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ADSORPTION OF NON-MEMBRANE PROTEINS ON THE SURFACE OF MODEL PHOSPHOLIPID MEMBRANES

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1. Two new methods are proposed for enhancement of the binding of hydrophilic proteins by liposomes. 2. An alkylating derivative of phosphatidic acid has been obtained by its reaction with *N,N,N'*-tris(2-chloroethyl)-*N'*-(*p*-formylphenyl)propylene-1,3-diamine. The alkylating activity of this derivative is very low due to the electron-acceptor effect of the formyl residue. Phosphatidylcholine liposomes which contain this alkylating derivative in the lipid bilayer may be obtained. The compound residing in the outer monolayer may be reduced by $NaBH_4$. Upon reduction, the formyl residue is transformed into a hydroxymethyl residue. Therefore, the alkylating group of the compound is activated, and proteins may be attached covalently to the outer monolayer by alkylation with such chemically reactive liposomes. 3. Reaction of alkylating liposomes with myoglobin results in covalent binding of this hydrophilic protein. Complement-mediated leakage of such myoglobin-carrying liposomes may be induced by antibodies against myoglobin. 4. Modification of hydrophilic proteins with dansyl chloride results, even at small extents of modification, in a dramatic increase of the affinity of such proteins to phosphatidylcholine liposomes.

Introduction

Irreversible fixation of proteins on the surface of phospholipid membranes is useful for many purposes. Such fixation takes place readily with membrane proteins, and with some other proteins which have a high affinity to membranes due to the proper distribution of hydrophobic amino acid residues on their surface.

However, there is a great number of hydrophilic proteins which are not adsorbed by membranes. In order to achieve coupling of such proteins with membranes, it is necessary to modify either the proteins or the membranes by chemical methods. The following requirements for coupling procedures may be outlined. Firstly, these procedures must change, but to smallest possible extent, the enzymatic or antigenic properties of proteins. Secondly, the membrane must retain its structural integrity after the binding with proteins. Thirdly, it is desirable to effect coupling by reactions which do not affect substances present inside the liposome shell.

Obviously, in principle, there are two approaches to chemical coupling. The first approach is preparation of chemically reactive liposomes followed by reaction of such liposomes with proteins (see, for example, Ref. 1). The second approach is introduction of hydrophobic residues into protein molecules in order to increase their affinity to the lipid bilayer (see, for example, Ref.

Abbreviations: RCl_3 (in this article), *N,N,N'*-tris(2-chloroethyl)-*N'*-(*p*-formylphenyl)propylene-1,3-diamine; PARCl, *N*-(2-chloroethyl)-*N*-(*p*-formylphenyl)-*N'*-(2-hydroxyethyl)-*N'*-(phosphatidylethyl)propylene-1,3-diamine.

2). Two chemical coupling procedures are proposed in the present paper, one based on the first, and the other on the second approach.

Materials and Methods

Dansyl chloride (1-dimethylaminonaphthalene-5-sulphanile chloride) was purchased from Merck. [α-^{32}P]UTP was purchased from Amersham International; its specific radioactivity was 0.1 Ci/mmol. Phosphatidic acid was purchased from the Far East State University, Vladivostok. Phosphatidylcholine was isolated from hen eggs using the method described in Ref. 3. *N,N,N'*-tris(2-chloroethyl)-*N'*-(*p*-formylphenyl)propylene-1,3-diamine (RCl_3) was synthesized as described in Ref. 4. Bovine serum albumin was purchased from Sigma, ribonuclease and hemoglobin from Reanal, deoxyribonuclease from Worthington. Myoglobin was isolated from human heart according to the method described in Ref. 5.

Modification of phosphatidic acid. Phosphatidic acid was modified as follows. 20 mg phosphatidic acid were dissolved in 630 μl chloroform/methanol/1 M borate buffer (pH 9.3) (3:4:1, v/v). 10 mg RCl_3 in 370 μl of the same solvent was added to this mixture, followed by 20 μl 1 M NaOH, and the solution was kept for 2 h at 50°C. The unreacted alkylating compound was removed by extraction with water acidified to pH 4.0. The organic phase was evaporated and the residue dissolved in pentane, and after this the extraction of traces of unreacted RCl_3 with water was repeated. The pentane fraction contained the reaction product, *N*-(2-chloroethyl)-*N*-(*p*-formylphenyl)-*N'*-(2-hydroxyethyl)-*N'*-(2-phosphatidylethyl)propylene-1,3-diamine (PARCl).

Modification of proteins by dansyl chloride. This was performed under the conditions given in Ref. 6. The reaction mixture contained 5–10 mg protein/10–25 μl dansyl chloride in 20–50 μl acetone/0.2–0.5 ml 0.5 M $NaHCO_3$ (pH 9.8). The reaction mixture was kept for 30 min at 37°C, and excess reagent removed by gel-filtration on Sephadex LH-20.

Tritium label. Label was introduced into proteins according to Ref. 7 by reductive alkylation with formaldehyde-NaB^3H_4.

Coupling of proteins with alkylating liposomes. Liposomes were obtained by ultrasonic treatment of a lipid composition (phosphatidylcholine, phosphatidic acid, cholesterol and PARCl taken in a molar ratio 10.2:3:5:1.5, respectively, in 0.01 M borate buffer (pH 9.3)/0.15 M NaCl. 1 mg $NaBH_4$ was added to 250 μl of such liposomes (5 μmol lipid), and the mixture was kept for 15 min at room temperature. Excess of $NaBH_4$ was inactivated by addition of 20 μl acetone. Tritium-labelled ribonuclease or tritium-labelled hemoglobin was added to this mixture to a final concentration $4 \cdot 10^{-5}$ M, and the mixture was kept for 2 h at 37°C. Non-bound protein was removed by gel-filtration on a column with Biogel A-5m (0.4 × 30 cm) equilibrated with 0.01 M borate buffer (pH 9.3)/0.15 M NaCl. The rate of elution was 9 ml/h; 450-μl fractions were corrected, and their radioactivities counted using a Nuclear Chicago Mark-III scintillation counter.

Binding of dansylated proteins with liposomes. Liposomes were obtained by ultrasonic treatment of phosphatidylcholine in 0.15 M NaCl/0.02 M Tris-HCl (pH 7.0). The binding was performed by incubation for 2 h at room temperature of a mixture of liposomes and the dansylated protein in the same buffer. Non-bound protein was removed by gel-filtration on Sepharose 4B equilibrated with 0.15 M NaCl/0.02 M Tris-HCl (pH 7.0).

Antiserum against myoglobin. Antiserum was obtained by immunization of rabbits with human heart myoglobin in Freund's adjuvant as described in Ref. 8.

Antigenic properties. Antigenic properties of modified and labelled proteins were tested using double immunodifussion techniques [9] in 1% agar gel.Activities of DNAase and RNAase were determined by methods described in Ref. 10 and 11, respectively.

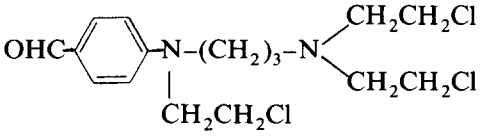
Immunospecific lysis of liposomes. Multilammelar liposomes were obtained according to Ref. 12 by shaking the lipid composition in 0.02 M borate buffer (pH 8.8), which contained 0.14 M NaCl and 0.001 M ATP. Non-entrapped ATP was removed by dialysis against the same buffer which did not contain ATP. The lipid composition of liposomes was the same as in the case of the chemical coupling [^{3}H]hemoglobin and [^{3}H]ribonuclease. Coupling of myoglobin was performed according to

the scheme described above for these proteins, except than non-bound myoglobin was removed by a few washings of the liposome precipitate by buffer with centrifugation at 18000 rev./min for 15 min. Immunospecific lysis of liposomes obtained was performed by incubation of 10 μl liposome suspension (0.2 μl lipid) and 22 μl antiserum against myoglobin for 15 min at 37°C. 40 μl fresh guinea-pig serum (as source of complement) were added to this mixture, followed by 140 μl 0.1 M Tris-acetate (pH 7.5)/0.002 M EDTA/0.07 M NaCl/50 μl ATP-monitoring reagent (LKB, Sweden). Leakage of ATP from liposomes was monitored by means of the luciferin-luciferase test [13] using the standard kit and a Luminometer from LKB.

Results and Discussion

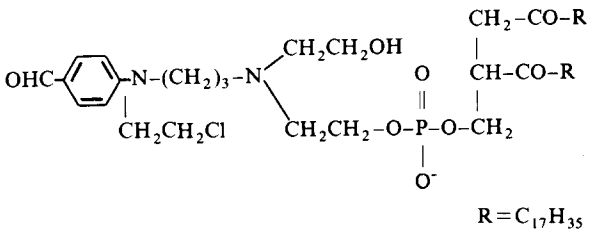
Alkylating liposomes

Gall et al. [4] have recently described an alkylating reagent, *N,N,N'*-tris(2-chloroethyl)-*N'*-(*p*-formylphenyl)propylene-1,3-diamine (RCl_3):



This reagent has three alkylating chloroethylamino groups, two at a nitrogen atom with aliphatic substituents, and one at a nitrogen atom with an aromatic substituent. The 'aliphatic' chloroethylamino groups are highly reactive and readily alkylate water and nucleophilic groups which occur in nucleic acids and proteins. As for the 'aromatic' chloroethylamino group, its alkylating reactivity is very small due to the electron-acceptor effect of the formyl residue, but may be made some 1000-times greater by reduction of the formyl group with $NaBH_4$. This reagent has been used in order to introduce latent alkylating ('aromatic' chloroethylamino) groups into biopolymer molecules, and such biopolymers become alkylating after $NaBH_4$ treatment [14]. In the present studies, we decided to use the reagent to prepare alkylating liposomes.

To this end, we reacted RCl_3 with phosphatidic acid, and thus obtained a latent alkylating derivative thereof (PARCl):



The structure of this compound follows from the method of its synthesis, and is confirmed by data of physical studies and chemical behaviour. The compound migrated in thin-layer chromatography on Kiselgel in chloroform/methanol/water (65 : 25 : 4) as a single ultraviolet-absorbing, iodine-stained spot with an $R_F = 0.72$ which is intermediate between those of the starting compounds (phosphatidic acid, $R_F = 0.17$; RCl_3, $R_F = 0.94$). The ultraviolet spectrum (Fig. 1) is close to the sum of the spectra of the starting compounds; it has a maximum at 335 nm, characteristic of benzaldehydes, which disappears upon reduction with $NaBH_4$.

PARCl was subjected to sonication together with a composition of natural lipids in an appropriate buffer solution. Liposomes obtained were purified by gel-filtration.

Such liposomes exhibited a characteristic absorption at 335 nm. Studies of the kinetics of their

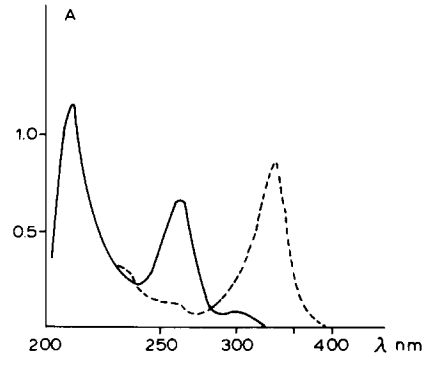


Fig. 1. Absorption spectrum of the product of modification of phosphatidic acid (PARCl). ------, starting PARCl. ———, PARCl after reduction with $NaBH_4$. Reduction was performed directly in the spectrophotometric cell in 2 ml methanol by addition of 1 mg sodium borohydride. The spectrum was measured using Specord UV-Vis spectrometer (Carl Zeiss, Jena) with a 1 cm cell.

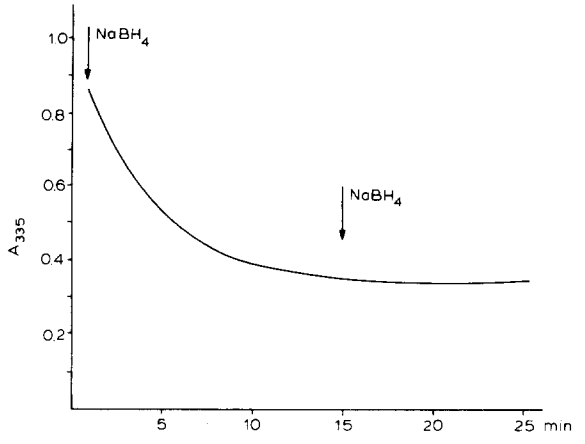


Fig. 2. Kinetics of the reduction of PARCl within the membrane lipid bilayer. Liposomes were prepared as described under Materials and Methods. 20 μl liposomes were diluted by 2 ml 0.01 M borate buffer (pH 9.3)/0.15 M NaCl. Reduction was performed directly in the spectrophotometric cell by addition of 1 mg $NaBH_4$. After 15 min of reduction, 1 mg more $NaBH_4$ was added. Absorbance was recorded by means of Specord UV-Vis (Carl Zeiss, Jena) at 1-min intervals. A solution of liposomes which contained no PARCl (20 μl liposomes, 20 μmol/ml, in 2 ml borate buffer) was present in the reference cell.

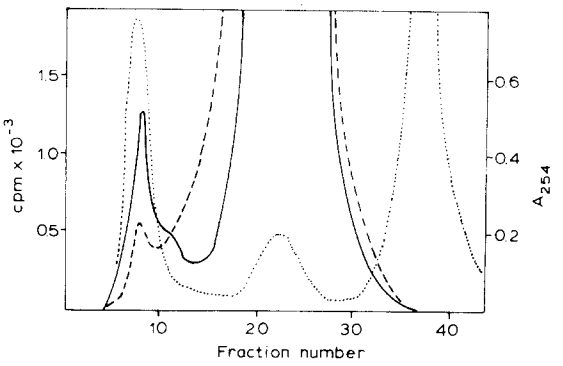


Fig. 3. Gel-filtration of alkylating liposomes on Biogel A-5m after their activation and incubation with [^{3}H]hemoglobin. ------, radioactivity in control (explanation see under Results and Discussion); ———, radioactivity in experiment; · · · · · ·, absorbance. The incubation conditions are presented under materials and Methods. Gel-filtration was run on a 0.4×30 cm column. The volume of sample applied was 300 μl; the rate of elution was 9 ml/h. 450 μl fractions were collected. 400 μl from each of the fractions were withdrawn to count radioactivity. The absorbance was recorded by means of an ultraviolet densitometer.

reduction with $NaBH_4$ showed that approx. 60% of their aldehyde groups react readily (Fig. 2). It is known that $NaBH_4$ does not penetrate through phospholipid membranes [15]. Hence, the aldehyde groups of PARCl which are not reduced by $NaBH_4$ in liposomes are presumably those which reside within the inner lipid monolayer. It follows that the 2-chloroethylamino groups of PARCl-containing liposomes are activated by $NaBH_4$ reduction only in the outer layer of liposomes, and the latter thus become alkylating liposomes.

Activated liposomes were treated with ^{3}H-labelled horse hemoglobin as described under Materials and Methods. Gel-filtration of the reaction mixture (Fig. 3) showed that labelled hemoglobin is bound by alkylating liposomes. Control liposomes which were mixed with labelled hemoglobin for 2 h after $NaBH_4$ reduction and lost their chloroethylamino groups due to hydrolysis also bound hemoglobin, but to an extent which was 3-times smaller.

The value of 'nonspecific' binding with inactivated liposomes was $2 \cdot 10^{-5}$ mol protein per mol lipid, in a good accord with the data of other workers [16].

In order to estimate the effect of the reaction with alkylating liposomes upon enzymatic activity, we studied the coupling of RNAase. With this enzyme, the extent of the nonspecific binding was $3 \cdot 10^{-5}$ mol per mol lipid, whereas the extent of the binding with alkylating liposomes was $1 \cdot 10^{-4}$ mol/mol. No measurable decrease of the RNAase activity took place.

Finally, it was shown by special experiments that the protein-binding procedure with alkylating liposomes does not lead to leakage of liposome-entrapped ATP.

Introduction of hydrophobic groups into protein molecules

The second method of coupling was based on the introduction of hydrophobic groups into protein molecules. A convenient procedure to this end appeared to be dansylation.

Fig. 4A shows the gel-filtration pattern of tritium-labelled bovine serum albumin treated with dansyl chloride. It is seen that the modified protein is eluted in the same fractions as the native one. Hence, moderate modification does not lead to aggregation of the protein.

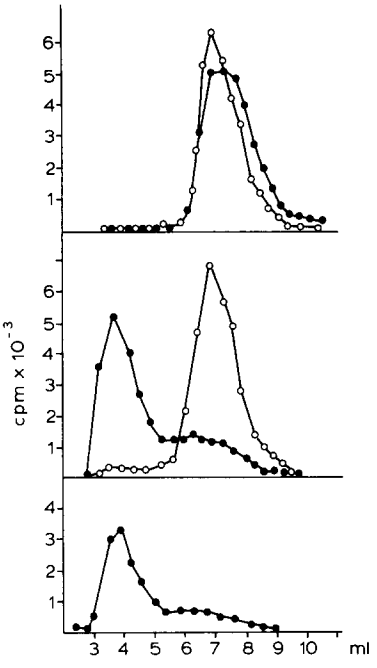


Fig. 4. Gel-filtration of dansylated and non-dansylated ^{3}H-labelled bovine serum albumin and of a mixture of this protein with liposomes. (A) 100 μl of ^{3}H-labelled bovine serum albumin (○——○) or dansylated ^{3}H-labelled bovine serum albumin (●——●) in 0.14 M NaCl/0.02 M Tris-HCl (pH 7.0); gel-filtration was performed on a 0.7×20 cm Sepharose 4B column in the same buffer; the radioactivity was counted in dioxan scintillating liquor. (B) 100 μl of the mixture of ^{3}H-labelled bovine serum albumin (○——○) or dansylated ^{3}H-labelled bovine serum albumin (●——●) with phosphatidylcholine liposomes were subjected to gel-filtration as described above (A); mixture of 1.3 nmol protein/6.5 mg/ml lipid/0.14 M NaCl/0.02 M Tris-HCl (pH 7.0) was kept for 24 h at 20°C. (C) 100 μl of peak 1 (b) were subjected to repeated gel-filtration under the conditions presented in the same figure.

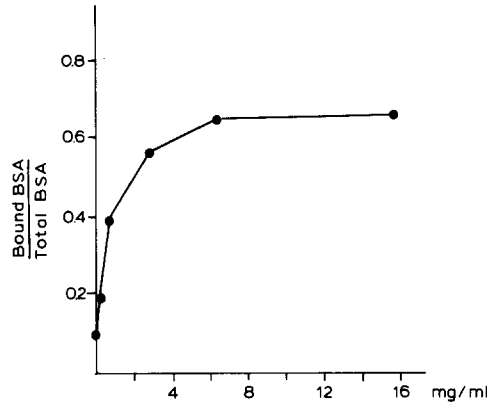


Fig. 5. Dependence of the extent of binding of dansylated ^{3}H-labelled bovine serum albumin with monolammelar liposomes upon the concentration of lipid. Mixture of 6 μM dansylated ^{3}H-labelled bovine serum albumin with different amounts of liposomes in 0.14 M NaCl/0.02 M Tris-HCl (pH 7.0) were kept 2 h at 20°C. and 100 μl aliquots were applied to Sepharose 4B. Conditions the same as in Fig. 4. The ratio of the radioactivity of liposomes peak to total radioactivity obtained from elution profile is plotted on the ordinate axis. The abscissa is the concentration of phosphatidylcholine.

Fig. 4B shows the result of gel-filtration of the same non-dansylated and dansylated labelled bovine serum albumin in the presence of liposomes. It is seen that dansylation results in binding of the major part of the protein with liposomes. As seen in Fig. 4C, repeated gel-filtration does not result in dissociation of the complex of dansylated bovine serum albumin with liposomes.

The extent of the binding of dansylated bovine serum albumin with liposomes depends on the lipid-to-protein ratio (Fig. 5). At the ratio when greater than $1.2 \cdot 10^3$ mol lipid per mol bovine serum albumin (concentration of lipid greater than 6 mg/ml in Fig. 5), about 70% of the modified protein is bound. The reason of the uncomplete binding is unknown. A possible explanation might be non-uniform modification of different protein molecules.

Adsorption of dansylated bovine serum albumin on liposomes does not lead to disruption or

TABLE I

CHARACTERISTICS OF MODIFIED PROTEINS AND EXTENT OF THEIR BINDING WITH LIPOSOMES

Protein	dansyl/protein (mol/mol)	Retention of enzymatic activity or antigenic properties by protein	binding (mol protein to mol lipid) ($\times 10^4$)
Bovine serum albumin	8.0	presence of antigenic properties	8.3
DNAase	1.5	presence of enzymatic activity	1.3
Myoglobin	2.0	loss of antigenic properties	9.1

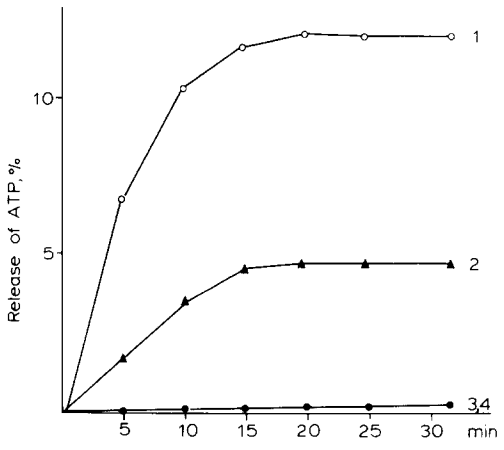


Fig. 6. Immunospecific lysis of liposomes. (1) Reaction in complete lysing system, as described under Materials and Methods; (2) myoglobin solution at a concentration $1 \cdot 10^{-7}$ M was introduced instead of Tris-acetate buffer; myoglobin was dissolved in the same buffer; (3) the incubated mixture was supplemented with non-immune serum, instead of antiserum; (4) the incubation mixture was supplemented by guinea pig serum with complement inactivated by heating for 30 min at 56°C. Ordinate: yield of leakage of ATP into solution (% of that entrapped by liposomes); abscissa: time of incubation after addition of complement.

destabilization of the latter. This follows from the results of experiments with liposomes loaded with radioactive UTP (data not shown)—adsorption of dansylated bovine serum albumin does not lead to leakage of UTP.

Introduction of dansyl residues enhances affinity of bovine serum albumin as well as of other proteins to lipid membranes. It is seen in Table I that dansylation of DNAase and myoglobin results also in an increase of their adsorption to liposomes. It is noteworthy that DNAase retains enzymatic activity after dansylation, that dansylated bovine serum albumin forms precipitates with antibodies against the non-modified protein; however, dansylated myoglobin loses its antigenic activity.

Hence, the above evidence shows that proteins which contain a few dansyl groups per molecule acquire the ability to be irreversibly bound by membranes without disruption or destabilization of the latter.

Immunospecific lysis of liposomes

Many workers have reported that liposomes which carry different haptens are lysed by complement in the presence of hapten-specific antibodies [12,17–19]. This finding may be of some practical importance, because the phenomenon may be used as a basic for ultra-sensitive immunoassay. Therefore we studied the immunospecific lysis of liposomes which were coupled with myoglobin by alkylation.

To this end, we prepared alkylating liposomes loaded with ATP, treated these liposomes with myoglobin, and studied their complement-induced lysis in the presence of antibodies against myoglobin. To monitor the leakage of ATP, we used the sensitive luciferin-luciferase test. It is seen in Fig. 6 that the lysis is immunospecific. No lysis occurs when one of the components of the system (antibody or complement) is absent. On the other hand, myoglobin acts as an inhibitor of the immunospecific lysis.

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