the blood was determined by assuming the total volume of blood was 7.3% of the total body weight (5).

RESULTS AND DISCUSSION

We have previously shown that small (d≤200 nm) sonicated liposomes composed of DOPE and OA can be stabilized by incubation with normal plasma or serum (2). These liposomes contain a large amount of serum protein (~0.5 mg protein/mg lipid). In order to further test the stability of the plasma-stabilized liposomes, we have incubated them in relatively harsh conditions and examined the loss of protein from liposome and the release of entrapped calcein (Table 1). Incubation in alkaline (pH 10) or high salt (0.5M KCl) solutions for 1 hour at room temperature did not cause any loss of protein from liposomes. Only a small fraction (<30%) of the entrapped calcein was released during incubation. Incubation of liposome in an acidic (pH 3) solution caused about 30% loss of protein, but less than 20% release of calcein. These results indicate that the stabilized liposomes are quite stable even in relatively harsh conditions. This is to be compared with the original DOPE:OA liposomes which show a complete release of entrapped calcein at pH below 5 (2).

Table 1. Stability of plasma-stabilized liposomes

Treatment[a]	Protein Content[b] (%)	Calcein Release[c] (%)
pH 7.4	100 (n=9)	0
pH 3.0	73.8 ± 2.8 (n=9)	17.9 ± 1.3
pH 10.0	99.9 ± 6.7 (n=12)	21.0 ± 5.9
KCl	98.3 ± 11.2 (n=9)	27.3 ± 3.5
Trypsin	33.0 ± 6.0 (n=12)	7.9 ± 3.5

[a]Plasma-stabilized liposomes (2.5 μmole in 0.5 ml PBS) were incubated with either equal volume of PBS with a final pH of 7.4, 3.0, 10.0 or with PBS containing 1 M KCl at room temperature for 1 hour or with PBS containing 1.0 mg/ml trypsin for 1 hour at 37°C.

[b]Normalized to the protein content of the plasma stabilized liposomes treated with the pH 7.4 buffer which was approximately 0.5 mg protein/mg lipid.

[c]The pH was adjusted back to 7.4 and calcein release was measured fluorometrically immediately after the treatment (n=4).

Table 2. Fluorescence depolarization of DPH in liposomes

Liposome Treatment	Fluorescence Anisotropy[c]	Microviscosity[f] (poise)
PBS	0.121 ± 0.002	1.205 ± 0.013
Plasma[a]	0.158 ± 0.004[d]	1.859 ± 0.026[d]
Plasma then trypsin[b]	0.160 ± 0.005[e]	1.901 ± 0.034[e]

[a]Liposomes containing 0.2 mol % DPH were incubated with PBS or 50% plasma for 1 hr at 37°C, followed by chromatography through a Bio-Gel A1.5m column.
[b]Plasma-treated liposomes were further incubated with trypsin (0.5 mg/ml final) for 1 hr at 37°C. Trypsin inhibitor (2.5 mg/ml final) was added to stop the reaction. Liposomes were gel-filtered through a Bio-Gel A1.5m column to remove free proteins.
[c]Measured in 5 determinations.
[d]$p < 0.0002$ when compared with values for PBS treatment.
[e]$p < 0.0001$ when compared with values for PBS treatment.
[f]Calculated according to Shinizky and Barenholz (9).

The stability of the stabilized liposomes is apparently not strongly dependent on the associated proteins, because the liposomes released less than 10% calcein even when 67% of the protein was removed by a trypsin treatment (Table 1). SDS-PAGE of trypsin-treated liposomes has indicated that all of the major proteins originally associated with liposomes had been removed except some very low molecular-weight peptides which did not resolve well in the gel (data not shown). These results suggested that most proteins associated with the stabilized liposomes are accessible to trypsin, and the integrity of liposome does not depend on these proteins. Furthermore, the trypsin-resistant, residual peptides may be tightly associated with the lipid membrane, perhaps inserted into the lipid bilayer. These peptides could be important for liposome stabilization.

If peptides or a portion of serum proteins are inserted into the lipid membrane of liposomes, the microviscosity of the membrane is expected to increase significantly. This phenomenon has been observed in the interactions with lipid bilayer of several bilayer-inserting proteins, such as cytochrome b_5 (6), mellitin (7), and apolipoproteins (8). We have examined this possibility

by measuring the fluorescence depolarization of a hydrophobic probe, DPH, which is widely used to measure the microviscosity of a lipid membrane (for review see 9). As can be seen in Table 2, a large increase of fluorescence polarization of DPH was observed in the plasma-stabilized liposomes. The microviscosity of the lipid core, calculated according to Shinizky (9), had increased from 1.2 to 1.9 poise by the plasma treatment. This result indicates that the mobility of DPH in the membrane of plasma-treated liposome is significantly reduced, probably due to the insertion of plasma proteins into the lipid bilayer. Importantly, the removal of most of the liposome-associated proteins by trypsin did not reduce the membrane microviscosity (Table 2). This result is consistent with the notion that trypsin does not remove the inserted peptides or proteins which could be essential for the stability of liposome.

Two important features of liposome stabilization by plasma or serum are that (1) only small ($d \leq 200$ nm) liposomes are stabilized, and (2) only PE-, but not PC-, based liposomes are stabilized (2). We have interpreted these observations by the facts that (1) DOPE molecule has a small and poorly hydratable head group (10) and relatively bulky acyl chains, (2) amphipathic molecules like DOPE are poorly packed in a concave surface such as the outer monolayer of a small liposome of high curvature (11), (3) lipids and proteins having a richly hydrated head group and relatively non-bulky hydrophobic portion can be easily inserted into the outer monolayer of the small liposome enriched with DOPE, bringing about liposome stabilization. Thus, we suspect that the apolipoproteins in the plasma or serum are prime candidates of the liposome stabilizer, because they contain amphipathic helices which interact strongly with phospholipids to form discoid structures (8,12). Insertion of the amphipathic helix to the outer monolayer of the small liposomes would promote tighter molecular packing and hence more stable bilayer. These helices would be resistant to trypsin digestion because they are partly "buried" in the membrane. They would also increase the microviscosity of the lipid core of the liposome membrane due to lipid-protein interactions. We are currently testing these hypotheses.

Table 3. Biodistribution of liposomes in mouse[a]

Tissue	DOPE:OA (2:1)			PC:chol:PS (10:5:1)
	Untreated (n=5)	Serum Treated (n=5)	Serum Treated and Trypsinized (n=5)	Untreated (n=3)
Blood	8.02±0.90	8.31±1.06	7.07±0.41	2.73±0.72
Spleen	2.90±0.36	3.93±0.42	2.68±0.32	3.55±0.68
Liver	31.44±0.68	43.85±1.18	40.88±1.41	24.77±3.22
Lung	0.35±0.06	0.33±0.10	0.28±0.03	0.09±0.02
Chest	1.81±0.30	2.19±0.34	1.92±0.2	0.52±0.16
Intestine	2.05±0.50	2.19±0.20	2.15±0.33	0.94±0.07
Heart	0.19±0.04	0.15±0.05	0.13±0.03	0.34±0.52
Trunk	1.49±0.45	2.15±0.31	2.55±0.44	1.05±0.40
Head	2.80±0.67	2.51±0.29	2.08±0.15	1.70±0.15
Upper Limb	1.23±0.31	1.15±0.29	0.82±0.20	0.90±0.47
Lower Limb	1.24±0.26	1.30±0.13	0.84±0.19	0.76±0.44
Kidney	1.67±0.26	0.96±0.09	0.86±0.07	0.48±0.03
Skin	2.26±0.25	2.09±0.63	1.65±0.43	6.62±2.54
Tail	1.82±0.61	2.47±0.98	6.19±3.2	0.94±0.06
% Recovered	60.24±2.9	74.75±2.58	70.23±2.75	45.38±4.19
Res/Blood[b]	4.34±0.60	5.80±0.68	6.18±0.49	11.00±3.72[c]

[a]Data expressed as % injected dose. Liposomes were labeled with [^{125}I]tyraminyl inulin.

[b]RES/blood = % doses in liver and spleen/% dose in blood.

[c]Significantly different from the other three, $p < 0.0001$ (F test).

While the molecular identity of the stabilizer is still unknown, we have tested a biological property of the stabilized liposome, i.e. biodistribution *in vivo*. Since we used mouse as an animal model, the liposomes were stabilized with mouse serum which had given a stabilization activity identical to that of human plasma. The stabilized liposomes were injected into the tail veins of Balb/c mice and the biodistribution of liposome was measured 5 hours later by following the distribution of an entrapped aqueous marker, [^{125}I] tyraminyl inulin. This radioactive marker is not metabolizable by mammals and free unencapsulated marker is rapidly ($t_{\frac{1}{2}}$=few minutes) excreted from the mouse (3). As can be seen in Table 3, DOPE:OA liposomes mainly accumulated in liver (about 31% of injected dose). Blood was the second highest compartment for liposome distribution with approximately 8%. The rest had spread in various other organs and tissues. DOPE:OA liposomes stabilized with mouse serum and then injected into mouse showed a similar biodistribution with a higher level of liver uptake.

Serum-stabilized liposomes which were trypsin-treated before injection also showed a similar biodistribution. The uptake of liposomes in liver and spleen is generally regarded as uptake by the reticuloendothelial system (RES) (for recent review see 1). The RES/blood ratio is a sensitive index for the relative affinity of liposome to the RES, because lower level of RES uptake is usually accompanied with a higher level in the blood (13). The RES/blood ratios for the three DOPE:OA liposome preparations are not significantly different from each other. This result is not surprising because the DOPE:OA liposomes would be coated with the same proteins, once injected into the blood of the animal, as the ones stabilized with serum in vitro. SDS-PAGE of the serum-stabilized liposomes treated with trypsin and exposed to serum again, showed nearly identical protein bands on the gel (data not shown). If some of the associated proteins serve as opsonins for RES uptake, as has been suggested by other investigators (13,14), similar degrees of RES uptake of these three liposome preparations are expected. On the contrary, the biodistribution of liposome of a commonly used lipid composition, i.e. egg PC:cholesterol:PS (10:5:1), showed a significantly higher affinity for RES with a RES/blood ratio significantly greater than those of the other three. These results indicate that the serum-stabilized liposomes originally composed of DOPE:OA show a relatively low affinity to RES and hence stay in blood for a relatively long period of time. Recently, Allen et al (15) and Gabizon and Papahadjopoulos (13) have independently developed liposomes which would stay in circulation for a relatively long period of time. One of the important ingredients of liposome composition is glycolipids such as ganglioside GM_1. The present liposome system does not contain any glycolipids. Nevertheless, the liposomes already show the signs of prolonged circulation time. This is an important result because liposomes with prolonged circulation halflife generally show a relatively high level of tumor accumulation (13). Thus, the drug delivery potential of the DOPE:OA liposomes looks favorable and will be further evaluated in future experiments.

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