Biochimica et Biophysica Acta, 943 (1988) 53–62
Elsevier

BBA 74084

Syntheses of lipophilic amino and carboxyl components for the functionalization of liposomes

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(Received 1 February 1988)

Key words: L-Lysine derivative; Palmitoyl derivative; Functionalized liposome; Free flow electrophoresis; Antibody coupling;

L-Lysine, 2-hydroxyethylpalmitoylamide and DL-glyceric acid were used as starting material for the synthesis of N^2-palmitoyl-L-lysine methyl ester; N^2-palmitoyl-N^6-succinoyl-L-lysine; $N^{2,6}$-dipalmitoyl-L-lysine; *N*-(2-succinoylethyl)palmitoylamide and DL-2,3-dipalmitoylglyceric acid. By means of the detergent dialysis method, the lipophilic amino-acid and carboxylate components were incorporated into liposomal membranes in such a way that one liposome carried 500–2000 functional residues on its outer side. The resulting unilamellar liposomes had hydrodynamic diameters of 60–80 nm and a population homogeneity of 22–44%. Both parameters remained nearly constant for 60 days. Being polycations, liposomes functionalized with different amounts of amino components exhibited distinct mobilities in free-flow electrophoresis. The amino functions of the liposomes were derivatized with maleinimido residues to which sulfhydryl components like 2-mercaptoethanol as a model compound were covalently linked. Preliminary results showed that liposomes functionalized with carboxylate components, could be coupled to antibodies in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

Introduction

Antibodies or antibody fragments can direct liposomes with incorporated drugs to target cells carrying the correspondent antigens [1–3]. The covalent linkage of the antibodies to liposomes has been the subject of a number of studies [3,4].

One coupling method [4] involves periodate oxidation of lipid residues incorporated into the liposome surface to form reactive aldehyde functions. Antibodies were then linked to the surface aldehyde groups through Schiff-base formation, followed by a reduction step.

By incorporation of lipophilic thioreactive groups into the liposome surface thioreactive liposomes were obtained. These functionalized liposomes were reacted with reduced Fab' fragments of IgG [5], or with thiolated antibodies [6–9].

According to another concept antibodies were attached via their amino groups to liposomes functionalized with *N*-hydroxysuccinimidyl residues [10].

The conjugation of the carboxyl groups of the antibodies to liposomes containing either amino

Abbreviations: PC, phosphatidylcholine; PBS, phosphate-buffered saline (150 mM NaCl/1 mM NaH_2PO_4); MPS, *N*-(3-maleinimidopropionyloxy)succinimide; PE, phosphatidylethanolamine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

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residues [11] or carboxyl residues [12] was performed using water-soluble carbodiimide as condensation agent.

Furthermore, liposomes were prepared containing differently functionalized cholesteryl derivatives to which the binding of human IgG was ascertained [13].

Apart from the advantages and disadvantages of these different concepts, it has not been cleared yet whether the methods are practicable on a preparative scale or result in defined liposomes being unaffected by storage over a longer period of time. We tried to work out simple methods for the preparation of non-toxic, functionalized liposomes with defined quality on a preparative scale. In the following we report on the synthesis of lipophilic amino and carboxylate components and their incorporation into the membrane of unilamellar liposomes.

Materials and Methods

Materials

N^6-Benzyloxycarbonyl-L-lysine · HCl [14], *N*-(2-hydroxyethyl)palmitoylamide [15] were synthesized according to methods already published. Palladium (10%) on carbon, DL-glyceric acid hemicalcium salt, palmitoylchloride, palmitic acid anhydride, succinic acid anhydride and L-lysine · HCl were purchased from Aldrich; egg phosphatidylcholine, phosphatidylethanolamine from Lipid Products. Cholesterol, stearylamine, MPS and DTNB were obtained from Fluka; [^{3}H]cholesterol was from Amersham.

Pyridine was distilled over KOH. Distilled methylene chloride, tetrachlorocarbon and pyridine were stored over molecular sieves. All solid reagents were dried over P_4O_{10} before use.

Methods

TLC was performed on Merck silica-gel 60 F_{254} plates. Merck silica-gel 60 (230–400 mesh) and Sephadex G-50 (Pharmacia) were used for the column chromatography. Concentration of the reaction mixtures and of the pooled fractions was effected at 50 °C with rotation evaporation under aspiration. The specific rotation of the optically active compounds was measured on a Perkin Elmer polarimeter 241.

N^6-Benzyloxycarbonyl-L-lysine methyl ester · HCl (1)

To a well-stirred mixture of N^6-benzyloxycarbonyl-L-lysine · HCl (22 g, 69.5 mmol) in dry CH_3OH (300 ml) freshly distilled thionyl chloride (18.7 ml, 258 mmol) was added dropwise during 45 min while the temperature was kept below −55 °C. The reaction mixture was stirred for 18 h at room temperature and evaporated to dryness. The residue was treated three times with CH_3OH/C_6H_6 (1 : 1, v/v) and evaporated each time, then dissolved in $CHCl_3$ (50 ml) and chromatographed on a silica-gel column (see Table I, run No. 1). Fractions containing the required product were evaporated to dryness to give 14.5 g (63%) of analytically pure (**1**), having an m.p. of 117 °C; **1** was homogeneous (R_F 0.82) by TLC using $CHCl_3$/EtOH (9 : 1, v/v).

N^2-Palmitoyl-L-lysine methyl ester · HCl (2)

To a stirred solution of (**1**) (20 g, 61 mmol) dissolved in dry CH_2Cl_2 (320 ml) was added palmitic acid anhydride (37 g, 75 mmol) at room temperature in portions. The pH of the reaction mixture was adjusted to 8–9 with triethylamine.

TABLE I

CONDITIONS OF THE COLUMN CHROMATOGRAPHIC PURIFICATION OF THE LYSINE DERIVATIVES ON SILICA-GEL USING MIXTURES OF $CHCl_3$/EtOH AS THE ELUENTS

Run no.	Column length × i.d. (cm)	Step no.	Eluent $CHCl_3$/EtOH (v/v)	Vol. (l)	Isolated product no.
1	27×9	1 [a]	90/10	3.5	**1**
2	27×9	1	100/0	2.0	–
		2	97/3	2.5	–
		3 [a]	90/10	2.5	–
3	40×5	1	95/5	2.0	–
		2	90/10	2.0	–
		3 [a]	80/20	2.5	**2**
4	30×5	1	97/3	2.0	–
		2 [a]	90/10	2.0	**4**

[a] Desired product was isolated at this step.

After 2.5 h the mixture was evaporated to a gum in vacuo, dissolved in ethyl acetate (1 l) and subsequently extracted with 1 N citric acid (200 ml), 1 N $NaHCO_3$ (200 ml) and H_2O (200 ml) in turn. The organic layer was evaporated in vacuo to a gum which was dissolved in $CHCl_3$ (50 ml) and chromatographed on a silica-gel column according to the conditions given in Table I, run No. 2. Fractions containing the required product were evaporated to dryness to give 23 g (71%) of N^2-palmitoyl-N^6-benzyloxycarbonyl-L-lysine methyl ester · HCl (**A**), which was homogeneous (R_F 0.92) by TLC using $CHCl_3$/EtOH (9:1, v/v) and having an m.p. of 80°C. For the hydrogenolytic cleavage of the benzyloxycarbonyl residue, **A** (16 g, 30.0 mmol) was dissolved in $CHCl_3$ (150 ml) and 10% Pd/C (1 g) was added. While shaking, H_2 was introduced into the suspension at a slightly increased pressure until no more H_2 was absorbed. During the hydrogenolytic cleavage the pH was kept at 5 by adding HCl. The catalyst was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in $CHCl_3$ (25 ml) and chromatographed on a silica-gel column (see Table I, run No. 3). Fractions containing the required product were evaporated to dryness to give 11.1 g (85%) of analytically pure **2**, having an m.p. of 167°C and a specific rotation of $[\alpha]_D^{20} = -0.189$ ($c = 1$, $CHCl_3$/MeOH, 1:1). **2** was homogeneous (R_F 0.48) by TLC using $CHCl_3$/EtOH (9:1, v/v).

$C_{23}H_{46}N_2O_3Cl$ (434.1):

	C	H	N	Cl
calc.:	63.64	10.68	6.45	8.17
obsv.:	63.21	11.01	6.24	8.00.

N^2-Palmitoyl-N^6-succinoyl-L-lysine (3)

Succinic acid anhydride (0.5 g, 5 mmol) was added to (**2**) (2 g, 46 mmol) and dissolved in dry pyridine (50 ml). After 3 h of stirring at room temperature, additional succinic acid anhydride (100 mg) was added. After 18 h of stirring the solution was evaporated to dryness. The residue was dissolved in $CHCl_3$ (100 ml) and extracted with 10% citric acid (50 ml) once and twice with H_2O (200 ml). The evaporated $CHCl_3$ layer yielded a white powder (2.2 g, 88%) of N^2-palmitoyl-N^6-succinoyl-L-lysine methyl ester. The methyl ester was removed by treating the white powder (1.95 g) dissolved in MeOH (32 ml) with 4 N NaOH (10.6 ml). The end of the reaction was detected by TLC. The reaction mixture was added to $CHCl_3$ (200 ml) and extracted with 10% citric acid (200 ml) and subsequently with 2% $NaHCO_3$ (200 ml). The $NaHCO_3$ layer was acidified with 1 N HCl and extracted three times with $CHCl_3$ (150 ml). The combined $CHCl_3$ layers were evaporated to dryness, yielding 1.2 g (49.5%) of analytically pure **3**, having an m.p. of 118–119°C and a specific rotation of $[\alpha]_D^{20} = +0.2198$ ($c = 1$, $CHCl_3$/MeOH, 1:1). **3** was homogeneous (R_F 0.19) by TLC using $CHCl_3$/EtOH (1:1, v/v).

$C_{26}H_{48}N_2O_6$ (484.7)

	C	H	N
calc.:	64.43	9.98	5.78
obsv.:	64.78	9.59	5.80.

$N^{2,6}$-Dipalmitoyl-L-lysine (4)

Palmitic acid chloride (20 g, 70 mmol) was added dropwise for 1 h at room temperature to L-lysine · HCl (5.48 g, 30 mmol) dissolved in H_2O (100 ml). The pH value of the mixture was adjusted with 0.1 N NaOH to 10–11 and maintained for 5 h. After stirring for 14 h at room temperature the solution was extracted three times with $CHCl_3$ (1 l). The $CHCl_3$ layers were evaporated to yield a white powder (15.4 g) which was extracted in a soxhlet extractor with Et_2O for 48 h. The residue (7.01 g) was suspended in $CHCl_3$ (100 ml) and chromatographed on a silica-gel column (see Table I, run No. 4). Fractions containing the required product were evaporated to dryness, yielding 6.26 g (32.9%) of analytically pure **4**, having an m.p. of 115–125°C and a specific rotation of $[\alpha]_D^{35} = +0.1099$ ($c = 1$, $CHCl_3$/DMF, 1:1). **4** was homogeneous (R_F 0.53) by TLC using $CHCl_3$/EtOH (4:1, v/v).

$C_{38}H_{44}N_2O_4 \cdot 0.5\ H_2O$ (632.0)

	C	H	N
calc.:	72.21	11.96	4.43
obsv.:	72.45	12.44	4.19.

N-(2-Succinoylethyl)palmitoylamide (6)

A mixture of *N*-(2-hydroxyethyl)palmitoylamide (**5**) (5 g, 16.5 mmol) and succinic acid anhydride (3.3 g, 33 mmol) was melted and then stirred for 3 h at 120 °C. Succinic acid anhydride which sublimated during the reaction was given back to the melt. To the cooled reaction mixture water was added. The mixture was refluxed for 1 h and evaporated to dryness. The residue was crystallized from EtOH yielding 6 g (92%) of analytically pure **6**, having an m.p. of 114–115 °C.

$C_{22}H_{41}NO_5$ (399.6)

	C	H	N
calc.:	66.13	10.34	3.51
obsv.:	66.34	10.47	3.47.

DL-2,3-Dipalmitoylglyceric acid pyridinium salt (8)

Palmitic acid chloride (4.2 g, 14.5 mmol) dissolved in dry CCl_4 (10 ml) was added to DL-glyceric acid (**7**) (1.5 g, 14 mmol) dissolved in a mixture of pyridine (3 ml) and dry CCl_4 (20 ml). **7** was obtained by converting DL-glyceric acid hemicalcium salt to the free acid by elution through a cation-exchange column (pyridinium$^+$ form). The reaction mixture was stirred for 12 h at 40 °C. After the addition of Et_2O (200 ml) the solution was extracted with 1% $NaHCO_3$, 1 N HCl and 1% $NaHCO_3$, respectively. The organic layer was dried over Na_2SO_4 and evaporated to a gum. Crystallization from EtOH (200 ml) gave 5.5 g (66%) of analytically pure **8**, which was homogeneous (R_F 0.40) by TLC using $CHCl_3$/EtOH (4:1, v/v).

$C_{34}H_{67}O_6 \cdot C_5H_6N$ (652.0)

	C	H	N
calc.:	71.84	11.29	2.15
obsv.:	71.73	11.09	1.93.

Preparation of liposomes

Liposomes containing the lipophilic components **3**–**9** (see Table II) were composed of 20 mg egg phosphatidylcholine, 2 mg cholesterol and 1.3 μmol (0.05 mol parts as referred to eggPC) of the corresponding lipophilic amino or carboxylate components.

The positively charged liposomes (see Table II) containing stearylamine or the lysine derivative **2** were composed of 40 mg eggPC, 4 mg cholesterol labelled with a trace of [^{3}H]cholesterol and increasing amounts (no more than 0.1 mol part referred to eggPC) of stearylamine or **2**.

From the mixture of the lipophilic components, liposomes were obtained by solubilization with sodium cholate in 1 ml PBS (pH 7.4), resulting in micellar solutions. The molar ratio of the total of the lipophilic components to the detergent was 0.6. The micellar solutions were dialysed against 10 liters PBS, (pH 7.4) for 15–20 h as described previously [16]. Hydrodynamic diameters (D) and population homogeneity (*H*%) of the liposomes (see Table II) were determined by laser light-scattering measurements as described before [17]. Measurements were made immediately after liposome preparation and after storage for 60 days at 4 °C. The calculation of incorporated molecules per liposome [18] was based on the following values: spherical vesicles with 3.7 nm bilayer thickness, the volume of one eggPC molecule being 1.253 nm^3 and the diameters obtained from the laser light scattering measurements.

Coupling of MPS to the functionalized liposomes (see Fig. 2)

Liposomes functionalized with phosphatidylethanolamine (PE) or with **2** were composed of 2 mol parts eggPC and cholesterol (10:1, w/w) and 1 mol part of PE or **2**. The liposomes suspended in PBS were incubated with a 1.5 molar excess (in respect to PE or **2**) of a 1.0 mg/ml solution of MPS in 10 mM NaCl/0.3 mM NaH_2PO_4 (pH 7.0) for 30 min at 25 °C. Unbound MPS was separated from the liposome suspensions at 4 °C using a Sephadex G-50 column (10 × 1 cm) pre-equilibrated and eluated with PBS (pH 6.0). Liposome containing fractions were deoxygenated with nitrogen and concentrated by ultrafiltration on an Amicon membrane (PM 30).

The amount of MPS linked via the amino groups of the functionalized liposomes was indirectly determined from the coupling of 2-mercaptoethanol to the maleinimide function of incorporated MPS. To 0.5 ml of MPS-liposomes (10–20 μmol eggPC) in PBS were added 10 μl of a 0.1 M solution of 2-mercaptoethanol, and the

suspension was kept at 25 °C for 20 min. Then 0.5 ml 0.2 M Tris-HCl (pH 8.2) and 50 µl of a 10 mM solution of DTNB [19] were added. After 10 min, the absorption of appropriately diluted aliquots was measured at 420 nm against a PBS blank. Liposomes without coupled MPS were equally treated to obtain the 100% value of the reaction with 2-mercaptoethanol. The amount of the coupled 2-mercaptoethanol was calculated from the difference of A_{420} units measured after the incubation of liposomes (100%) and liposomes derived (up to 100%) with 2-mercaptoethanol. All solutions used were carefully deoxygenated with nitrogen.

Free-flow electrophoresis of positively charged liposomes

Free-flow electrophoresis of the liposomes containing PE or the lysine derivative **2** was performed according to De Cuyper et al. [20] on a Desaga FF-48 instrument. For free-flow electrophoresis the liposome suspensions were dialyzed against a 300 mM glucose solution in order to remove PBS. The liposomes (1.0 ml) were injected continuously at a rate of 0.06 ml/min into a laminar flow sheet (flow rate, 4.8 ml/min of a 300 mM glucose solution). A 60 mM phosphate buffer (pH 7.4) served as electrode buffer. An electric field of 65 V/cm was applied perpendicular to the flow of the separation buffer, corresponding to a current of 150 mA at 20 °C. At the bottom of the separation chamber the buffer stream was separated into 48 fractions. The collected fractions were analyzed for [^{3}H]cholesterol radioactivity in a Packard 460 CD liquid scintillation counter. The electrophoretic migration (see Table III) of the liposomes is defined as migrated distance of the peak concentrations relative to the cathode side of the flow chamber.

Results

The syntheses of lipophilic components carrying amino (**2**) or carboxylate groups (**3**, **4**, **6**, **8**) were performed according to the scheme of Fig. 1. The amino component N^2-palmitoyl-L-lysine methyl ester · HCl (**2**) was prepared with N^6-benzyloxycarbonyl-L-lysine methyl ester · HCl (**1**) as the starting material. The α-amino group (N^2) of

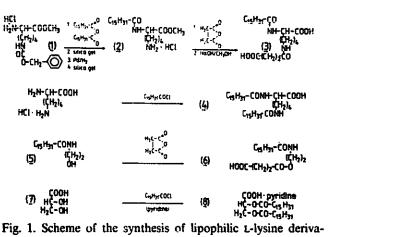

Fig. 1. Scheme of the synthesis of lipophilic L-lysine derivatives, **2**–**4**, the *N*-(2-succinoylethyl)palmitoylamide, **6**, and the DL-2,3-dipalmitoylglyceric acid pyridinium salt, **8**.

1 was acylated with palmitic acid anhydride in dry methylene chloride. The ε-amino group (N^6) of the purified and fully protected L-lysine derivative was cleaved by hydrogenation. The reaction mixture was chromatographed on a silica-gel column and **2** was isolated in 62% yield. The syntheses of lysine derivatives carrying carboxylate residues were performed by acylation of the amino groups of lysine with reactive carbonic acid derivatives. N^2-Palmitoyl-N^6-succinoyl-L-lysine (**3**) was obtained by acylation of the ε-amino group of **2** with succinic acid anhydride in dry pyridine and subsequent alkaline cleavage of the methyl ester group. By a two-fold acylation of unprotected L-lysine · HCl with palmitoyl chloride in water and by subsequent purification of the reaction mixture on a silica-gel column, $N^{2,6}$-dipalmitoyl-L-lysine (**4**) was obtained in 30% yield.

Besides the easily available lysine derivatives (**3**, **4**) carrying carboxylate functions, two other lipophilic carboxyl components (**6**, **8**) for the functionalization of liposomes were synthesized, starting from *N*-(2-hydroxyethyl)palmitoylamide (**5**) or DL-glyceric acid (**7**). Treatment of **5** with succinic acid anhydride yielded 92% of *N*-(2-succinoylethyl)palmitoylamide (**6**). By acylation of **7** with palmitoyl chloride, DL-2,3-dipalmitoylglyceric acid (**8**) was obtained in 66% yield.

The different steps of derivatization can easily be checked by means of TLC and the corresponding spray reagents. Ultraviolet-absorbing compounds were visualized at 254 nm. Primary amino groups were detected on the TLC with ninhydrin

spray [21], palmitoyl residues with 2,7-dichlorofluorescein spray [22,23] and carboxylate functions with Bromocresol-green spray [24]. The amide bond was visualized by using the chlorotolidin reaction [25]. The results thus obtained were controlled by elementary analysis, ^{13}C-NMR and IR spectroscopy, and mass spectrometry and always confirmed. The data obtained are not quoted here.

Each of the lipophilic amino and carboxylate components described here could be incorporated into unilamellar liposomes. Liposomes functionalized with amino groups were obtained by incorporation of **2** into the membranes of unilamellar liposomes. The incorporation of **3**, **4**, **6**, **8** resulted in liposomes functionalized with carboxylate residues. The variously functionalized liposomes showed only minor differences in hydrodynamic diameters (*D*) and population homogeneity (*H*) (see Table II). Liposome sizes ranged from 59 to 84 nm for fresh preparations. After storage for 60 days at 4°C, sizes of 48 to 77 nm were observed. Values of the homogeneity parameter (*H*) of 26–44% indicated homogeneous liposome preparations. The *H* and *D* values of the obtained liposomes indicated that the lipophilic components used for functionalization did not significantly affect size or stability of the liposomes. Liposomes functionalized with the carboxyl component **4**, however, became inhomogeneous during storage and partially precipitated. All other functionalized liposomes were equal to liposomes functionalized with natural PE in regard to size, homogeneity and stability. For this reason the more easily available lipophilic amino and carboxyl components, **3**, **6**, **8**, represent a simple alternative to the corresponding phosphatidylethanolamine derivatives used so far. The calculated amount of carboxylate residues provided by the incorporated components **3**, **4**, **6**, **8** ranged from 1800–2200 per liposome of a mean diameter of 70 nm and 1.3 μmol (0.05 mol parts referred to eggPC as the matrix lipid) component incorporated. The amounts of amino residues per one liposome provided by the different amounts of incorporated **2** or stearylamine ranged between 1000 and 4000 molecules. Assuming a 1:1 distribution between the outer and inner bilayer side of the lipid membrane, on the average 500 to 2000 functional

TABLE III

RESULTS OF FREE-FLOW ELECTROPHORESIS OF POSITIVELY CHARGED LIPOSOMES BY INCORPORATED STEARYLAMINE (A) OR N^2-PALMITOYL-L-LYSINE METHYL ESTER (B)

Electrophoretic migration [a] from cathode (mm)		Amount of (A) or (B) (mol%)
A	B	
60	53	2
84	80	5
107	103	10

[a] The migration of uncharged liposomes was 28 mm.

TABLE II

HYDRODYNAMIC DIAMETERS (*D*) AND POPULATION HOMOGENEITY (*H*) OF LIPOSOMES FUNCTIONALIZED WITH LIPOPHILIC COMPONENTS (**2–4**, **6**, **8**) (FIG. 1) AND DERIVATIZED BY THE MPS RESIDUE (**2a**, **9**; FIG. 2)

Liposome no.	Lipophilic component	Liposome properties			
		after 1 day		after 60 days [a]	
		D (nm)	*H* (%)	*D* (nm)	*H* (%)
2	N^2-palmitoyl-L-lysine methyl ester·HCl	84	26	75	27
2a		76	44	77	41
3	N^2-palmitoyl-N^6-succinoyl-L-lysine	63	38	58	30
4	$N^{2,6}$-dipalmitoyl-L-lysine	81	28	68	47
6	*N*-(2-succinoylethyl)palmitoyl amide	73	25	68	21
8	DL-2,3-dipalmitoylglyceric acid pyridinium salt	59	22	48	36
9	phosphatidylethanolamine	59	39	56	42

[a] Storage at 4°C.

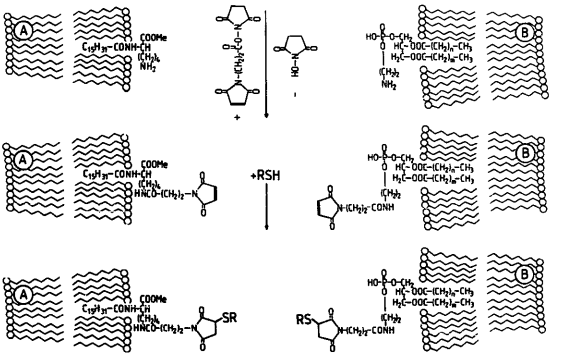

Fig. 2. Scheme of the coupling of *N*-(3-maleinimidopropionyloxy)succinimide to the amino groups of liposomes functionalized with: (A) N^2-palmitoyl-L-lysine methyl ester; (B) phosphatidylethanolamine and of the subsequent addition of 2-mercaptoethanol to the maleinimido residues of the derivatized liposomes.

groups were located on the liposome surface, thus providing the liposomes with an excess of functional groups for antibody coupling.

The incorporation of **2** or stearylamine resulted in positively charged liposomes as demonstrated with free-flow electrophoresis. In Table III the increasing positive liposome net charge on vesicle migration from cathode to anode in an electrical field is summarized. By increasing the amounts of incorporated amino components the migration distance toward the anode increased. Equal amounts of the positively charged components **2** or stearylamine incorporated gave rise to comparable vesicle polycation charge.

Liposomes functionalized with amino groups can be coupled with MPS. The resulting derivatized liposomes were able to bind sulfhydryl-containing components to the maleinimide residues by an addition reaction (see Fig. 2). The addition reaction was demonstrated using 2-mercaptoethanol as a sulfhydryl-containing model component. Uncoupled 2-mercaptoethanol reacted with DTNB (Ellman's reagent), releasing a yellow reaction product which was photometrically determined. Liposomes functionalized with **2** and derivatized with MPS (liposome A, Fig. 2) bound 23% of the 2-mercaptoethanol added. The amount of 2-mercaptoethanol bound to liposomes functionalized with PE (liposome B, Fig. 2) was found to be only 11%.

Discussion

In regard to a therapeutic application of the derivatized liposomes, we have guaranteed that all components used for functionalization of the liposomes are non-toxic, analytically characterized and easily available. In addition, it was postulated that the functionalized liposomes were obtainable as small unilamellar liposomes in reproducible population homogeneity and hydrodynamic diameters and could be stored over a long period of time. With the starting materials and the route of synthesis applied, these requirements could be realized to a high degree.

The naturally occurring amino acid, L-lysine, was chosen for the functionalization of liposomes carrying amino groups. L-Lysine could be converted into the lipophilic amino component **2** by practicable chemical reactions not limited to an analytical scale. By varying the amount of **2**, the functionalization of the resulting unilamellar liposomes could be influenced within broad limits.

The likewise easily available lipophilic stearylamine which has recently been proposed for functionalization of liposomes by amino groups [7] was excluded because of its toxic effects [27]. Since the authors using stearylamine describe only the functionalization of large unilamellar and oligolamellar liposomes, it was furthermore questionable whether incorporation of stearylamine would lead to the desired small unilamellar vesicles. Moreover, the functional amino group is located directly at the lipophilic part of the molecule, which is integrated in the liposomal membrane. Consequently, the possibility of steric hindrance during derivatization of the amino groups of liposomes has to be regarded. In N^2-palmitoyl-L-lysine, on the other hand, the functional amino group is linked to the lipophilic palmitoyl residue which is integrated into the liposome membrane via the amino-acid residue serving as spacer. The derivatization of these liposomes and preliminary investigations of the coupling of antibodies indicated that the amino groups of liposomes derivatized with **2** are easily accessible.

Steric hindrance, which possibly occurs in the case of stearylamine, also has to be expected on the introduction of amino groups into liposomes via PE. PE, which is often used for derivatization of liposomes, is non-toxic like L-lysine, but in regard to a preparative application it has various disadvantages compared to the lysine derivatives. The determination of the molecular structure is complicated because PE isolated from natural sources has two fatty acyl chains of varying length and saturation.

For this reason, substitution of natural by synthetic PE which – as a rule – contains two saturated, equally long fatty acyl chains, is attempted in order to obtain defined molecular structures. However, it is not yet clear to what extent the stability of liposomes changes as natural PE is substituted by synthetic PE or even derivatives of synthetic PE are used. As is well known, the length of the hydrocarbon chains as well as the number of unsaturated bonds significantly influence the stability of the liposomes. Results obtained for natural PE cannot be extended generally to synthetic PE or its derivatives.

For example, the chemical synthesis of PE from glycerol requires six steps compared to only three steps needed for the proposed synthesis of the lipophilic lysine derivative, **2**, starting from L-lysine. Apart from a high expense of synthesis, PE has the disadvantage that the hydrocarbon chains are linked via alkali-labile ester bonds while, for lysine derivatives, the palmitoyl residue is coupled via a more stable amide bond. Therefore, in comparison with the PE derivatives, the lysine derivatives bring about not only simplification of synthesis but also more favorable indications for a long-term stability.

The results obtained for the derivatization of the functionalized liposomes with MPS also support the functionalization of liposomes using **2** instead of PE. The yield (23%) of the addition of 2-mercaptoethanol to liposomes A functionalized with **2** and derivatized with MPS was 2-times higher than to liposomes B, which carried PE instead of **2**. The higher binding value of 2-mercaptoethanol for liposomes A might be explained by a higher distribution rate of **2** on the outer liposome surface or because the maleinimido moiety protrudes further out of the membrane surface as compared to liposome B. Because of the covalent coupling of 2-mercaptoethanol to the derivatized liposomes, it can be expected that antibodies or their fragments carrying free sulfhydryl groups, or being thiolated, can equally be added to the maleinimido residues of the liposomes.

In our opinion, the direct incorporation of maleinimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine, which was described recently [8], has the disadvantage that the reactive groups are incorporated into the outer as well as the inner part of the bilayer membrane of the unilamellar liposomes. Apart from their being uneconomical, it cannot be excluded that these liposomes, which carry reactive groups in the inner membrane, cause undesired side-reactions during further application. In our two-step method of derivatizing func-

tionalized liposomes, only the functional groups on the outer side are converted into reactive derivatives and the degree of derivatization can be varied within broad limits.

The disadvantages in respect to a preparative preparation of liposomes arising from the use of PE as starting material also apply to the recently described carboxy acyl derivatives of PE [12]. Therefore we have chosen as starting material for the synthesis of lipophilic carboxylate components L-lysine and the components N^2-palmitoyl-L-lysine methyl ester (**2**) and 2-hydroxyethylpalmitoylamide (**5**), which can easily be synthesized and have a defined molecular structure. After the unproblematic acylation of the hydroxyl group of **5**, the lipophilic carboxylate component **6** was obtained. The acylation of **2** and the subsequent removal of the ester function yielded the one-fold palmitoylated lysine derivative, **3**. Upon acylation of lysine with palmitoyl chloride in water the two-fold palmitoylated lysine derivative, **4**, was obtained, which melted at 115–125°C and the C, H, N values determined in elementary analysis correspond to the calculated values. Recently, Kiwada et al. [27] published without experimental details a complicated synthesis of dipalmitoyl-L-lysine resulting in a product having m.p. 101–103°C and a C value 1.3% below the calculated one. Furthermore, it has to be mentioned that liposomes functionalized with dipalmitoyl-L-lysine were the only ones to be stable over only a short period of time. Probably the liposome membrane was destabilized due to the unfavorable configuration of the two palmitoyl residues. Therefore, it is not clear whether the liposome-like vesicles proposed by Kiwada et al., which are composed of palmitoyl amino acids, remain stable over a longer period of time, since the authors have not given details on this issue.

Though the coupling of antibodies to the functionalized or derivatized liposomes is to be reported in detail elsewhere and thus not object of the investigation reported here, we want to summarize some preliminary results already presented [28–30]. Liposomes functionalized with **4** could not be considered for antibody coupling due to their lack of stability. By means of EDC, monoclonal antibodies (IgG; Tü 7, Tü 43) were coupled to the carboxylate groups of liposomes which were functionalized by incorporation of **3** or **6** and additionally loaded with prodrugs of 1-β-arabinofuranosylcytosine (ara C) without losing their activity. Linkage to liposomes functionalized by incorporation of **8** was not successful, probably because of steric reasons. This result agrees with investigations [12] showing that PE amide of succinic acid incorporated into liposomes does not bind antibodies on account of steric hindrance.

Quantitative analysis of our condensation reactions, the conditions of which were varied within a wide range showed that the yield of the coupling reaction depended essentially on the type of antibody coupled and the molar ratios of reactants chosen and ranged between 2 and 31%. To each liposome, eight antibodies could be linked at the most. Excess antibody could be regained by column chromatography without loss of activity and thus be reused. For a cell-targeting of the liposome-prodrug-IgG (Tü 7) complexes in vitro; however, one to two immobilized antibodies were sufficient. The comparatively low efficiency of coupling which is usually accomplished using EDC is compensated for by the advantage that the coupling can be performed without prior derivatization of the antibody. Other coupling methods involving disulfide or thioether bonds, for example, are possibly more efficient. On the other hand, these methods require either the exposure of sulfhydryl groups via partial hydrolysis of the antibodies or the introduction of the sulfhydryl groups in a preliminary derivatization of the antibody. These reactions not only result in low yields, but can also lead to loss of activity for the antibody.

According to our experience it is useful if a broad variety of differently functionalized liposomes are available. Thus, for each antibody the method of coupling can be chosen individually so that maximum coupling efficiency is accomplished. Moreover, a high multiplicity of differently derivatized liposomes contributes to the possibility of synthesizing new liposome preparations.

The determining factor for a therapeutic application of the antibodies-drug-liposome complex is their actual effect in vivo which is dependent on several varying parameters. In our case, eggPC and cholesterol were used as matrix lipids which usually compose biodegradable liposomes of suffi-

cient stability. To what extent the additional functionalized lipid components influence the in vivo stability of the liposomes is the object of further investigation.

Acknowledgement

This work was supported by the Deutsche Krebshilfe and the Krebsliga, Kanton Zürich, Switzerland.

References

1 Ghose, T., Blair, A.H., Uadia, P., Kulkarni, P.N., Guandalkar, A., Mezei, M. and Ferrone, S. (1985) Ann. N.Y. Acad. Sci. 446, 213–227.
2 Gregoriadis, G., Senior, J., Wolff, B. and Kirby, C. (1985) Ann. N.Y. Acad. Sci. 446, 319–340.
3 Ford, C.H.J. and Casson, A.G. (1986) Cancer Chemother. Pharmacol. 17, 197–208.
4 Heath, T.D., Macher, B.A. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 640, 66–81.
5 Martin, J.J. and Papahadjopoulos, D. (1982) J. Biol. Chem. 257, 286–288.
6 Barbet J., Machy, P. and Lesermann, L.D. (1981) J. Supramol. Struct. Cell Biochem. 16, 243–258.
7 Goundalkar, A., Ghose, T. and Mezei, M. (1984) J. Pharm. Pharmacol. 36, 465–466.
8 Derksen, J.T.P. and Scherphof, G.L. (1985) Biochim. Biophys. Acta 14, 151–155.
9 Hashimoto, K., Loader, J.E. and Kinsky, S.C. (1986) Biochim. Biophys. Acta 856, 556–565.
10 Kinsky, S.C., Hashimoto, K., Loader, J.E. and Benson, A.L. (1984) Biochim. Biophys. Acta 769, 543–550.
11 Endoh, H., Suzuki, Y. and Hashimoto, Y. (1981) J. Immunol. Methods 44, 79–85.
12 Kung, V.T. and Redemann, C.T. (1986) Biochim. Biophys. Acta 862, 435–439.
13 Carrol, T.R., Davison, A. and Jones, A.G. (1986) J. Med. Chem. 29, 1821–26.
14 Eckstein, H., Sievers, R.E. and Bayer, E. (1973) Liebigs Ann. Chem. 1467–1470.
15 D'Alelio, G.F. and Reid, E.E. (1937) Am. Chem. Soc. 59, 111–112.
16 Milsmann, M.H.W., Schwendener, R.A. and Weder, H.G. (1978) Biochim. Biophys. Acta 512, 147–155.
17 Riddles, P.W., Blakely, R.L. and Zerner, B. (1979) Anal. Biochem. 94, 75–81.
18 Huang, C. and Mason, J.T. (1978) Proc. Natl. Acad. Sci. USA 75, 308–310.
19 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
20 De Cuyper, M., Joniau, M. and Dangereau, H. (1983) Biochemistry 22, 415–420.
21 Spackmann, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190–1206.
22 Malins, D.C. and Mangold, H.K. (1960) J. Am. Oil. Chem. Soc. 37, 576–578.
23 Duphy, P.J., Whittle, K.J. and Pennock, J.F. (1965) Chem. Ind. 1217–1218.
24 Bryant, F. and Overell, B.T. (1953) Biochim. Biophys. Acta 10, 471–476.
25 Hanes, C.S. and Isterwood, F.A. (1979) Nature 164, 1107–1112.
26 Yoshihara, E. and Nakae, T. (1986) Biochim. Biophys. Acta 854, 93–101.
27 Kiwada, H., Akimoto, M., Araki, M., Tsujie, M. and Kato, Y. (1987) Chem. Pharm. Bull. 35, 2935–2942.
28 Schwendener, R.A., Schott, H., Leitner, B. and Hengartner, H. (1987) 19th Annual meeting of the USGEB/USSBE, Lausanne, Switzerland.
29 Uchanska-Ziegler, B., Feller, A., Bödewadt, B., Schott, H. and Ziegler, A. (1987) III. Frühjahrstagung der Gesellschaft für Immunologie, Lübeck, F.R.G.
30 Schott, H., Heß, W., Schwendener, R.A., Uchanska-Ziegler, B. and Ziegler, A. (1987) J. Steroid Biochem. 28, Suppl. 46 S.