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COMPARATIVE STUDIES ON COVALENT AND NONCOVALENT IMMOBILIZATION OF PROTEIN MOLECULES ON THE SURFACE OF LIPOSOMES

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SUMMARY Methods of enzyme molecules covalent immobilization on the surface of liposomes are suggested. The methods permit more protein molecules to be bound than traditional methods of non-covalent immobilization by means of adsorption or incorporation. The liposome membranes preserve their integrity during immobilization. At the same tame, the enzyme bound with the liposome surface via the "spacer" groups completely preserves its ability to interact with a specific macromolecular compound.

In recent years liposomes have become increasingly more important as carriers in drug transport *in vivo* (1,2). Liposome-sequestered drugs do not come into contact with the blood and for this reason they cause no harmful side-effects and suffer no rapid biodegradation. The problem of target selectivity of liposome-entrapped drug transport can be solved by creating liposomes possessing a specific affinity to the target area. For this purpose the molecules of high affinity towards the target area should be bound with the liposome surface (1-4).

Recent studies have shown that glycolipid-containing liposomes can specifically recognize and bind to lectins (5), antibodies (6), and cells (7). Ganglioside-containing liposomes can specifically bind to liver cells containing lectine-like molecules in their membranes (8).

Even more advantageous seems to be the use of specific immunoglobulins against a variety if chemical compounds - typical components of the appropriate organs (9), or other specific proteins, for example, enzymes.

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The main difficulty is to find a way of binding the protein with the liposome surface so that the following requirements are met: 1) a sufficient quantity of the protein must be bound with the liposome; 2) the liposome-protein bond should be stable; 3) the protein specific binding properties should remain unchanged; 4) the liposome integrity during the immobilization procedure should be preserved.

The protein-liposome binding methods by means of adsorption or incorporation into the membrane described in the scientific literature only meet these requirements to a small extent. The firm binding of the protein is only possible where there are strong hydrophobic interactions between the nonpolar regions of the protein and the liposome membrane (10). It is clear that only part of the proteins is able to form such a hydrophobic binding (5, 10-12).

On the other hand, membrane incorporated proteins may undergo a noticeable decrease in their binding ability towards high molecular weight ligands because of steric hindrances (13, 14) or denaturation caused by the action of the membrane's nonpolar components (10-15).

In this report we suggest a method of protein binding with liposome membranes which does not have the above mentioned shortcomings. It is a covalent attachment of the protein molecules to the membranes via "spacer" groups.

MATERIALS AND METHODS

Materials. Crystalline α-chymotrypsin with a specific activity of 67%, as determined by spectrophotometric titration (16), was obtained from "Sigma" (USA). 2,4,6-Trinitrobenzene sulphonic acid, trypsin inhibitor from beef pancreas, cholesterol (99%) and dipalmitoyl L-α-phosphatidyl ethanolamine (98%) were supplied from "Sigma" (USA). N-acetyl-L-tyrosine ethyl ester was obtained from "Koch-Light Laboratories Ltd" (Great Britain). N-trans-cinnamoyl imidazole (titrant for the enzyme active centers), dimethyl suberimidat·2HCl and Triton X-100 were products of "Serva" (Germany). Glutaraldehyde (as 25% water solution) was

manufactured by "Merck" (Germany). [^{3}H] 2-deoxy-D-glucose (8.5 Ci/mmol) and [^{14}C] cholesterol (59.2 Ci/mol) were obtained from "Radiochemical Centre Amersham" (Great Britain). The dialysis tubes were produced by "Union Carbide Corp." (USA). Egg yolk phosphatidyl choline "Reakhim" (USSR) was purified by column chromatography on silica gel with a $CHCl_3/CH_3OH$ gradient. Thin layer chromatography of the purified product on Silufol with the mixture of $CHCl_3$-CH_3OH-H_2O (65:25:4 per volume) yielded a single spot. Sepharose 4B was obtained from "Pharmacia" (Sweden). All the other reagents (inorganic salts, buffer solution components, acids etc.) were analytical grade preparations.

Preparations of liposomes. Chloroform solutions of egg yolk phosphatidyl choline, cholesterol, dipalmitoyl L-α-phosphatidyl ethanolamine in a molar ratio of 7:2:1 (40 mg total) and a trace amount of [^{14}C] cholesterol were mixed together, and the lipid film was formed by solvent evaporation in a rotary evaporator. Then 4 ml of 10^{-2}M borate buffer pH 8.5 containing 0.145M NaCl and a small amount of [^{3}H] 2-deoxy-D-glucose was added to the film, and liposomes were formed by ultrasonic irradiation at 25°C. Liposome dispersion was dialysed against the same buffer to separate nonentrapped deoxy glucose.

Liposome concentration was determined observing [^{14}C] cholesterol radioactivity. The liposome integrity was controlled observing the efflux of [^{3}H] 2-deoxy-D-glucose. The measurements of the [^{14}C] and [^{3}H] compound concentrations were made by means of Mark III 6880 Liquid Scintillation System, Searle Analytic Inc. (USA).

The electron microscopy studies were performed on a JEM-100C electron microscope (Japan). The suspension of liposomes was supplied on a grid covered with formwar film. The liposome samples were negatively contrasted with 5% aqueous uranyl acetate. According to the electron microscopy data multilamellar liposomes of an average diameter of 800 Å were obtained.

Noncovalent coupling of α-chymotrypsin. (a) For the enzyme adsorption on the surface of the liposomes the liposome dispersion (10 mg of lipids per 1 ml) was incubated at 4°C for 48 h with $1.6 \cdot 10^{-4}$M α-chymotrypsin. (b) For the enzyme incorporation into the membrane, the enzyme ($1.6 \cdot 10^{-4}$M) was added to the borate buffer solution, in which the liposomes were formed. After coupling procedure, the reaction mixtures were applied to a Sepharose 4B column, equilibrated with 0.145M NaCl to separate the liposomes from the free protein.

Covalent coupling of α-chymotrypsin. (a) The liposome suspension (10 mg of lipids per 1 ml) in 10^{-2}M borate buffer (pH 8.5) with 0.145M NaCl was mixed with $1.6 \cdot 10^{-4}$M α-chymotrypsin and then dimethyl suberimidate was introdused in small portions in cold (10°C), with pH being constant, up to 1.5 mg/ml final concentration. Then the mixture was incubated in the same conditions for 30 min and dialyzed against the buffer at 4°C. The reaction conditions are typical for enzyme modification with imidates (17). (b) Glutaraldehyde was added to the same liposome suspension up to 15 mM final concentration and the mixture was incubated for 5 min at 20°C. The excess of the glutaraldehyde was removed by the dialysis against the borate buffer. The titration of the liposomal phosphatidyl ethanolamine amino groups with trinitrobenzene sulphonic acid (18) in the presence of 0.4% of Triton X-100 have demonstrated that glutaraldehyde modifies about 70% of the amino groups. $1.6 \cdot 10^{-4}$M solution of α-chymotrypsin was introduced into the suspension of activated liposomes and

Table 1. Properties of α-chymotrypsin bound with liposomes by different methods

Immobilization method	Moles of the bound enzyme per mole of lipids	2-Deoxy-D-glucose preserved inside the liposomes after the enzyme association procedure (%) *	The ratio of apparent constants of inhibition (M) of immobilized and native α-chymotrypsin with the trypsin pancreatic inhibitor
Adsorption	$2.0 \cdot 10^{-5}$	70	1
Incorporation	$4.7 \cdot 10^{-5}$	80	5
Covalent binding via the diimidate	$4.9 \cdot 10^{-5}$	100	1
Covalent binding via the dialdehyde	$7.1 \cdot 10^{-5}$	75	1

* In comparison with the liposomes treated in the fashion described above (see Methods), but in the absence of the enzyme and modifiers.

the mixture was incubated at 4°C for 48h with the subsequent Sepharose 4B column chromatography. The quantity of the immobilized α-chymotrypsin was determined, observing the catalytic activity of the liposome suspension after Sepharose 4B column chromatography.

Measurements of α-chymotrypsin catalytic activity. α-Chymotrypsin catalytic activity was measured in a TTT-1c pH-stat "Radiometer" (Denmark) by determinations of the enzymatic hydrolysis initial rates of the specific substrate N-acetyl-L-tyrosine ethyl ester (0.01M) in 0.145M NaCl at pH8.0 and 37°C. To determine the quantity of liposome-sequestered enzyme, the liposomes were destroyed by the addition of 0.1% of Triton X-100 and the total enzyme activity was measured in the fashion described above. The preliminary experiments have shown that the enzyme activity of α-chymotrypsin is not affected by 0.1% of Triton X-100. The apparent constant of α-chymotrypsin inhibition with a trypsin pancreatic inhibitor was determined measuring the enzyme activity in the presence of the inhibitor different concentrations. The results were calculated in modified Dixon's coordinates (19).

RESULTS AND DISCUSSION

The results of comparative studies on different methods of model enzyme α-chymotrypsin immobilization on the surface of liposomes are shown in the Table 1. The latter shows that only two methods - incorporation and covalent binding make it possible to bind noticeable quantities of the enzyme with liposomes.

The highest quantity of the protein can be bound with liposomes by covalent attachment through the glutaraldehyde. The real covalent binding of the enzyme in this case is supported by the fact of low enzyme binding in the case of adsorption.

In all the experiments the liposomes remain intact. This fact is proved by the high level of [^{3}H] radioactivity preservation in the liposomes.

All the methods including adsorption give a firm binding of the enzyme with liposomes - after the incubation of enzyme-containing liposomes at 20°C for 3 days and subsequent rechromatography on Sepharose 4B column 80-100% of the enzyme remain in the bound state.

During the enzyme incorporation into the liposome membrane noticeable quantities of the enzyme are trapped within the aqueous interior of the liposome. We have shown that after destroying the liposomes with Triton X-100, the enzyme activity in the reaction solution increases by 4-5 times in comparison with the intact liposomes. The method of covalent binding is completely free from this drawback.

We have studied the binding ability of α-chymotrypsin immobilized by different methods, towards a specific macromolecular inhibitor (pancreatic trypsin inhibitor) as a model of antigen-antibody interaction (Fig.1). In the case of the covalent attachment, most of the immobilized enzyme molecules (80%) are inhibited by this inhibitor. This means that the active sites of the enzyme are located on the liposomal surface. At the same time, about half of the α-chymotrypsin incorporated into the membrane molecules, is inaccessible for the trypsin pancreatic inhibitor.

It is important that the ability of the covalently coupled enzyme to bind the high molecular weight protein inhibitor is

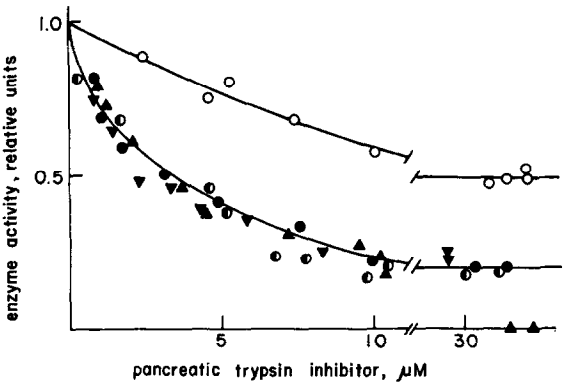

Figure 1. Inhibition with the pancreatic trypsin inhibitor of native α-chymotrypsin ▲ and α-chymotrypsin immobilized on the surface of liposomes by incorporation into the membrane ○, by adsorption ▼, or by covalent binding via glutaraldehyde ◐ and dimethyl suberimidate ●.

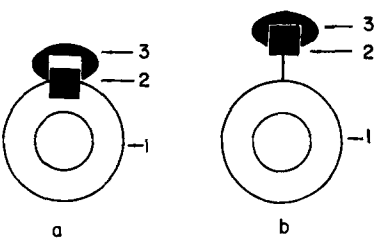

Figure 2. A schematic representation of liposomes /1/ with the immobilized enzyme or antibody /2/ on their surfaces. In the case of incorporated protein /a/ high affinity binding with macromolecular antigen or substrate-similar substance /3/ is difficult. The protein covalently bound through a spacer group /b/ preserves the binding ability.

like that of the native enzyme (see Table 1). In contrast, the apparent binding ability of the α-chymotrypsin incorporated into the membrane is poorer (Table 1), probably because of steric hindrance (Fig.2).

CONCLUSION REMARKS

1. The covalent attachment of high affinity proteins to the surface of liposomes via "spacer" groups seems to be a highly promising method. For this purpose, different reactive lipids

can be introduced into the liposome membrane to make possible the protein attachment to different functional groups:-NH_2, -COOH and others (20).

2. In the case of the covalent attachment of target-specific proteins to the liposome the bound protein should preserve the specific binding ability. In this case, the drug should not drip out the liposome and should not contain high affinity molecules in the liposome as an impurity.

3. The results obtained may be useful in attaching protein markers to model membranes.

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