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# Covalent modification of serum transferrin with phospholipid and incorporation into liposomal membranes

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**A method is described for incorporation of water-soluble proteins into liposomal membranes using covalent protein-phospholipid conjugates in detergent solution. A disulfide derivative of phosphatidylethanolamine containing a reactive *N*-hydroxysuccinimide ester group is synthesized, and the derivative is reacted with serum transferrin in deoxycholate-containing buffer. Disulfide-linked transferrin-phosphatidylethanolamine conjugates containing up to 6 mol phospholipid /mol protein are prepared. The amphiphilic conjugates have solubility properties very similar to integral membrane proteins. The conjugates self-associate to form protein micelles of narrow size distribution (Stokes radii 6–7 nm), and in the presence of excess phospholipid (egg phosphatidylcholine), they readily incorporate into liposomal membranes upon removal of detergent. Stable incorporation into liposomes requires the introduction of two molecules of phosphatidylethanolamine into the transferrin. Using the disulfide linker to release transferrin from the liposomes, evidence is presented for a function of the phosphatidylethanolamine as an anchor-molecule into the liposomal lipid. Optimal conditions for preparation of homogeneous liposomes with diameters in the range 30–125 nm and with a varying content of transferrin are defined. The liposomes appear well suited for studies on liposome–cell membrane interactions.**

## Introduction

Several methods have been developed for coupling of water-soluble proteins to the surface of liposomes in attempts to prepare stable liposomes with high binding-affinity for cell membranes [1]. Such proteoliposomes may serve as efficient carriers for transference of drugs, enzymes and nucleic acids into cells [2–7]. A particular useful protein-liposome coupling procedure involves covalent modification of functional groups on the proteins with lipid residues. The proteins hereby obtain an increased hydrophobicity and affinity for liposomal membranes [8–14]. Using this type of approach to prepare homogeneous proteoliposomes with defined properties, it is essential to understand in more detail how lipid-modified proteins interact with each other in aqueous solution and incorporate into liposomal membranes. Here an efficient method for covalent coupling of phospholipid to protein in detergent solution using a cleavable disulfide protein-phospholipid linker is presented. We report on the solubility properties of phospholipid-modified serum transferrin, and optimal conditions for liposomal binding of the protein are defined. Serum transferrin is a globular glycoprotein ($M_r$ 80 kDa) [15,16], and it is of particular use as a model protein because of high aqueous solubility, low affinity for lipid, and ability to bind to specific receptors on cell membranes [17].

## Materials and Methods

### *Materials*

Human serum transferrin (98%, substantially iron-free) was obtained from Sigma Chemical Co. (St. Louis, U.S.A.), and dithiobis(succinimidyl propionate) from Pierce Chem. Co. (Rockford, U.S.A.). Egg phosphatidylethanolamine and egg phosphatidylcholine were from

Abbreviations: DOPE, dioleoylphosphatidylethanolamine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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Serdary Research Laboratories (London, Canada). 1,2-Dioleoyl-L-3-phosphatidyl[2-$^{14}$C]ethanolamine (50 Ci/mol) and *N*-succinimidyl[2,3-$^{3}$H]propionate (105 Ci/mmol) were obtained from Amersham International (Amersham, U.K.). Rabbit immunoglobulin to human transferrin was from Dakopatts (Copenhagen, Denmark).

*Synthesis of a N-hydroxysuccinimide ester derivative of PE*

A disulfide derivative of PE containing a reactive *N*-hydroxysuccinimide ester group was synthesized analogously to the procedure described by Kinsky et al. [18]. 3.3 mg egg PE dissolved in 0.5 ml chloroform containing 10 μCi [$^{14}$C]DOPE and 20 mM triethylamine was added dropwise to a stirred solution of 11 mg dithiobis(succinimidyl propionate) in 0.5 ml chloroform. 10 μl of acetic acid was added after a 1 h incubation and the reaction mixture was then applied to a 0.5 × 5 cm silica column (silica gel 60, 230–400 mesh) washed with methanol and equilibrated with chloroform before use. The column was eluted with chloroform/methanol/acetic acid mixtures; 5 ml of 100:2.5:0.6 (v/v) and 5 ml 100:5.0:0.6 (v/v), followed by 5 ml 100:20:0.6 (v/v) to elute the desired *N*-hydroxysuccinimide ester derivative of PE. The yield on the basis of [$^{14}$C]PE was 80–90%, and the purity of the derivative was > 90% as judged by TLC in the solvent system chloroform/methanol/acetic acid (30:5:2.5, v/v). TLC $R_F$ values: PE, 0.18; PE derivative, 0.64. A PE-derivative ($R_F$ 0.70) without a reactive *N*-hydroxysuccinimide ester group was the major impurity.

*Coupling of PE to serum transferrin*

0.5–4 μmol dry PE-derivative was dissolved in 1 ml 20 mM Na-Hepes buffer (pH 8.3) containing 2% (w/v) deoxycholate and 0.02% azide. 150 nmol $^{3}$H-labeled serum transferrin (apotransferrin or iron-saturated transferrin) in 1 ml 20 mM Na-Hepes (pH 7.4) was added and the mixture incubated in darkness for 5 h. Transferrin with covalently linked PE was then separated from the reaction mixture by gel filtration on Sephadex G-200 (3 × 40 cm) equilibrated and eluted (12 ml/h) at room temperature with 150 mM NaCl, 20 mM Tris-HCl (Buffer I) (pH 8.1) containing 0.15% (w/v) deoxycholate and 0.02% azide. Fractions collected from the column were pooled on the basis of their absorbance at 280 nm. The concentration of transferrin was determined using $A^{1\%}_{1cm} = 11.4$ for apotransferrin and 14.1 for iron-saturated transferrin [19]. [$^{14}$C]PE was quantitated by liquid scintillation counting using the specific activity of the starting PE-derivative. The isolated transferrin-PE conjugates were stable for more than one month when stored at 4°C.

Human serum transferrin was saturated with iron as described by Bates and Schlabach [20]. $^{3}$H-labeled transferrin (100–200 mCi/mmol) was prepared by reaction of the protein with *N*-succinimidyl[2,3-$^{3}$H]propionate in 20 mM Na-Hepes buffer (pH 7.4) for 1 h at 20°C.

*Cleavage of transferrin-PE conjugates*

The disulfide link between protein and phospholipid in the transferrin-PE conjugates was cleaved during a 2 h incubation at 37°C with either 5 mM dithioerythritol or 10 mM cysteine in Buffer I (pH 7.4). [$^{14}$C]PE derivative released from the transferrin was extracted into chloroform by the addition of 2.5 ml methanol. 2 ml chloroform and 1 ml 2 M KCl to 1 ml reaction mixture.

*Deoxycholate removal by gel filtration*

Deoxycholate was removed from the transferrin-PE conjugates by gel filtration [21] on 0.9 × 55 cm columns with Sephadex G-200 equilibrated and eluted with Buffer I (pH 7.4) at room temperature (flow rate 2 ml/h).

*Incorporation of transferrin-PE into liposomes by deoxycholate dialysis*

1 mg of egg PC in chloroform was dried under a stream of nitrogen, then under vacuum, and finally rehydrated in 1.5 ml of Buffer I (pH 8.1) containing transferrin-PE conjugates (0.1–0.8 mg of transferrin) and 0.15% (w/v) deoxycholate. Liposomes were formed during removal of the deoxycholate by dialysis against 100 mM NaCl, 10 mM Tris-acetate, 0.1 mM EDTA buffer (pH 8.1) for 3 days at room temperature, with 3 changes of 100 ml buffer a day. Liposomes with incorporated transferrin were separated from free transferrin by flotation in sucrose gradients.

*Flotation of liposomes in sucrose gradients*

Liposome suspensions (1 ml) in 35% (w/w) sucrose were layered at the bottom of 12 ml linear 0–30% (w/w) sucrose gradients in Buffer I (pH 7.4) with 1 mM EDTA and centrifuged at 35000 rpm for 16 h in a Beckman SW 40 Ti rotor at 4°C. Fractions collected from the bottom of the tubes were analyzed for radioactivity, PC and transferrin. Sucrose concentrations were determined by refractometry. The liposomes were concentrated by ultrafiltration [22]. Trapping of [$^{14}$C]sucrose in the liposomes was carried out as described in Ref. 11.

*Analysis for transferrin and phospholipid*

SDS-PAGE was carried out in 11.6% (w/v) slab gels with 0.1% (w/v) SDS [23]. Staining for protein was made with Coomassie brilliant blue R 250.

Phospholipid was quantitated as lipid phosphorus by the method of Bartlett [24], after extraction of the lipid into chloroform [25], and TLC in the solvent system chloroform/methanol/water (32:13:2, v/v).

Immunoadsorption of transferrin-containing liposomes to Sepharose beads with bound anti-(human transferrin) IgG was carried out in Buffer I (pH 7.4) with 0.05% (w/v) serum albumin. The beads were extracted for phospholipid to determine binding of liposomes. Control experiments were carried out in which liposomes without transferrin were used.

*Electron microscopy*

Liposomes were negatively stained with 1% uranyl acetate (uncorrected pH) on 400 mesh formvar and carbon coated nickel grids subjected to vacuum glow discharge just before use. Electron microscopy was carried out in a Philips 201c electron microscope operated at 60 kV.

For immunoelectron microscopy grids were floated on droplets of transferrin-containing liposomes for 10 min. The grids were then transferred through droplets containing: 1, Buffer I (pH 7.4) (10 min); 2, bovine serum albumin (1% in Buffer I) (10 min); 3, rabbit anti-(human transferrin) IgG (1 : 1000 dilution in Buffer I with 1% serum albumin) (1 h); 4, Buffer I (2 × 10 min); 5, protein A-gold complex (diluted in Buffer I to an absorbance of 0.1 at 490 nm) (30 min); 6, Buffer I (2 × 15 min); and 7, uranyl acetate (1%) (5 min). In order to test the specificity of the gold labeling, control experiments were performed in which the anti-transferrin IgG was omitted or a non-immune serum was used. Protein A was conjugated to 5 nm colloidal gold particles as described in Ref. 26.

*Cell binding of transferrin-containing liposomes*

A human small cell lung cancer cell line (OC-NYH) was maintained as described in Ref. 27. Subconfluent cells were washed twice in phosphate-buffered saline (150 mM NaCl, 50 mM phosphate pH 7.2), and resuspended to $(5–10) \cdot 10^6$ cells/ml in culture medium RPMI 1640 (GIBCO, Scotland) with 0.5% (w/v) ovalbumin. The cells were incubated at 4°C for 2 h with lipcsomes prepared with iron-saturated $^3$H-transferrin-PE conjugates (0.12 mg transferrin/mg PC) and a trace of hexadecyl [$^3$H]cholesteryl ether (50 μCi/mg PC), washed twice with 5 ml of icecold phosphate-buffered saline, and then analyzed for $^3$H-transferrin and [$^3$H]cholesteryl ether. Hexadecyl [$^3$H]cholesteryl ether (1.2 Ci/mmol) was synthesized as described in Ref. 28.

## Results

*Covalent coupling of phosphatidylethanolamine to serum transferrin*

In attempts to couple phospholipid covalently to serum transferrin through a cleavable linker, we have synthesized a disulfide derivative of phosphatidylethanolamine (PE) containing a reactive *N*-hydroxysuccinimide ester moiety (Fig. 1). The *N*-hydroxysuccinimide ester group reacts readily and under mild conditions with free amino groups in proteins to form stable amide bonds [29,30]. Serum transferrin contains 59 amino groups [31]. The desired PE-derivative was obtained in high yields by reaction of egg PE with the bifunctional reagent dithiobis(succinimidyl propionate) in the presence of triethylamine [18]. A radiolabeled derivative was prepared using [$^{14}$C]PE.

The PE-derivative reacted with serum transferrin in 20 mM Hepes buffer containing 1% deoxycholate, and transferrin with covalently attached [$^{14}$C]PE could be isolated from the reaction mixture by gel filtration on Sephadex G-200 equilibrated with buffer containing 0.15% deoxycholate. The transferrin-PE conjugates eluted in a narrow peak at 0.60–0.64 × bed volume, near the position of a transferrin standard (0.65 × bed volume), and well separated from unreacted PE-derivatives and other components of the reaction mixture. Less than 5% of the [$^{14}$C]PE in the conjugates could be extracted into chloroform-methanol mixtures. However, the disulfide bond in the linker between PE and transferrin was cleavable by reaction with dithioerythritol and cysteine, and almost quantitative extraction of PE was then obtained. These results together confirmed a covalent link between transferrin and phospholipid.

When the concentration of PE-derivative in the reaction mixture was varied, it was possible to prepare transferrin-PE conjugates with a different content of covalently attached phospholipid. Transferrin with an average degree of substitution by PE up to $N_{av} = 6.1$ (mol PE/mol transferrin) was prepared with apotransferrin. The yield of protein-bound PE on the basis of [$^{14}$C]PE derivative was 30–35%. When the coupling reaction was carried out with iron-saturated transferrin, the yield was only 8–12% and an $N_{av}$ up to 1.8 was obtained. This difference may in part reflect the higher reactivity of amino groups in apotransferrin [32].

*Self-association of transferrin-PE conjugates*

Samples of the transferrin-PE conjugate preparations were subjected to filtration on a Sephadex G-200 column equilibrated with a deoxycholate-free buffer in order to remove deoxycholate from the conjugates. As shown in Fig. 2, higher molecular weight forms of the conjugates eluted from the column in broadened peaks (0.45–0.50 × bed volume) in addition to monomeric transferrin (0.63 × bed volume), indicating aggregation

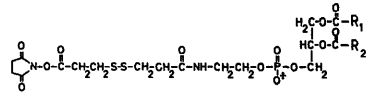

Fig. 1. *N*-Hydroxysuccinimide ester derivative of phosphatidylethanolamine.

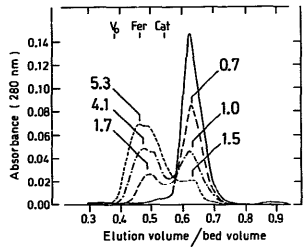


Fig. 2. Elution profiles for apotransferrin-PE conjugates on Sephadex G-200 without deoxycholate. $N_{av} = 0$ (———); 1.59 (– – –); 3.42 (·–·–·) and 6.06 (– – – – –). PE/transferrin ratios (mol/mol) in peak fractions are given by the numbers in the figure. Recoveries of PE and transferrin: 65–80%. Standard proteins: Ferritin (Fer) and catalase (Cat). $V_o$, void volume.

of the phospholipid-modified transferrin. The proportion of aggregated transferrin increased with $N_{av}$. Also the monomeric transferrin recovered from the column contained covalently linked PE, but had a lower degree of substitution than the aggregated protein (values included in Fig. 2). Recoveries of [$^{14}$C]PE and transferrin were only 65–80%, indicating absorption of aggregated transferrin in the Sephadex gel matrix. Absorption effects also accounted for the finding that the PE/transferrin ratio in the aggregate eluted from the column was lower than that expected from $N_{av}$ of the starting conjugate sample.

The average Stokes radii of the transferrin aggregates were estimated to 6–7 nm when the Sephadex column was calibrated with ferritin, catalase, and apotransferrin (Stokes radius 6.7 nm, 5.2 nm, and 4.0 nm respectively). The aggregates dissociated during refiltration on a second Sephadex G-200 column eluted with deoxycholate-containing buffer (0.15%).

*Incorporation of transferrin-PE conjugates into liposomes*

In order to incorporate the transferrin-PE conjugates into phospholipid liposomes, conjugate samples dissolved in 0.15% deoxycholate were mixed with egg PC, and liposomes were then formed during removal of the deoxycholate, either by gel filtration or by dialysis. Both methods are routinely used for insertion of isolated membrane proteins into liposomes.

Fig. 3 shows the elution profiles obtained by gel filtration (in deoxycholate-free buffer) of samples containing egg PC and $^3$H-labeled transferrin with a varying degree of substitution by PE ($N_{av} = 0$–4.4). The percentage of $^3$H-labeled transferrin coeluting with the PC-liposomes in the void volume increased with $N_{av}$ (83% at $N_{av} = 4.4$). More than 80% of the transferrin in the void volume was associated with liposomes (density 1.06–1.14 g/ml) as judged by flotation in sucrose gradients. Liposomes containing up to 0.12 mg transferrin/mg PC were prepared using the gel filtration method.

When liposomes were formed by deoxycholate-dialysis, maximal liposomal incorporation of transferrin was 25–35% using conjugates with $N_{av} = 1$–2. Liposomes containing up to about 0.25 mg transferrin/mg PC were obtained (Table I). Conjugates prepared with iron-saturated transferrin appeared to incorporate better than the conjugates with apotransferrin. A different membrane affinity of the two conjugates may be due to differences in the structure of the transferrins [33], as well as in the orientation of the PE molecules on their surface.

The encapsulation capacity of the liposomes, as determined with [$^{14}$C]sucrose, decreased with increased transferrin content, and ranged from about 1.2 to 0.5 μl/mg PC for transferrin-free control liposomes and liposomes with 0.26 mg transferrin/mg PC. SDS-PAGE analysis on the liposomes revealed a single protein band with a molecular weight close to a transferrin standard. Immunoprecipitation of more than 90% of the liposomes with anti-transferrin IgG bound to Sepharose beads showed that the majority of liposomes contained transferrin, and that it was incorporated in part into the outer surface of the liposomes. During incubation of the liposomes with cysteine, cleavage of the disulfide bonds in the linker between transferrin and PE was 60–70% after 2 h at 37°C as judged by extraction of [$^{14}$C]PE into chloroform. Flotation of the incubated liposomes in sucrose gradients showed that up to 40% of the transferrin had been released from the liposomes.

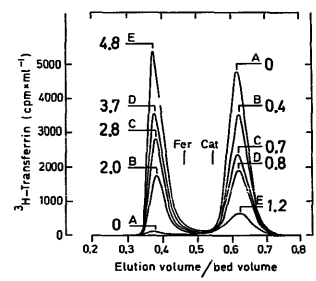


Fig. 3. Elution profiles for apotransferrin-PE conjugates with egg PC on Sephadex G-200. Samples (500 μl) contained 3 mg egg PC, 0.75 mg deoxycholate and 0.5 mg $^3$H-apotransferrin with covalently linked [$^{14}$C]PE. $N_{av} = 0$ (A); 0.81 (B); 1.53 (C); 2.33 (D) and 4.37 (E). Filtrations were carried out in deoxycholate-free buffer. [$^{14}$C]PE/$^3$H-transferrin ratios (mol/mol) in peak fractions are given in the figure. Recoveries of [$^{14}$C]PE and $^3$H-transferrin: > 90%. Standard proteins: Ferritin (Fer) and catalase (Cat).

*Electron microscopy and cell binding of liposomes*

Electron micrographs of negatively stained liposomes with a varying content of transferrin are shown in Fig. 4. Liposomes containing 0.04 and 0.26 mg transferrin/mg PC appeared unilamellar and were of narrow size distribution: mean diameter 76 nm (range 45–125 nm) and 47 nm (range 35–90 nm), respectively (Figs. 4A and 4B). Negative staining of liposomes with a higher protein to lipid ratio (0.52 mg transferrin/mg PC) revealed only irregularly shaped sheet-like structures 25–200 nm in size (Fig. 4C). The density of particles, approximately 5 nm in size, on the outer surface of the liposomes increased with protein to lipid ratio, and may represent transferrin molecules incorporated into the liposomal membranes. Localization of transferrin at the liposome surface was confirmed by immunoelectron microscopy using anti-transferrin IgG and colloidal gold particles conjugated to protein A (Fig. 4D).

Binding of the transferrin-containing liposomes to small cell lung cancer cells (OC-NYH) in suspension was determined at 4°C to minimize endocytosis and degradation of the liposomes [34,35]. Using hexadecyl [$^3$H]cholesteryl ether as a nontransferable marker for the liposomal lipid [28], binding was found to increase with liposome concentration up to 200 μg PC/ml as shown in Fig. 5. Addition of iron-saturated transferrin in the assay in order to prevent binding to transferrin receptors on the cells, reduced cell-binding of the liposomes to the level obtained with transferrin-free control liposomes. Specific (transferrin-inhibitable) binding of

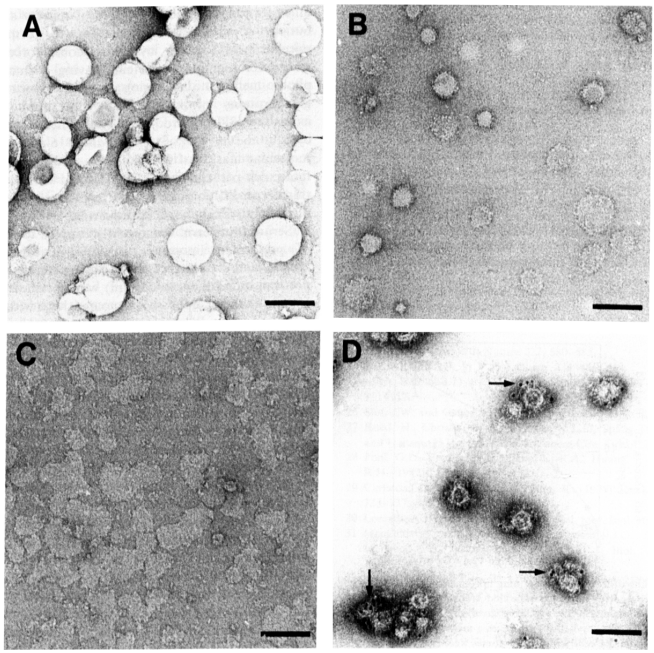


Fig. 4. Electron micrographs of negatively stained liposomes with a varying content of transferrin. Liposomes were prepared by deoxycholate-dialysis with iron-saturated transferrin-PE conjugates ($N_{av}$ = 1.6) and contained 0.04 (A), 0.26 (B and D) and 0.52 (C) mg transferrin/mg PC. Immunogold labeling of transferrin on the liposomes (D) was carried out using anti-transferrin IgG and protein A-gold (5 nm). Bars, 100 nm.

TABLE I

*Incorporation of transferrin-PE conjugates into liposomes*

Liposomes were formed by deoxycholate-dialysis of egg PC (1 mg) and $^3$H-transferrin (0.8 mg) with a varying degree of substitution by [$^{14}$C]PE. Dialysis and isolation of liposomes by flotation in sucrose gradients was carried out as described under Materials and Methods.

| Transferrin-PE conjugate $N_{av}$ [a] | Liposome composition | | Yield [b] (%) |
|---|---|---|---|
| | $^3$H-transferrin/PC (mg/mg) | [$^{14}$C]PE/$^3$H-transferrin (mol/mol) | |
| 0 [c] | 0.02 | 0 | 2 |
| 0.72 [c] | 0.10 | 1.7 | 13 |
| 1.59 [c] | 0.19 | 2.5 | 24 |
| 3.42 [c] | 0.15 | 4.1 | 19 |
| 6.06 [c] | 0.08 | 4.7 | 10 |
| 1.61 [d] | 0.26 | 2.3 | 33 |

[a] Average degree of substitution in the transferrin-PE conjugates (mol [$^{14}$C]PE/mol $^3$H-transferrin).

[b] Liposome-incorporated transferrin compared to total transferrin in the dialysis mixture.

[c] Apotransferrin.

[d] Iron-saturated transferrin.

liposomes showed saturation and was estimated to 0.4 μg PC/$10^6$ cells. The apparent association constant ($K_a$) for cell binding of liposome-incorporated $^3$H-transferrin was $5.3 \cdot 10^6$ $M^{-1}$ or about 10-fold lower than for the unmodified transferrin monomer ($K_a = 7.1 \cdot 10^7$ $M^{-1}$). $K_a$ for binding of the transferrin-PE aggregate (2.2 mol PE/mol transferrin) isolated by gel filtration was $3.3 \cdot 10^7$ $M^{-1}$.

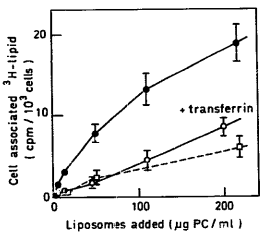


Fig. 5. Binding of transferrin-containing liposomes to OC-NYH cells. Liposomes prepared with iron-saturated transferrin-PE conjugates ($N_{av} = 1.4$) contained 0.12 mg transferrin/mg PC and 50 μCi hexadecyl [$^3$H]cholesteryl ether/mg PC as a marker for the liposomal lipid. Incubations of cells with the liposomes were carried out for 2 h at 4°C in the absence (●) and in the presence (○) of 1 mg/ml of iron-saturated transferrin to prevent receptor-mediated binding. Control incubations with transferrin-free liposomes (□------□) were carried out in the absence of transferrin. Bars, S.E. ($N = 3$).

## Discussion

We have successfully used a *N*-hydroxysuccinimide ester derivative of PE to prepare cleavable disulfide-linked transferrin-PE conjugates with a varying content of phospholipid. Noncleavable conjugates can be prepared using disuccinimidyl suberate instead of dithiobis(succinimidyl propionate) as the protein-lipid linker (results not shown). The coupling reaction between phospholipid derivative and protein is efficient, and proceeds under mild conditions without covalent protein-protein crosslinking. The conjugates readily incorporate into PC liposomes, and appear to have solubility properties very similar to integral membrane proteins containing both hydrophilic and hydrophobic portions. The gel filtration data demonstrate that the conjugates, in the absence of detergent and excess phospholipid, associate with each other forming soluble aggregates of narrow size-distribution. A similar self-association, to form protein micelles held together by hydrophobic forces, is observed for membrane proteins having a small hydrophobic membrane anchoring domain: membrane spike glycoproteins of viruses [36], intestinal sucrase/isomaltase [37], erythrocyte acetylcholinesterase [38], and cytochrome $b_5$ [39]. Stokes radii of 6–7 nm suggest molecular masses of 300–450 kDa and 4–6 transferrin molecules per aggregate. The defined behavior of the transferrin-PE conjugates will facilitate further studies on their physical-chemical properties.

Serum transferrin was recently coupled to the surface of preformed liposomes containing reactive lipids [40,41], and coupling of about 30 transferrin molecules per liposome (diameter 60 nm) was obtained [41]. The 50 nm diameter liposomes prepared here with 0.26 mg transferrin/mg PC are estimated to contain about 50 transferrin molecules/liposome, assuming $2 \cdot 10^4$ PC molecules/liposome [42]. Serum transferrin is 6 nm × 2.5 nm in size [43], and up to approximately 15% of the liposome surface is calculated to be covered by transferrin. The liposomes appear unstable as the protein to lipid ratio becomes larger (Fig. 4C), suggesting that the transferrin-PE complex affects the organization of PC molecules into vesicles.

The finding that reductive cleavage of the disulfide bond in the linker between transferrin and PE by cysteine releases transferrin from the liposomes, strongly indicate that the protein is located at the surface of the lipid bilayer, and that it is associated to the lipid through the PE molecules acting as a hydrophobic anchor. A spectrum of transferrin molecules with different content of PE are present in the conjugate preparations in agreement with a random reaction between the transferrin and the *N*-hydroxysuccinimide ester derivative of PE. Only the transferrin molecules into which 2 (or more) molecules of PE have been introduced incorporate into liposomes. Multiple hydrophobic interac-

tions therefore appear to be required for stable association of the large and hydrophilic transferrin molecule to lipid bilayers. For IgG ($M_r$ 150 kDa) modified with *N*-hydroxysuccinimide esters of long-chain fatty acids, incorporation into liposomes is obtained with two fatty acid residues per IgG molecule [11], and a single phospholipid molecule is sufficient to anchor the Fab' fragment of IgG ($M_r$ 50 kDa) to liposomes [44]. It is also relevant to note that several cell membrane proteins contain covalently attached phospholipid (phosphatidylinositol), and that the lipid appears to contribute to the membrane anchoring of the proteins [45]. Analysis on isolated erythrocyte acetylcholinesterase indicates a content of 2 mol phospholipid per mol of the dimeric protein ($M_r$ 150 kDa) [46,47].

In the present work we have defined optimal conditions for preparation of homogeneous proteoliposomes using phospholipid-modified serum transferrin as a model protein. The advantages of using the *N*-hydroxysuccinimide ester derivative of PE as a hydrophobic anchor are two-fold: (a) two adjacent fatty acid residues can be introduced into a protein with modification of a single amino group; (b) a flexible spacer (phospholipid backbone plus dicarboxylic acid linker) between protein and fatty acid facilitates insertion of the fatty acid hydrocarbon chains into lipid bilayers. The level of protein-liposome coupling obtained with the phospholipid modified transferrin during detergent dialysis compares with that obtained for IgG coupled with palmitic acid *N*-hydroxysuccinimide ester (0.1–0.4 mg protein/mg lipid) [11,12]. In the most efficient protein-liposome coupling procedures where proteins are reacted with preformed liposomes containing reactive lipids, coupling ratios up to 0.3–0.8 mg protein/mg lipid have been obtained [44,48].

The affinity of the transferrin for its receptor on cell membranes is preserved, at least in part, during derivatization and incorporation of the protein into liposomes. Specific binding of 0.4 μg liposomal lipid/$10^6$ cells corresponds to the binding of $1.5 \cdot 10^4$ liposomes/cell for 50 nm diameter liposomes. The OC-NYH cells have about $1 \cdot 10^5$ transferrin receptors/cell. The transferrin-coated liposomes appear well suited for basic in vitro studies on the interactions of proteoliposomes with cell surfaces. We have developed a procedure for efficient loading of the liposomes with the anthracycline antitumor agent doxorubicin using disulfide-linked doxorubicin-PE conjugates (unpublished), and studies are underway to determine transference of the membrane-bound doxorubicin from the liposomes to cell membranes.

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