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Carboxyacyl derivatives of cardiolipin as four-tailed hydrophobic anchors for the covalent coupling of hydrophilic proteins to liposomes

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Two carboxyacyl derivatives of cardiolipin, *O*-succinyl- and *O*-glutarylcardiolipin, were synthesized with the aim of using them as artificial membrane anchors for the immobilization of hydrophilic proteins to liposomes. Four adjacent fatty acid residues can be introduced into a protein with only one single amino group being blocked, by reacting the cardiolipin derivatives with the protein amino groups after carbodiimide activation. α-Chymotrypsin, used as a model protein, and modified with on average two molecules of *O*-succinylcardiolipin was incorporated into liposomes, which had been prepared by different methods, with very high yield. If incorporated in preformed liposomes, the carboxyacyl cardiolipin anchors were also efficient in binding proteins to liposomal surfaces. Up to 350 μg chymotrypsin / μmol lipid were coupled to small unilamellar vesicles, preserving reactivity of the enzyme towards specific macromolecular inhibitors. Human IgG could also be bound to anchor-containing liposomes with high protein to lipid coupling ratio as well as high coupling yield.

Introduction

Liposomes with covalently attached hydrophilic ligands on their outer surface have become important for a variety of applications, e.g. as drug-carriers grafted with receptor-recognizing ligands, or for the preparation of immunogenic liposomes bearing peptide epitopes or proteins on their surface [1].

In the main, there are two different concepts of the covalent immobilization of hydrophilic proteins to liposomes:

(i) native or modified proteins are coupled to preformed vesicles containing lipid derivatives with activated functional groups, or

(ii) proteins are first modified with hydrophobic molecules serving as membrane anchors, and then incorporated into liposomal membranes.

During recent years a number of coupling techniques belonging to these two categories, and using different membrane anchors, have been suggested (reviewed in Refs. 2–4) . So far, cholesterol derivatives, long chain fatty acids or phospholipid derivatives with two fatty acid residues have been employed as membrane anchors.

In this paper we describe the synthesis of four-tailed membrane anchors (carboxyacyl derivatives of cardiolipin) and their capacity to attach hydrophilic proteins to liposomes, testing both main immobilization strategies.

Abbreviations: PC, phosphatidylcholine; CL, cardiolipin; *O*-succ-CL, *O*-succinyl-CL; *O*-glut-CL, *O*-glutaryl-CL; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; ATEE, *N*-acetyl-L-tyrosine ethyl ester; SDS, sodium dodecyl sulfate; DMAP, dimethylaminopyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; L/D ratio, molar ratio of lipid to detergent; SUV, small unilamellar vesicle; REV, reverse-phase evaporation vesicle; STI, soybean trypsin inhibitor; DMSO, dimethylsulfoxide; DOC, sodium deoxycholate; TCA, trichloroacetic acid.

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Materials and Methods

Materials

Egg phosphatidylcholine (PC) and bovine heart cardiolipin (CL, sodium salt, ethanolic solution) were produced by the Kharkov bacterial preparations company

and kindly donated by Prof. Torchilin (Moscow, U.S.S.R.). Cholesterol was purchased from ICN Pharmaceuticals, Inc., Life Science Group, Plainview, NY. Succinic acid anhydride and glutaric acid anhydride were obtained from VEB Jenapharm, Laborchemie Apolda and [1,4-^{14}C]succinic acid anhydride (spec. act. 348 MBq/mmol) was a product of the Central Institute of Nuclear Research of the Academy of Sciences of the G.D.R., Dresden. [^{3}H]Cholesterol (spec. act. 185 GBq/mmol) was from Amersham International, Amersham, U.K. 4-(*N*,*N*-Dimethylamino)pyridine (DMAP) was supplied by Merck, Darmstadt. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), human polyclonal IgG, dialysis bags, *n*-octyl β-D-glucopyranoside (octyl glucoside) and calcein were obtained from Serva, Heidelberg. Chymotrypsin A_4 from bovine pancreas (EC 3.4.21.1), salt-free, with a content of active sites of 88% as determined with *trans*-cinnamoylimidazole was from Boehringer, Mannheim and its specific substrate, *N*-acetyl-L-tyrosine ethyl ester (ATEE), was supplied by Koch-Light Laboratories, Colnbrook Bucks, U.K. Soybean trypsin inhibitor (STI) was from Reanal, Budapest, Hungary and Aprotinin (Contrycal®) from AdW, Dresden. Metrizamide (Amipaque®) was purchased from Nyegaard & Co. AS, Oslo, Norway and Sepharose CL-4B from Pharmacia, Uppsala, Sweden.

Methods

Analytical methods and assays. Analytical TLC was performed on Silica gel 60 F_{254} plates (E. Merck, Darmstadt) of 0.25 mm thickness. $CHCl_3/CH_3OH/25\%$ NH_4OH (130:64:15, v/v) was used as a solvent system for analytical as well as preparative TLC and lipid spots were stained by phosphomolybdic acid spray. Protein was determined according to Lowry et al. [5] on a microscale [6] in the presence of DOC (0.43% final concn.) or, in the case of IgG-liposomes, in the presence of SDS (0.85% final concn.). In samples of proteoliposomes, protein measurements were corrected for lipid blanks from a calibration curve made in the presence of DOC. All vesicles were prepared with a molar phospholipid/cholesterol ratio of 65:35. Trace amounts of [^{3}H]cholesterol (185 GBq/mmol) were used as the radioactive lipid marker. The catalytic activity of α-chymotrypsin was determined with the substrate ATEE by automatic titration at constant pH according to Wilcox [7]. For determination of specific activities of native, hydrophobized and immobilized enzyme samples the esterolytic activity was measured in 0.01 M ATEE, 0.15 M NaCl in a total volume of 10 ml at 25°C at the respective pH-optimums in the presence of 0.1% (w/v) Triton X-100. When no Triton X-100 was added to proteoliposome samples only the activity at the outer surface could be measured. Independently, we found (not shown) that the specific activity of α-chymotrypsin is not significantly influenced by Triton X-100 at a concentration of 0.1% (w/v).

Anchor synthesis. The anchors were prepared from CL (25 mg), dried from ethanol by nitrogen and then kept under vacuum overnight. Succinic acid anhydride (71.5 mg) or glutaric acid anhydride (81.2 mg) was then added, together with DMAP (6.5 mg) in 3 ml anhydrous pyridine and the mixture stirred under nitrogen in the dark. As monitored by TLC, the reaction proceeds to completion in 10–15 h. At the end of this period the solvent was evaporated, the residue dissolved in $CHCl_3$ and then washed with 0.1 M $NaHCO_3$ to remove excess anhydride. The product was isolated by preparative TLC on silica gel G plates of 0.5 mm thickness using the solvent system mentioned above. The main phosphate positive band was scraped off the plates and extracted with $CHCl_3/CH_3OH$ (1:1, v/v). The concentration of the modified cardiolipin was estimated after ashing the phospholipid according to Ames [8]. The cardiolipin derivatives produced by this procedure were stored in dry chloroform under nitrogen at −70°C. A radiolabeled derivative was prepared using [1,4-^{14}C]succinic acid anhydride.

Coupling of α-chymotrypsin with O-succinylcardiolipin (O-succ-CL). To modify α-chymotrypsin according to Ref. 9 0.58 μmol of dry *O*-succ-CL were suspended in 100 μl DMSO, followed by addition of 500 μl 0.15 M NaCl. After brief sonication 10 μl 0.01 M HCl and then 20 mg EDC were added. After stirring for 5 min at room temperature, 4.6 mg (0.18 μmol) α-chymotrypsin in 500 μl 0.1 M borate /NaOH buffer (pH 8.5) were added and the mixture incubated under stirring overnight at 4°C. The modified protein was separated from the reaction mixture by gel filtration on a Sepharose CL-4B column presaturated with protein (2 × 6.5 cm, equilibrated and eluted with 0.15 M NaCl, sample volume 0.5 ml), collecting opalescent drops, and was quantitated by protein determination. The increase in hydrophobicity of the modified α-chymotrypsin was shown by phase separation in Triton X-114 solution according to Bordier [10].

Determination of the degree of covalent modification of α-chymotrypsin by O-succ-CL. The following procedure was used to remove non-covalently bound anchor: After mixing 500 μl of the chromatographically separated hydrophobized protein, modified with ^{14}C-labeled anchor, with an equal volume of 95% ethanol, the protein was precipitated by addition of 400 μl of 1.2 M TCA and carefully washed with $CHCl_3/CH_3OH/25\%$ NH_4OH (10:10:1, v/v) several times until the radioactivity in the supernatent was negligible. The precipitated protein was dissolved by brief sonication in NaOH solution and the degree of covalent modification of chymotrypsin was calculated from protein concentration and radioactivity.

Incorporation of the hydrophobized α-chymotrypsin into liposomal bilayers. (a) Reverse-phase-evaporation method. Proteo-REVs were prepared as described in Ref. 9 according to the procedure of Szoka and Papahadjopoulos [11] using 4.6 mg lipid dissolved in 3 ml diethyl ether and 1 ml aqueous solution of the hydrophobized protein. The resulting proteoliposome dispersions were extruded through 0.4 μm polycarbonate membranes (Nuclepore, Pleasanton, U.S.A.).

(b) Detergent removal by dialysis from mixed micelles. Dry lipids were solubilized either in cholate-buffer solution, 0.15 M NaCl, 5 mM borate (pH 8.5) containing hydrophobized protein (final lipid concentration 5.6 mg/ml, molar ratio of lipid to detergent $L/D = 0.4$) or in octyl glucoside-buffer solution (final lipid concentration 3.1 mg/ml, $L/D = 0.1$). Solubilization of the mixed micelle suspension was supported by brief sonication. Detergent was removed by dialysis at 4°C for 2 h against borate buffer first and then followed by 40 h dialysis against 0.15 M NaCl.

The formation of proteoliposomes was studied by density gradient centrifugation in sucrose gradients (0–30% w/v) in 0.15 M NaCl. Liposomes in 35% (w/v) sucrose were layered at the bottom of the gradients and centrifuged at $150000 \times g$ for 16 h at 4°C. The fractions of the gradients were analyzed for enzyme activity and lipid content. Sucrose concentrations were determined by refractometry.

To determine the percentage of enzyme molecules, localized on the outer vesicle surface, the catalytic activity was measured before and after addition of Triton X-100 (final concentration in the assay solution: 0.1% w/v).

In order to determine the molar protein to lipid ratio, in some cases the proteoliposomes were isolated by discontinuous density gradient centrifugation, as will be described below for IgG-REVs.

Immobilization of α-chymotrypsin to anchor-containing SUVs. Preparation of liposomes: SUVs, consisting of 35 mol% cholesterol and 65 mol% phospholipid (0–5 mol% *O*-succ-CL and PC) were prepared in 0.15 M NaCl by sonication with a tip sonifier (Branson sonifier B-12) followed by centrifugation at $100000 \times g$ for 1 h to remove titanium particles and larger lipid aggregates.

Protein coupling: 10 μl 0.01 M HCl and then 2 mg EDC dissolved in 10 μl water were added to 200 μl of liposomes (4.8 mM lipids) in 0.15 M NaCl. Following incubation at 23°C for 5 min, the mixture was supplemented with 100 μl of chymotrypsin solution in 0.1 M borate buffer (pH 8.5) (0.8–32 mg protein/ml) and incubated for 16 h at 4°C under stirring.

Unbound protein was separated from liposomes by gel chromatography on Sepharose CL-4B (7 × 2 cm) presaturated with lipid and protein. Fractions collected from the column were assayed for protein and lipid content.

A possible loss of membrane integrity caused by the immobilization of the protein was evaluated by monitoring the leakage of entrapped calcein fluorimetrically during the coupling procedure. Leakage measurements and the fluorimetrical determination of trapped volume according to Oku [12] were performed with a Shimadzu UV 300 spectrophotometer (Kyoto, Japan). The trapped volume per mole lipid was used as a measure of the apparent vesicle diameter by simple geometric calculations similar to those in Table I of Mimms et al. [13].

Interaction of the immobilized chymotrypsin with specific protein proteinase inhibitors. The inhibitors used were Aprotinin and STI. The degree to which the enzyme, immobilized to preformed SUVs, could be inhibited by the different protein inhibitors was compared with the degree to which the free enzyme could be inhibited. The remaining esterolytic activity was measured at pH 7.4 and 30°C after preincubation of the enzymes with increasing quantities of each inhibitor at pH 7.4 (5 mM PBS) and room temperature for either 15 min (STI) or 60 min (Aprotinin).

Antibody coupling to anchor-containing REVs. REVs (containing 2.5 mol% of either *O*-succ-CL or *O*-glut-CL) were prepared according to Ref. 11 and antibody coupling was performed as described for α-chymotrypsin. Isolation of the proteoliposomes from the reaction mixture was performed by flotation in a discontinuous metrizamide gradient as in Refs. 14 and 15 with minor modification (40/30/0% w/v metrizamide; 1.5/3.0/0.5 ml at $150000 \times g$ for 2 h). The liposomes collected from the 30/0% interface were assayed for protein and lipid content after removal of metrizamide by dialysis.

Freeze-fracture electron microscopy. The samples were studied by freeze-fracture electron microscopy after quenching rapidly (10^4 K s^{-1}) from room temperature (22°C) according to the sandwich technique with liquid propane [16]. The specimens were fractured and shadowed in a Balzers BAF 400 D freeze-fracture device at −120°C. The cleaned replicas were examined in a Jeol JEM 100 B electron microscope.

Results

Synthesis of carboxyacyl derivatives of CL

Under all conditions described, a conversion of CL into one major phosphate positive product (67% as calculated by phosphorous content) and two side products was found. Using ^{14}C-labeled succinic acid anhydride the main product was identified as *O*-acylated CL (Fig. 1) as shown by calculation of succinic acid residue to phosphate ratio. Value obtained was 0.51 ±

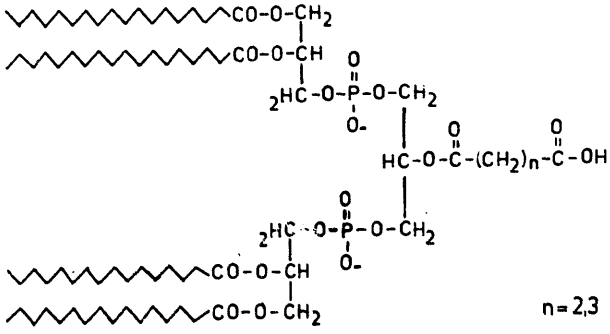

Fig. 1. Carboxyacyl derivatives of cardiolipin.

0.02, $n = 5$, which agrees with the theoretical one. TLC R_f values found are 0.50 and 0.23 for CL and for *O*-succ-CL/*O*-glut-CL, respectively. The two phosphate positive and ^{14}C-containing side products had R_f values of 0.10 and 0.04. The desired products were purified by preparative TLC. Phosphate analyses indicated a 50–60% yield of acylated CL, which was chromatographically pure. To test the reactivity with amines, stearylamine was coupled with *O*-succ-CL in organic solution upon activation with dicyclohexylcarbodiimide. A new, faster running product appeared with a R_f value of 0.60.

Coupling of α-chymotrypsin with O-succ-CL

In the presence of water soluble carbodiimides *O*-succ-CL reacts with free amino groups of proteins to form amide bonds. Brief sonication was used to solubilize the anchor in saline containing DMSO leading to vesicle formation with diameters of around 40 nm (Fig. 2). The opalescent vesicle dispersion formed by this process becomes more turbid under reaction with protein. Nevertheless, the hydrophobized protein could be separated from the reaction mixture by gel filtration on Sepharose CL-4B (without addition of detergent to the eluant) as an opalescent liquid. Approx. 35% of the protein was associated with the isolated phospholipid.

The amount of covalently linked anchor was determined by extraction of non-covalently bound ^{14}C-labeled anchor from the precipitated modified protein by organic solvents. Control experiments with non-covalently bound anchor showed that sufficient delipidation could only be achieved using an alkaline $CHCl_3/CH_3OH$ mixture. Over 95% of the initial ^{14}C-radioactivity could be removed in the control experiments. The molar ratio of covalently bound *O*-succ-CL to chymotrypsin was determined to be 2.1 : 1.

An increase in hydrophobicity was demonstrated by measuring the partition of the modified protein in a solution of Triton X-114 after phase separation: 92.5% of the isolated modified enzyme was found in the detergent rich phase in contrast to native α-chymotrypsin, which was nearly completely recovered in the detergent depleted phase.

The loss of specific activity caused by modification was not higher than 25%. Before the addition of Triton X-100 90% of the enzyme activity could be measured.

Incorporation of the hydrophobized protein into liposomal bilayers

Under optimal conditions, up to $1.64 \cdot 10^{-3}$ mol α-chymotrypsin (hydrophobized with 2 mol anchor/mol enzyme) per mol of liposomal lipid (corresponding to 41 μg protein/μmol lipid) could be incorporated into REVs. This corresponds to a yield of about 99% as shown in Table I. Although many attempts were made, it was not possible to prepare REVs with an essentially higher protein to lipid ratio. By increasing the initial molar ratio of protein to lipid, heavy precipitation occurred during liposome preparation.

The trapped volume, determined fluorimetrically, was found to be 9.6% of the total amount of the calcein solution. This means that approx. 10.3 l aqueous phase are entrapped by one mole of lipid. Thus the relatively high trapping efficiency of REVs for water-soluble compounds [11] is not decreased by the incorporation of hydrophobized protein. The apparent vesicle diameter, evaluated as detailed in Methods, was 300 nm. This value could be confirmed by electron microscopy. Vesicles are visible with diameters ranging from 100 to 350 nm (Fig. 3a). The fracture faces of the

Fig. 2. Freeze-fracture electron micrograph of small vesicles, prepared from *O*-succinylcardiolipin, showing diameters of around 40 nm. Bar represents 100 nm and the electron micrograph is oriented with shadow direction from bottom to top.

TABLE I

Incorporation of hydrophobized α-chymotrypsin into liposomal bilayers

Incorporation procedure	Initial molar protein/lipid ratio	Vesicle bound enzyme activity (%)	Enzyme activity at the outer vesicle surface [a] (%, mean ± S.D.)	Preservation of the specific activity [b] (%, mean ± S.D.)
Reverse phase evaporation technique	$5.22 \cdot 10^{-4}$	98.6		
	$6.49 \cdot 10^{-4}$	97.0		
	$1.00 \cdot 10^{-3}$	97.2	65.5 ± 2.1	77.6 ± 2.3
	$1.66 \cdot 10^{-3}$	98.8, 98.2		
Cholate dialysis [c]	$1.03 \cdot 10^{-3}$	94.9, 95.9	55.9 ± 2.8	91.7 ± 6.2
Octyl glucoside dialysis [d]	$1.08 \cdot 10^{-3}$	94.4, 94.2	60.2 ± 2.7	85.5 ± 2.5

[a] Determined by measuring the enzyme activity before and after addition of Triton X-100.
[b] Measured after disruption of the vesicles by addition of Triton X-100.
[c] Vesicles prepared at 5.6 mg/ml total lipid, $L/D = 0.4$.
[d] Vesicles prepared at 3.1 mg/ml total lipid, $L/D = 0.1$.

vesicles are smooth and without any incorporated protein particles.

Using different detergents, the hydrophobized chymotrypsin was attached to liposomal bilayers according to the detergent-dialysis method originally developed for the reconstitution of membrane proteins [17]. The yield of vesicle bound enzyme was in the same range as found for the REV-procedure (Table I).

From the data given in Table I, it can also be seen that the different incorporation procedures used impair the enzyme activity only to a small extent. As shown by enzyme activity measurements before and after destruction of the liposomal membranes with the aid of detergent, in all cases something more than 50% of the enzyme molecules become located at the outer vesicle surface.

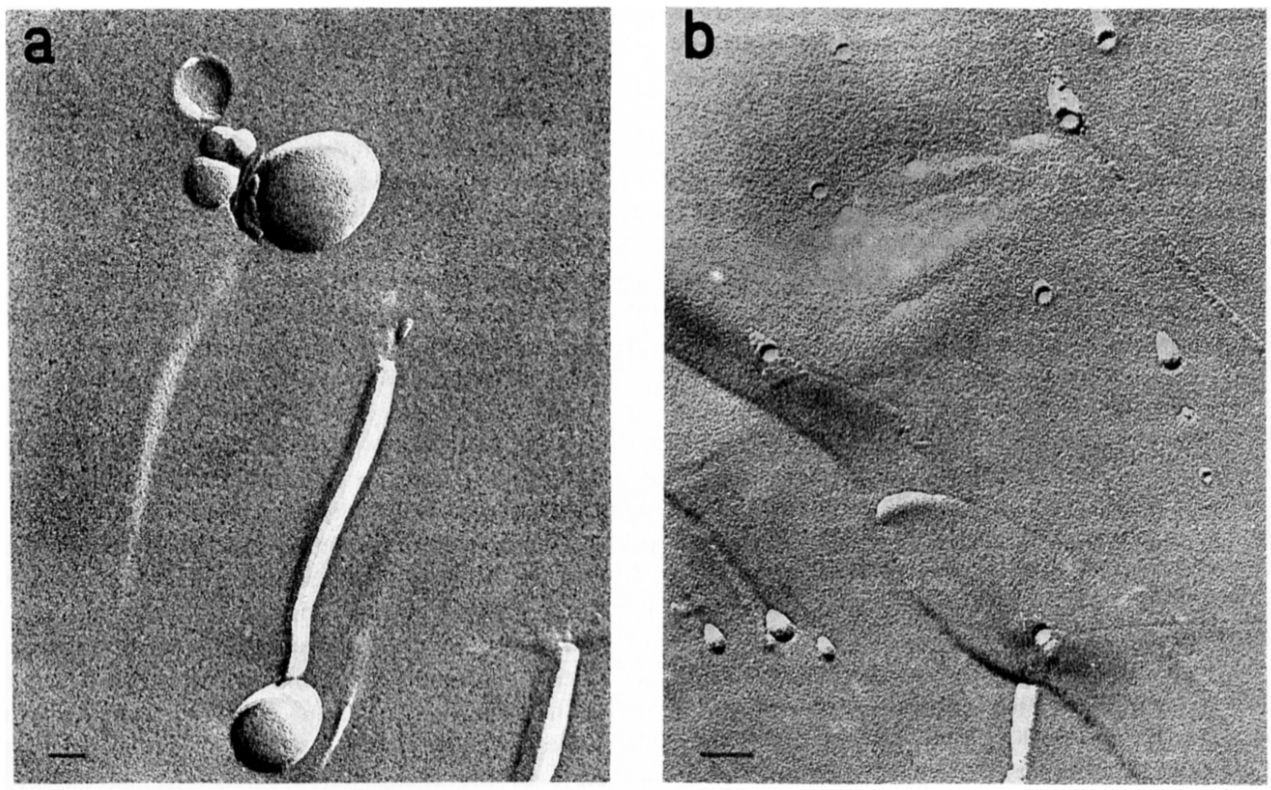

Fig. 3. Freeze-fracture electron micrographs of (a) proteo-REVs, loaded with hydrophobized α-chymotrypsin, showing diameters ranging from 100 to 350 nm and of (b) proteo-SUVs, loaded with unmodified α-chymotrypsin with diameters of around 35 nm. Bars represent 100 nm; shadowing direction is from bottom to top of the micrographs.

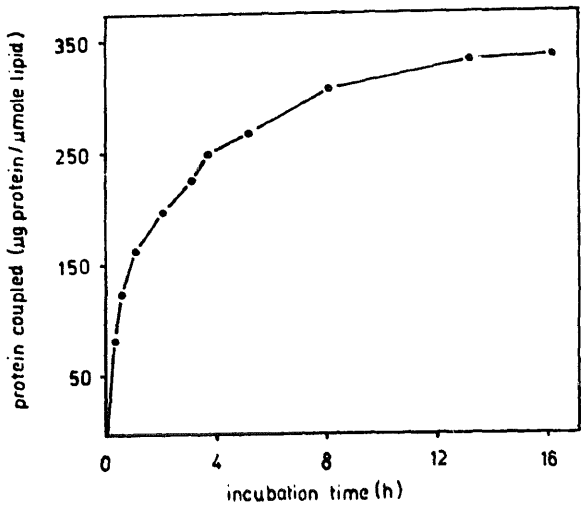

Fig. 4. Time course of the covalent coupling of protein to SUVs, activated by EDC. SUVs (5 mol% of *O*-succ-CL), activated by EDC as described under Methods, were mixed with chymotrypsin (liposomal lipid concentration: 3 mM; protein concentration: 8 mg/ml) and incubated at 4°C. After the time indicated, proteoliposomes were isolated by gel filtration.

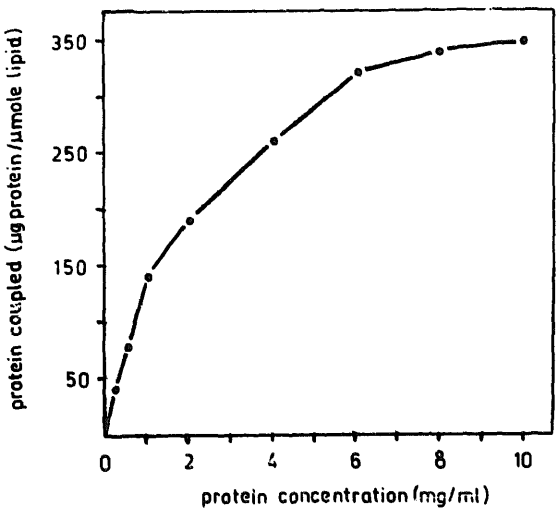

Fig. 6. Dependence of protein coupling to SUVs on protein concentration in the reaction mixture. SUVs (5 mol% *O*-succ-CL), activated by EDC as described, were mixed with chymotrypsin in various concentrations and incubated for 16 h at 4°C (liposomal lipid concentration: 3 mM).

Coupling of α-chymotrypsin to preformed SUVs

The amount of protein coupling to liposomes depends upon several parameters of the activation and the coupling step. Activation of liposomal carboxylic groups for 5 min in buffer free medium and with an optimal EDC-concentration (depending on liposomal lipid concentration and anchor content) gave the best results. Coupling of protein to activated vesicles was complete within 12–16 h at 4°C (Fig. 4). It was found that there is a nearly linear relationship between liposomal anchor content and protein coupling (Fig. 5). Also, the concentration of protein (Fig. 6) and lipid (not shown) in the reaction mixture influence the extent of protein conjugation. Under optimal conditions up to 350 µg protein/µmol liposomal lipid could be coupled to EDC-activated vesicles, whereas in a corresponding control experiment without EDC-activation only 1.3 µg protein/µmol liposomal lipid were attached.

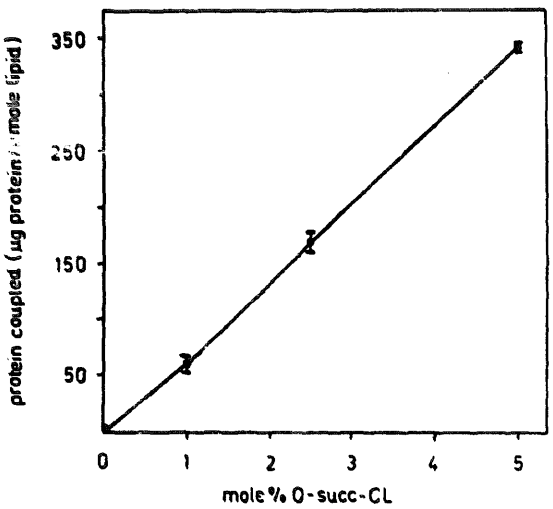

Fig. 5. Amount of chymotrypsin bound to SUVs, activated by EDC, versus anchor content of the liposomes. SUVs, activated by EDC as described, and chymotrypsin were allowed to react for 16 h at 4°C (liposomal lipid concentration: 3 mM; protein concentration: 8 mg/ml).

During storage at 4°C in 0.9% NaCl the stability of the protein-vesicle-linkage was tested by a two-fold rechromatography of the proteoliposomes on Sepharose CL-4B after 7 and 14 days. No release of protein could be detected.

As examined by turbidity measurements during the coupling procedure, no changes of particle size occurred upon immobilization of α-chymotrypsin, in contrast to the coupling of IgG to SUVs (not shown).

The mean captured aqueous volume of the SUVs (containing 5 mol% anchor) was 0.6 l/mol lipid, i.e. in the expected range of SUV preparations. From this value an average vesicle diameter of 32 nm was calculated, which is in good agreement with the electron microscopical results (Fig. 3b). Vesicles are visible with smooth fracture faces and diameters of around 35 nm. Aggregated vesicles could not be found.

Leakage measurements of entrapped calcein showed that the liposomal bilayer permeability is not significantly altered by the coupling procedure. Up to the end of the coupling procedure (16 h), the leakage of calcein was only 6.4% with more than the half (3.6%) in the first two hours. In control experiments, where protein was incubated with non-activated liposomes, 3.8% of the encapsulated calcein leaked out of the vesicles. Anchor-containing liposomes stored at 4°C over this period of time were quite stable (100% calcein latency).

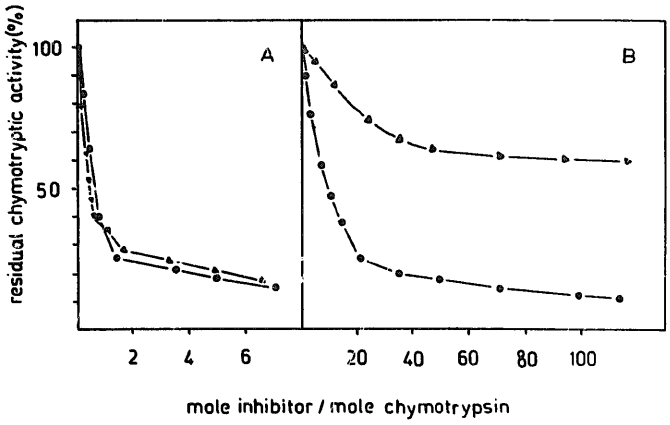

Fig. 7. Inhibition of chymotrypsins by Aprotinin (M_r 6500) and STI (M_r 22000). The capacity of Aprotinin (A) and STI (B) to inhibit ATEE hydrolysis by chymotrypsins was determined. ●———●, native enzyme; ▼———▼, enzyme, immobilized on the surface of SUVs ($3 \cdot 10^{-3}$ mol chymotrypsin/mol liposomal lipid) by covalent binding via carbodiimide activated *O*-succ-CL.

The loss of specific activity (ATEE as substrate) by the immobilization was 12.5% only Enzyme activity could be measured before and after the disruption of the vesicles by the addition of Triton X-100. Inhibition studies with Aprotinin and STI showed that the immobilized enzyme not only retained its ability to react with low molecular weight substrates, but also to interact with specific protein inhibitors. The immobilized chymotrypsin (M_r 25000) is inhibited by Aprotinin (M_r 6500) to the same extent as the soluble enzyme, whereas in the case of STI (M_r 22000) the residual esterolytic activity was 61.9% (Fig. 7), possibly caused by sterical hindrance of the interaction of some of the immobilized enzyme molecules with STI.

TABLE II

Coupling of IgG to preformed anchor-containing REVs

To 220 μl of REVs (4.8 mM lipids, 2.5 mol% CL-anchor), activated by EDC, 100 μl of a solution of human IgG (5.12 mg/ml) were added, and the resulting mixture (pH 8.5) was then incubated for 16 h at 4°C (final liposomal lipid concentration: 3 mM; final protein concentration: 1.6 mg/ml). Liposomes were separated from unbound protein by density gradient centrifugation. in control experiments non-activated anchor-containing liposomes were incubated with IgG under the same conditions. For further details, see Materials and Methods.

Cardiolipin derivative	Coupling efficiency [a] (%)	Coupling ratio (μg protein/μmol lipid)
	control	control
O-succ-CL	32.2 ± 1.4	192.5 ± 9.9
	6.1 ± 1.5	38.1 ± 6.4
O-glut CL	44.8 ± 1.1	237.5 ± 10.5
	7.1 ± 1.7	41.0 ± 8.4

[a] Protein bound to liposomes in % ± S.D. of total protein added to the reaction mixture.

Antibody coupling to preformed REVs

Coupling yield, as well as the amount of IgG coupled to preformed REVs containing 2.5 mol% of the anchor molecules, are shown in Table II. Covalent attachment led to significantly higher coupling yield and protein loading of the liposomes than nonspecific adsorption to anchor containing vesicles which were not activated by carbodiimide.

Discussion

In this paper we described the synthesis of carboxyacyl derivatives of CL and their application for covalent attachment of hydrophilic proteins to liposomal membranes. This new type of artificial membrane anchor possesses four adjacent hydrophobic acyl chains, at the same time providing for a spacer group. The modified lipid is able to self-associate to small liposomes, as shown by freeze-fracture electron microscopy (Fig. 2). After activation with water-soluble carbodiimide, the carboxylic group of the CL-derivatives reacted with free amino groups of proteins forming stable amide bond. This type of coupling reaction has been already successfully applied for coupling proteins or other ligands containing amino groups (peptides, aminosugars) using either carboxyacyl derivatives of PE [9,15,18–23] or synthetic lipophilic carboxylate components [24]. Either unmodified ligands were coupled to preformed liposomes [15,18–23,24], or hydrophobized proteins were incorporated into liposomal membranes during liposome preparation [9,22,23]. Using the CL-anchors we have tested both main immobilization strategies.

To demonstrate the effect of hydrophobization we chose α-chymotrypsin as a hydrophilic model protein. We carried out the protein modification with sonicated 'anchor vesicles' bearing carboxylic groups at their surface. Using the mean diameter of 40 nm (Fig. 2) it can be estimated that each of these vesicles contains about 10000 molecules of *O*-succ-CL. With this value we can express the number of anchor vesicles as 'moles' allowing to calculate a molar ratio of protein to vesicles of about 3000:1. Under these experimental conditions, two covalently attached CL-anchors were introduced per one chymotrypsin molecule only. Given the molar excess of protein over anchor vesicles, we assume for topological reasons that the activated carboxylic groups, reacting with the protein, are neighbouring in anchor vesicles, implying that the two moored CL-molecules are not randomly distributed on the surface of the protein molecules. This should be useful for the binding of artificially hydrophobized proteins to liposomes.

After hydrophobization the protein remains soluble. Gel filtration data (not shown) demonstrate that the anchor-protein-conjugates, in the absence of detergent and excess of phospholipid, associate with each other forming micelles. A similar self-association was clearly

shown by Afzelius et al. [25] for artificially hydrophobized serum transferrin.

The hydrophobized protein is readily incorporated into liposomes prepared by detergent dialysis from mixed micellar solutions of lipids, hydrophobized protein and detergent (either sodium cholate or octyl glucoside). In the second incorporation procedure used, the protein incorporation was achieved into REVs without detergent, just by adding the modified protein to the original two-phase emulsion [9]. At the same time, a high amount of a water-soluble compound could be encapsulated. As seen from smooth fracture faces of the REVs (Fig. 3a), no larger aggregates of the hydrophobized protein are incorporated into the hydrophobic interior of the bilayer. Since Triton X-100 in the concentration of 0.1% (w/v) does not significantly influence the esterolytic activity of the native enzyme or vesicle immobilized enzyme, we concluded from activity measurements before and after disruption of liposomes, in which the hydrophobized enzyme was incorporated (Table I), that about two thirds of the enzyme molecules are located on the outside of the REVs. On basis of the calculated average vesicle diameter, confirmed by electron microscopy, we estimated that under optimal conditions up to 800 chymotrypsin molecules are attached to the outside of a vesicle with a diameter of 300 nm.

The coupling ratio obtained by the reverse phase evaporation procedure using chymotrypsin modified with about two CL-anchors is in the same range as was obtained using chymotrypsin hydrophobized with 5–6 mol PE-anchor/mol chymotrypsin [9]. The introduction of 8 hydrophobic acyl chains by covalent modification of only 2 functional groups by CL-anchors led to a loss of specific enzyme activity of 25% only, whereas the modification by the PE-anchors reduced the activity to 46%.

Using preformed liposomes containing the CL-anchors, proteins can be coupled without prior modification. Under optimal conditions a coupling ratio up to 350 μg α-chymotrypsin/μmol lipid could be achieved, which corresponds to 53 chymotrypsin molecules bound to the outer surface of a single liposome with a diameter of 32 nm. The good coupling capacity of the CL-anchors could be confirmed in preliminary experiments where a human IgG-fraction was coupled to REVs. By variation of several parameters of the coupling step (liposomal anchor content, protein and lipid concentration in the reaction mixture) it was possible to control the liposome loading over a wide range.

The loss of specific activity towards ATEE of only 12.5% and the preservation of the ability of the enzyme to interact with specific high-molecular-weight inhibitors indicate that coupling of the native protein to liposomes is a mild procedure using the carbodiimide method. This result agrees with findings of Bogdanov et al. [18], where various lectins were immobilized to preformed liposomes containing *N*-glutaryl-PE without loss of lectin specificity towards saccharides. In contrast, immobilization using thioreactive lipids was found to be unacceptable in some cases due to the loss of binding specificity of lectins by the pretreatment with *N*-hydroxysuccinimidyl-3-(2-pyridyldithio)propionate.

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