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Palmitoyl derivatives of L-cysteine, cysteamine, L-cystine, cystamine and their incorporation into the bilayers of unilamellar liposomes

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The amino groups of the amino acids L-cysteine and L-cystine as well as their biogene amines cysteamine and cystamine were derivatized with palmitoyl residues. The obtained lipophilic R-SH and R-S-S-R components were incorporated into the bilayers of unilamellar liposomes. The resulting liposomes carrying about 2000 functional groups each remained stable and homogeneous during 60 days after incorporation of *N*-palmitoyl cysteamine and *N,N'*-dipalmitoyl cystamine. The incorporation of the lipophilic amino acid derivatives, however, destabilized the resulting liposomes. Via the thiol residues of the functionalized liposomes activated molecules can be linked to the liposomal surface by disulfide bonds.

Introduction

Antibodies or antibody fragments can direct liposomes loaded with drugs to target cells that possess the corresponding antigens. This cell-targeting, which has potential therapeutic applications, presupposes the linkage of the antibodies to the liposomal surface. Several methods have been devised for this purpose.

Leserman et al. [1,2] employed liposomes prepared with *N*-[3-(pyridyl-2-dithio)propionyl]phosphatidylethanolamine. These functionalized liposomes readily react with sulfhydryl residues of the antibodies resulting in disulfide-linked conjugates. Martin and Papahadjopoulos [3] as well as Derksen and Scherphof [4] prepared liposomes containing *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine. These liposomes were coupled to antibodies via thioether linkages formed by sulfhydryl addition to the maleimide double bond.

Hashimoto et al. [5] coupled sulfhydryl-containing antibodies to the liposome bilayers via thioether bonds. The liposomes were prepared with homologous series of saturated dimyristoylphosphatidylethanolamine derivatives in which a reactive iodoacetyl function was separated from the phospholipid amino group by either 0, 1 or 2 aminoethylthioacetyl spacers. A disadvantage of the phosphatidylethanolamine derivatives used is their complicated synthesis followed by high cost.

For these reasons Goundalkar et al. [6] prepared liposomes with *N*-[3-(pyridyl-2-dithio)propionyl]stearylamine and coupled these activated liposomes via disulfide bonds to sulfhydryl residues of antibodies according to the concept introduced by Leserman.

All these concepts applied comprise a principal disadvantage. Out of the multitude of activated sulfhydryl functions which are incorporated into

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the liposome bilayers only few are actually employed in the coupling of antibodies. Therefore the liposome-antibody complexes are still containing many non-used but activated sulfhydryl residues which are located on the outside as well as on the inner side of the bilayer membrane. When using such liposomes for cell-targeting it can not be excluded that these reactive liposomal groups may cause undesired side reactions. In regard to therapeutical application the possibility of toxical side reactions has to be considered.

In order to exclude these difficulties we have developed the following new strategy. We prepare liposomes which carry inactive sulfhydryl residues while the antibodies assigned for coupling are provided with the reactive sulfhydryl groups. To realize this concept we have derivatized the easily available amino acids L-cystine and L-cysteine as well as their biogene amines cystamine and cysteamine with palmitoyl residues. The resulting lipophilic derivatives were incorporated into the liposome bilayer. The synthesis of the lipophilic derivatives as well as size, homogeneity and stability of the thus functionalized liposomes are reported in the following.

Materials and Methods

L-Cysteine, L-cystine, cystamine and cysteamine · HCl, palmitic acid anhydride and dimethylaminopyridine were purchased from Aldrich. Palmitoylchloride, cholesterol and anhydrous zinc bromide were obtained from Fluka, rhodium from Merck, egg phosphatidylcholine from Lipid Products. 4,4′-Dimethoxytrityl chloride was synthesized according to the method already published [7]. Pyridine was distilled over KOH. Distilled triethylamine, methylene chloride, tetrahydrofuran and pyridine were stored over 4 Å molecular sieves. All solid reagents were dried over P_4O_{10} before use.

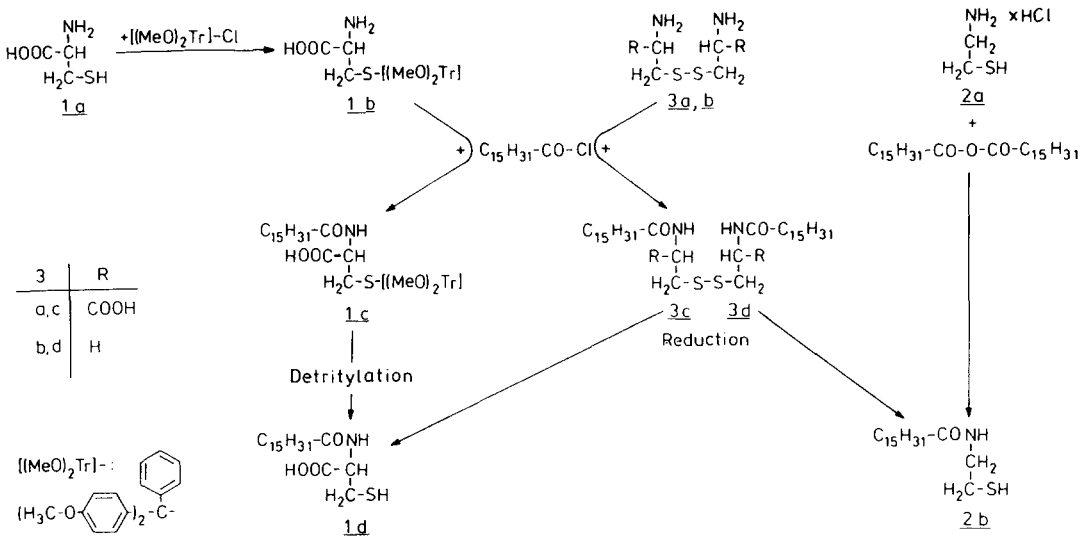
Thin-layer chromatography (TLC) was performed on Merck silica gel 60, F_{254} using $CHCl_3$/C_2H_5OH (4:1, v/v) as a solvent. The R_F values given were related to the R_F value of palmitic acid. Ultraviolet-absorbing compounds were visualized at 254 nm. Primary amino groups were detected on the TLC with ninhydrin spray [8], sulfhydryl functions with Ellman's reagent [9] and palmitoyl residues with 2,7-dichlorofluorescein spray [10,11]. The 4,4′-dimethoxytrityl residue showed an orange colour upon spraying with perchloric acid (70%)/C_2H_5OH (1:1, v/v). The amide bond was visualized by using the chlorotoluidine reaction [12].

Column chromatography was effected with Merck silica gel 60 (230–400 mesh) or with Sephadex LH-20 obtained from Pharmacia. Concentrations of the reaction mixtures or of the pooled fractions were effected at 40 °C or less with rotating evaporation under aspirator or mechanical oil-pump vacuum. Pyridine was removed from the reaction mixture by repeated coevaporation with toluene. The specific rotation of the optically active compounds was recorded on a Perkin-Elmer polarimeter 241.

N-Palmitoyl-L-cysteine 1d starting from L-cysteine 1a

To a solution of L-cysteine 1a (2.42 g, 20 mmol) and 4,4′-dimethoxytrityl chloride (7.45 g, 22 mmol) in dry pyridine (20 ml) *N,N′*-dimethylaminopyridine (3.66 g, 30 mmol) was added. The mixture was concentrated in vacuo to a volume of approx. 20 ml. After 24 h and again after 48 h the reaction volume was reduced by 5 ml. After a total reaction time of 60 h the mixture was evaporated to dryness. In order to remove pyridine, the residue was coevaporated from toluene repeatedly. The residue was dissolved in $CHCl_3$ (250 ml) and extracted with 1 M $NaHCO_3$ (200 ml) and H_2O (3 × 100 ml), respectively. The combined aqueous layers were extracted with $CHCl_3$ (100 ml). The pooled $CHCl_3$ layers were dried over Na_2SO_4 and evaporated to dryness in vacuo. The residue was dissolved in CH_2Cl_2 (5 ml) and chromatographed on a silica gel column as described in Table I (run No. 1). Fractions containing the required product were evaporated to a yellow powder (8.0 g); mp 138–140 °C; $[\alpha]_D^{20} = +29°$ ($c = 1$, CH_2Cl_2). TLC showed the major band ($R_F = 0$ to 0.3) to be *S*-(4,4′-dimethoxytrityl)-L-cysteine 1b contaminated by three unidentified side products with higher R_F values.

To a solution of 1b (7.68 g, 18.2 mmol) and triethylamine (2.58 ml, 35 mmol) dissolved in dry CH_2Cl_2 (30 ml), palmitoyl chloride (5 g, 18.2 mmol) dissolved in dry CH_2Cl_2 (10 ml) was added



Scheme I.

dropwise. The reaction mixture was stirred at room temperature for 1 h. After addition of a small amount of H_2O the mixture was evaporated to dryness (two additional times from ethanol, 30 ml). The residue was suspended in CH_2Cl_2 (300 ml) and extracted with 1 M $NaHCO_3$ (200 ml) and H_2O (3 × 100 ml), respectively. After being dried over Na_2SO_4 the CH_2Cl_2 layer was evaporated to yield 9.8 g of crude *S*-(4,4′-dimethoxytrityl)-*N*-palmitoyl-L-cysteine 1c. This residue was dissolved in $CHCl_3$ (15 ml) and chromatographed on a silica gel column according to the conditions described in Table I (run No. 2). Fractions of the second chromatographic step containing 1c were evaporated to dryness yielding 5.1 g, $[\alpha]_D^{20} = -229.5$, ($c = 0.5$, $CHCl_3/C_2H_5OH$, 95 : 5). 1c was homogeneous ($R_F = 0.8$) by TLC. Fractions of the third chromatographic step were

TABLE I

CONDITIONS OF THE COLUMN CHROMATOGRAPHY OF THE MIXTURES RESULTING FROM REACTION 1 TO 5 ON SILICA GEL

Run no.	Column length × i.d. (cm)	Conditions of the elution					Isolated product	
		Step No.	Mixing vessel		Reservoir		No.	contained within the elution vol. (l)
			eluent (v/v)	vol. (l)	eluent	vol. (l)		
1	45 × 9	1	CH_2Cl_2/C_2H_5OH (95 : 5)	4	–		1b	1.6 to 2.1
2	40 × 9	1	$CHCl_3$	2	–		–	
		2	$CHCl_3/CH_3OH$ (93 : 7)	4	–		1c	3.0 to 4.8
		3	$CHCl_3/CH_3OH$ (87 : 13)	2	–		1d	6.4 to 7.4
3	15 × 5	1	$CHCl_3$	2	CH_3OH	2	3c	1.2 to 1.7
4	35 × 9	1	CH_3Cl/C_2H_5OH (95 : 5)	4	–		2b	3.0 to 3.5
5	30 × 9	1	CH_2Cl_2	4.2	–		–	
		2	CH_2Cl_2	3	C_2H_5OH	3	3d	6.0 to 8.0

also evaporated and yielded 1.6 g of *N*-palmitoyl-L-cysteine 1d resulting from 1c by cleavage of the trityl group during the chromatographic purification. The side product 1d was identical with the product obtained by detritylation of 1c according to the following procedures.

1c (0.8 g, 1.2 mmol) was added to a solution of anhydrous $ZnBr_2$ (0.09 g, 40 mmol) in CH_2Cl_2/isopropanol (85 : 15, v/v). After being stirred at room temperature for 5 min the reaction mixture was evaporated to dryness. The residue was dissolved in 50% methanolic $CHCl_3$ (6 ml) and chromatographed on a Sephadex LH-20 column (90 × 4 cm) with 50% methanolic $CHCl_3$ as the eluent. *N*-Palmitoyl-L-cysteine 1d was eluted within a volume of 350 to 420 ml. The evaporation of the pooled fractions resulted in 400 mg of analytically pure 1d; $[\alpha]_D^{20} = -17.4°$ ($c = 1$, $CHCl_3/C_2H_5OH$, 1 : 1), 1d was homogeneous by TLC ($R_F = 0.35$).

$C_{19}H_{37}NO_3S$ (359.6)		C	H	N	S
	calc.	63.46	10.37	3.89	8.92
	obs.	63.20	10.42	3.55	8.67

N-Palmitoyl-L-cysteine 1d starting from L-cystine 3a

A suspension of L-cystine 3a (2.39 g, 10 mmol) in dry pyridine (50 ml) was stirred for 3 h at 30 °C. Palmitoyl chloride (5.5 g, 20 mmol) dissolved in dry tetrahydrofuran (50 ml) was added dropwise within 30 min at 0 °C. The mixture was kept stirring without cooling until it had attained room temperature. After the addition of water (10 ml) the reaction mixture was stirred (10 min) and then evaporated to dryness (three additional times from toluene (20 ml)). The residue was dissolved in $CHCl_3$ (5 ml) and chromatographed on a silica gel column according to the conditions listed in Table I (run No. 3). Fractions containing the desired *N,N'*-dipalmitoyl-L-cystine were evaporated yielding 2.8 g (39%) of analytically pure 3c, which was homogeneous by TLC ($R_F = 0.34$); $[\alpha]_D^{20} = -4.2$ ($c = 1$, $CHCl_3/C_2H_5OH$, 3 : 2).

$C_{38}H_{72}N_2O_6S_2$ (717.1)		C	H	N	S
	calc.	63.65	10.12	3.91	8.94
	obs.	63.41	10.38	3.49	8.82

For hydrogenolytic reduction a solution of 3c (2 g, 2.78 mmol) in $CH_3OH/CHCl_3$ (10 : 1) (150 ml) was saturated with N_2 and then rhodium (20 mg) was added. While shaking H_2 (≈ 1.1 liter) was added to this mixture at a slightly increased pressure. The catalyst was removed via filtration; the solution was dried over Na_2SO_4 and evaporated. The residue was dissolved in $CHCl_3$ (5 ml) and chromatographed on a Sephadex LH-20 column (90 × 4 cm) with 50% methanolic $CHCl_3$ as the eluent. *N*-Palmitoyl-L-cysteine 1d left the column within 350 to 420 ml. The evaporation of the fractions containing the desired product resulted in 1.7 g of analytically pure 1d which was identical to 1d resulting from the derivatisation of L-cysteine.

N-Palmitoyl cysteamine 2b starting from cysteamine · HCl 2a

To cysteamine · HCl 2a (3.41 g, 30 mmol), dissolved in a mixture of dry pyridine (5 ml) and dry dioxane (30 ml), *N,N'*-dimethylaminopyridine (7.5 g, 45 mmol) and palmitic acid anhydride (14.85 mmol, 30 ml) were added. After the mixture had been stirred at about 40 °C for 6 h it was evaporated to dryness. The residue was dissolved in CH_2Cl_2 (200 ml). The solution was extracted with 1% aqueous citric acid (400 ml) once, twice with 1 M $NaHCO_3$ (200 ml) and at last twice with H_2O. The CH_2Cl_2 layer was dried over Na_2SO_4 and evaporated to dryness (coevaporated several times from toluene). The residue was dissolved in $CHCl_3$ (5 ml) and chromatographed on a silica gel column according to the conditions listed in Table I (run No. 4). Fractions containing the required product were evaporated yielding 5.7 g of analytically pure 2b.

$C_{18}H_{37}NOS$ (315.6)		C	H	N	S
	calc.	68.52	11.82	4.44	10.16
	obs.	68.43	11.59	4.41	9.84

2b was homogeneous by TLC ($R_F = 1.07$) and identical to 2b resulting from the derivatization of cystamine 3b, as will be shown in the following.

N-Palmitoyl cysteamine 2b starting from cystamine 3b

Cystamine 3b (2.25 g, 10 mmol) was suspended in dry pyridine (30 ml) and subjected to sonication for about 10 min. The suspension was ren-

dered anhydrous by repeated coevaporation from dry pyridine. The resulting gum was dissolved in a mixture of dry pyridine (2 ml) and dry tetrahydrofuran (15 ml). During 30 min, palmitoyl chloride (5.5 g, 20 mmol) dissolved in dry tetrahydrofuran (15 ml) was dropped to the solution, kept stirring at 0 °C. Afterwards the reaction was allowed to attain room temperature, water (10 ml) being added. After stirring for additional 30 min the solution was concentrated in vacuo to a gum, which was coevaporated three times from toluene (20 ml). The residue was dissolved in CH_2Cl_2 (5 ml) and chromatographed on a silica gel column according to the conditions given in Table I (run No. 5). Fractions containing *N,N'*-dipalmitoyl/cystamine were evaporated yielding 4.48 g analytically pure 3d, which was homogeneous according to TLC ($R_F = 0.27$) and showing a sharp mp 124–125 °C.

$C_{36}H_{72}N_2O_2S_2$ (629.1)		C	H	N	S
	calc.	68.73	11.54	4.45	10.19
	obs.	68.39	11.43	4.16	9.78

For hydrogenolytic reduction a solution of 3d (0.9 g, 1.43 mmol) in $CH_3OH/CHCl_3$ (10:1, v/v) (100 ml) was saturated with N_2 and then rhodium was added (10 mg). While shaking about 500 ml H_2 were introduced into this mixture at a slightly increased pressure. The catalyst was filtered off and the filtrate concentrated to an oily residue. This oil was triturated with ether (40 ml) and kept at 4 °C in the refrigerator. The white precipitate was filtered off yielding 440 mg of 2b.

Preparation of liposomes

The liposomes were composed of 20 mg egg phosphatidylcholine (egg PC), 2 mg cholesterol and 1.3 μmol (0.05 mol parts as referred to egg PC) of the corresponding derivatives 1d, 2b, 3c, 3d (see Scheme I) and 4a,b [13] per 1 ml 150 mM NaCl, 1 mM NaH_2PO_4 (pH 7.4). The micelle solutions were dialyzed against 10 l of 150 mM NaCl, 1 mM NaH_2PO_4 (pH 7.4) during 15–20 h as described before [14,15]. Hydrodynamic diameters (*D*) and population homogeneity (*H* %) of the liposomes were determined by laser light scattering measurements as described before [16]. Measurements were performed immediately after the liposome preparation and again after 60 days of storage at 4 °C.

The calculation of incorporated 1d, 2b, 3c, 3d, 4a, 4b molecules per liposome was based on the following assumptions: spherical vesicles with 3.7 nm bilayer thickness, volume of one egg PC molecule 1.253 nm^3 and with the diameters obtained from the laser light scattering measurements [17]. The presence of the additional liposome components was not taken into account for the calculations.

Results

The methods applied for the synthesis of the lipophilic derivatives of the amino acids L-cysteine and L-cystine as well as their biogene amines cysteamine and cystamine are summarized in Scheme I. *N*-Palmitoyl-L-cysteine 1d was obtained either directly by derivatization of L-cysteine 1a or from *N,N'*-dipalmitoyl-L-cystine 3c by hydrogenolytic reduction. In an analogous way *N*-palmitoyl-L-cysteamine 2b was synthesized either from cysteamine · HCl 2a or from the derivatized cystamine 3d. The derivatization resulted in analytically pure products being homogeneous by TLC. Usually the reaction products were purified by column chromatography and isolated as foamy powders after evaporation of the product-containing fractions. Except for *N,N'*-dipalmitoylcystamine 3d the products did not show sharp melting points. All derivatives of the L-amino acids were optically active.

The different steps of derivatization could easily be checked by means of TLC and the corresponding spray reagents. In some cases the results thus obtained were controlled by ^{13}C-NMR, infrared or mass spectroscopy and always confirmed. Enumeration of the spectroscopical data was omitted especially since the syntheses were additionally confirmed by the hydrogenolytic reduction of the derivatized dimers. The corresponding derivatives of the monomers L-cysteine and cysteamine were identical with the products obtained via reductive cleavage of the derivatized dimers L-cystine and cystamine. In particular the derivatization of the different substances was realized as described in the following.

The synthesis of 1d starting from 1a required three steps. In the first instance the sulfhydryl residue of 1a was blocked by the 4,4′-dimethoxytrityl function. The main product of the reaction mixture migrated on TLC as an ultraviolet absorbing band, which was stainable with ethanolic perchloric acid. The orange colour indicated the introduction of the *N,N′*-dimethoxytrityl residue. The presence of the free amino function of 1b was verified by a positive ninhydrin reaction [7]. The blocking of the sulfhydryl residue was concluded from the negative reaction after treatment with Ellman's reagent [9]. After the column chromatographic purification on silica gel *S*-(4,4′-dimethoxytrityl)-L-cysteine 1b was obtained in 95% yield. Although 1b still exhibited minor contaminations on TLC the product was acylated in the following step.

After acylation of 1b with palmitoyl chloride in CH_2Cl_2/triethylamine, the reaction mixture was purified on a silica gel column. During this process, the acid-labile trityl group was partially cleaved from 1c, so that in addition to 25% *N*-palmitoyl-L-cysteine 1d only 42% of the desired *N*-palmitoyl-*S*-(4,4′-dimethoxytrityl)-L-cysteine 1c were obtained in a chromatographically pure form. The derivatization of 1c and 1d with the palmitoyl residue could be concluded from the negative reaction with ninhydrin and the positive reaction with 2.7-dichlorofluorescein spray reagent [10,11] as well as with the chlorotoluidine reaction [12]. The presence of the trityl group in 1c and its absence in 1d was indicated by the orange stain of 1c and the negative reaction of 1d with ethanolic perchloric acid. The free sulfhydryl group of 1d was detected with Ellmann's reagent, while 1c did not turn yellow. 1d was identical with the product obtained by cleaving of the protective trityl group from 1c. Without prior protection of the sulfhydryl group we did not succeed in directly acylating L-cysteine with palmitoyl chloride.

1c was detritylated with anhydrous $ZnBr_2$. After chromatographic purification on a Sephadex LH-20 column *N*-palmitoyl-L-cysteine was obtained in 91% yield. 1d was also identical with the substance obtained by hydrogenolytic reduction of *N,N′*-dipalmitoyl-L-cystine 3c, as is described below.

The preparation of 1d from L-cystine 3a (see Scheme I) required only two steps. In the first step 3a was acylated with palmitoyl chloride in dry pyridine. After the chromatographic purification on a silica gel column, 3c was isolated in 30% yield. The introduction of the palmitoyl residues was confirmed with fluorescein spraying reagent and the chlorotoluidine reaction. The low yield may be contributed to the fact that acylation of the slightly soluble L-cystine is incomplete in the heterogeneous phase. Via hydrogenolytic reduction, which was accomplished in methanolic $CHCl_3$ with catalytic amounts of rhodium, 3c was converted to 1d. After chromatographic purification on a Sephadex LH-20 column 1d was obtained in 85% yield.

To improve the yield a more sophisticated way of synthesis was experimented on by first converting L-cystine in L-cystine di-*tert*-butyl ester [13]. The introduction of the blocking group was performed with 67% yield. The subsequent acylation of this well soluble cystine derivative with palmitoyl chloride resulted in *N,N′*-dipalmitoyl-L-cystine di-*tert*-butyl ester 4a in a yield of 77%. By reduction of 4a the monomeric compound *N*-palmitoyl-L-cysteine *tert*-butyl ester 4b was obtained in 66% yield. This more complicated synthesis did not lead to a higher total yield of 3c. Additionally, the *tert*-butyl ester group had to be split off before the derivatives were incorporated into the liposome. Retaining of the protective group did not affect incorporation of the lipophilic derivatives 4a,b into the liposomes but the stability of the obtained liposomes was signfiicantly decreased, as is shown below.

While these investigations were under progress, H. Kiwada et al. [18] published the synthesis of *N,N′*-dipalmitoyl-L-cystine though without giving experimental details. The procedure is more complicated but leading to higher yields. Whether the *N,N′*-dipalmitoyl-L-cystine thus obtained was chromatographically pure, is unknown because only the m.p. and the values of the elementary analysis are given.

Acylation of cysteamine · HCl 2a and cystamine 3a was performed analogously to the derivatization of L-cysteine and L-cystine (see Scheme I). By reaction of 3b with palmitoyl chloride and subsequent column chromatographic purification on silica gel, 3d was obtained in 71% yield. With the spraying reagent fluorescein and the chloro-

toluidine test the palmitoylamide bond was identified.

Hydrogenolytic reduction of 3d resulted in *N*-palmitoylcysteamine 2b, which was obtained in 49% yield after being recrystallized from ether. The *N*-palmitoyl residue in 2b was detected with fluorescein and the chlorotoluidine reaction while with Ellman's reagent the free sulfhydryl group showed a yellow colour. 2b was identical with the product resulting from the reaction of 2a with palmitic acid anhydride and column chromatographic purification on silica gel in 60% yield. 2b was more easily and effectively accessible by acylation of 2a than by derivatizing 3b. Since the reductive cleavage of 3d to 2b was not quantitative, the higher yield achieved via acylation of 3b did not deserve further interest.

In Table II the physico-chemical characteristics of liposome preparations containing 1.3 μmol of the corresponding derivatives 1d, 2b, 3c, 3d, 4a,b are summarized. Liposome sizes range from 59 to 78 nm for fresh preparations. After storage during 60 days at 4°C only the liposomes carrying *N*-palmitoylcysteamine 2b or *N,N'*-dipalmitoylcystamine 3d remained stable over the observation period in terms of vesicle size and population homogeneity. The amino acid derivatives showed to destabilize the lipid bilayer membrane in a fashion that in the case of *N*-palmitoylcysteine 1d and *N,N'*-dipalmitoylcystine 3c liposomes of a much larger diameter were formed during storage. The increase in liposome size is probably due to membrane fusion resulting in large but still homogeneous liposomes (cf. *H*%-values after 60 days). Values of the homogeneity parameter *H* of 30 to 60% indicate homogeneous liposome populations, whereas at $H > 60\%$ a heterogeneous or more than one vesicle population are present. The incorporation of the L-cystine and L-cysteine derivatives (4a,b) with the carboxyl group protected by *tert*-butyl ester groups into liposomes resulted in the formation of unstable liposomes which precipitated between 2 and 10 days after preparation.

Discussion

In regard to a therapeutical application of derivatized lipsomes we have first of all guaranteed that all components used for derivatization of the liposomes are non-toxic, analytically characterized and easily accessible. Another demand to be met is that the functionalized liposomes are obtained as small unilamellar vesicles in a reproducible population homogeneity (*H*) and hydrodynamic diameters (*D*) and can be stored over a longer period of time. With the starting materials used and the routes of synthesis worked out these aims are realized to a great extent.

In regard to the stability the lipophilic derivatives of the biogene amines are best suited for the functionalization as *D* has not grown larger during 60 days. Probably the liposomes can be stored even longer, because during 300 days we have not observed significant changes for differently func-

TABLE II

HYDRODYNAMIC DIAMETERS (*D*) AND POPULATION HOMOGENEITY (*H*) OF LIPOSOMES FUNCTIONALIZED WITH LIPOPHILIC R-SH OR R-S-S-R COMPONENTS

No.	Lipophilic component	Liposome properties			
		after 1 day		after 60 days [a]	
		D in mn	*H* in %	*D* in nm	*H* in %
1d	*N*-palmitoyl-L-cysteine	69	35	374	55
2b	*N*-palmitoylcysteamine	67	46	76	50
3c	*N,N'*-dipalmitoyl-L-cystine	63	32	179	35
3d	*N,N'*-dipalmitoylcystamine	66	46	61	51
4a	*N,N'*-dipalmitoyl-L-cystine di-*tert*-butyl ester	78		75	unstable after
4b	*N*-palmitoyl-L-cysteine *tert*-butyl ester	59		39	≥10 days

[a] Storage at 4°C under nitrogen.

tionalized liposomes composed of the same basic lipids. For the increase of D during storage of the liposomes derivatized with palmitoylated amino acids probably steric and not ionic effects are responsible caused by the carboxylate residues of the amino acids. The assumption is supported by the fact that the liposomes become destabilized if the carboxylate residues of the amino acids incorporated are blocked by bulky *tert*-butyl ester group and thus the charge is neutralized. On the basis of our results it is questionable whether the liposome-like vesicles based on palmitoyl amino acids recently proposed by Kiwada et al. [18] are unaffected by storage which is a major presupposition for a practicable therapeutical application.

Incorporation of N,N'-dipalmitoyl cystamine results in functionalized liposomes the sulfhydryl functions of which are 'protected'. Thus an oxidative dimerisation of the sulfhydryl residues which can not be excluded in case of palmitoylcysteamine is made impossible. Therefore liposomes derivatized with N,N'-dipalmitoylcystamine can be kept even in the presence of oxidizing agents while liposomes functionalized with palmitoylcysteamine have to be stored under careful exclusion of oxygen. In case of need these 'protected' liposomes can be deprotected by using standard methods, e.g., reductive cleavage of the disulfide bonds via dithiothreitol. The palmitoylcysteamine residue remaining in the liposomes is available for derivatization.

The calculated amount of functional groups per liposome provided by an incorporation of 1.3 μmol of the lipophilic R-SH or R-S-S-R derivatives in our experiments range between 1800 and 2200 for liposomes of a mean diameter of 70 nm [17]. Assuming a 1 : 1 distribution between the outer and inner bilayer leaflet on the average 1000 binding sites are located on the liposome surface.

Liposomes functionalized with *N*-palmitoylcysteamine can be derivatized with antibodies even after storage for more than 6 weeks. Consequently a large amount of liposomes can be synthesized and used up gradually. The stability of the liposomes is remarkable as sulfhydryl components not immobilized to liposomes lose their reactivity after some time at neutral pH. As the coupling of 4 different human monoclonal antibodies (IgG) which was reproduced repeatedly is to be presented in detail elsewhere the results are briefly summarized in the following.

The linkage of antibodies to the sulfhydryl residues of the functionalized liposomes was performed according to the concept of Leserman et al. [1,2] which was simplified by us. Firstly, about 1.7 3-(2-pyridyldithio)propionate residues per antibody were incorporated on reaction with an 8-fold excess with *N*-hydroxysuccinimidyl-3-(2-pyridyldithio)propionate (SPDP). Selectivity of the derivatized antibody was controlled and confirmed. Then, these derivatized antibodies were radioactively labelled by ^{125}I and coupled to the sulfhydryl residues of liposomes functionalized with palmitoylcysteamine resulting in the liberation of α-thiopyridon. With an excess of derivatized antibody, which was afterwards removed from the antibody-liposome complex by column chromatography, 3–5 antibodies were coupled per liposome. Surplus uncoupled antibody could be regained without loss of activity. It results that only 0.3–0.5% of the sulfhydryl functions on the liposomal surface were derivatized with antibodies. The low yield in coupling is probably due to the fact that most of the sulfhydryl groups are located too deep inside the liposomal surface and thus sterically not accessible for the derivatized antibody. This assumption agrees with the results of Kung and Redemann [19]. The authors report that to liposomes functionalized with phosphatidylethanolamide of carboxyacyl derivatives antibodies could only be coupled if the lipophilic part of the molecule was separated from the carboxylate function by a long spacer.

The assumption that the majority of the sulfhydryl groups turned inactive during storage is contradicted by two arguments. Inactivation of the sulfhydryl groups because of oxidative crosslinking of the liposomes can be excluded as the liposomes were stored in the presence of nitrogen and did not precipitate during storage. Inactivation due to dimerization of the sulfhydryl groups on the liposomal surface is unlikely because these are immobilized and statistically far apart from each other.

In regard to a therapeutic application (immunotargeting) the efficiency of liposomal loading with expensive antibodies should be as low as possible in order to avoid high costs and prevent

unwanted immune reactions. The immobilization of 3–5 antibodies per liposome was sufficient for the in vitro cell-targeting of our antibody-liposome complexes. Different cell-binding experiments demonstrated that IgG-liposome complexes were much better bound by the target cells than by non-target cells which was clearly proven by ELISA and RIA. On the other hand, liposomes without antibodies showed negligible cell-binding.

How far these results can be transferred to in vivo conditions has to be the object of further experiments. The crucial point for in vivo experiments is that degradation of the biodegradable antibody-liposome complexes in blood and serum proceeds slower than the cell targeting. For the following three reasons we are optimistic that our approach can meet this requirement.

Firstly: On the average, one antibody is coupled to a liposome via 1.7 disulfide bonds. On the justified assumption that the disulfide bonds between antibody and liposome are sterically shielded it can be expected that the antibody-liposome linkage is comparable in stability to the disulfide bonds of proteins. Besides one liposome carries 3–5 antibodies. Thus, the antibody-liposome complex has several 'reserves' in case that in vivo disulfide bonds are cleaved and antibodies get lost before the target cell is reached. For a therapeutic success it is decisive that sufficient but not all liposomes find the target cell.

Secondly: Egg PC and cholesterol were used as matrix lipids which usually compose biodegradable liposomes of sufficient stability. To what extent the additional functionalized lipid components positively or negatively influence the in vivo stability of the complexes is the object of further investigation.

Thirdly: However, 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. Liposomes which can be synthesized in reproducible quality, as is the case here, can be adapted to various problems of the in vivo system because the stability and degree of derivatization can be determined by the nature and the composition of the lipids used for the synthesis of the liposomes.

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