Immobilization of α-chymotrypsin on sucrose stearate–palmitate containing liposomes

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Stable liposomes have been prepared from lipid mixture containing sucrose stearate–palmitate. $1.2 \cdot 10^{-4}$ mol of model enzyme α-chymotrypsin per mol of lipid have been coupled to prepared liposomes activated by periodate oxidation of sucrose units.

Liposome *Sucrose stearate–palmitate* *Immobilization* *α-Chymotrypsin*

1. INTRODUCTION

At present liposomes are considered to be one of the most promising drug carriers in drug targeting [1].

It was found to be useful to immobilize the specific macromolecules (e.g., proteins) on liposomal surface to enhance the affinity of liposomes to the target site. A variety of immobilization techniques are used, and among them chemical methods are the most effective [2]. Two different ways of protein immobilization on liposomes are known: (1) chemical modification of the protein with hydrophobic anchors with its subsequent incorporation into liposome membrane [3–5]; (2) chemical coupling of protein with activated liposomes. In the second case the reactive phospholipid derivatives are used most often [6,7]. However, any other membrane-compatible reactive compound, possessing a hydrophobic moiety, can be used for protein immobilization instead of phospholipids. From this point of view the sucrose derivatives acylated with fatty acids are of potential interest because of their low cost and commercial availability.

In our work we have shown that liposomes, containing sucrose stearate–palmitate included in the phospholipid bilayer upon their activation by periodate oxidation can easily bind a protein (model enzyme α-chymotrypsin in our study).

2. MATERIALS AND METHODS

Egg phosphatidylcholine was the product of the Kharkov bacterial preparations plant. Cholesterol and dicetylphosphate were Sigma products. Sucrose stearate–palmitate 15 was obtained from Serva.

α-Chymotrypsin (chymotrypsin A 4) was obtained from Boehringer. Calcein was a Koch–Light product and was purified by Sephadex LH 20 chromatography as in [8]. Radioactive materials were obtained from Amersham International.

2.1. *Sucrose stearate–palmitate purification*

Commercially obtained SSP was purified from traces of fatty acids and coloured impurities by chromatography on DEAE–Sephadex A 25 (Pharmacia). 200 mg of SSP were dissolved in diisopropyl ether : methanol : water (89 : 10 : 1) and applied to a small column packed with

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Abbreviations: SSP, sucrose stearate–palmitate; PC, phosphatidylcholine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; ATEE, *N*-acetyl-L-tyrosine ethyl ester; BBS, borate buffered saline

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Table 1

Changes in the trapped volume of liposomes with time[a]

Incubation time (h)	Trapped volume (%)[b]	
	SPS-containing liposomes	SPS-free liposomes
–	14.2 ± 0.7	19.3 ± 0.6
24	12.2 ± 0.6	17.4 ± 0.8
48	11.3 ± 0.8	16.6 ± 0.6
72	9.9 ± 0.7	15.8 ± 0.7

[a] Calcein-loaded liposomes were incubated in calcein-containing isotonic buffer solution at 4°C for the time periods indicated. 55 nmol of the total lipid were used for each determination carried out as in [13]

[b] Trapped volume is presented as the mean ± SE of six determinations

DEAE–Sephadex A 25 and eluted with the same solvent system. Eluate was collected and solvents were removed on a rotary evaporator. Purity of SSP was followed by TLC performed on Alufol (Merck) plates in chloroform:methanol:water (65:25:4) and further staining with α-naphthol reagent [9].

2.2. *Preparation of liposomes*

Liposomes (PC:SSP:cholesterol:dicetylphosphate, 8:2:2:1 molar ratio) were prepared by reverse-phase evaporation [10] from diisopropyl ether/water emulsions. In some experiments traces of [^{14}C]cholesterol or ^{14}C-labeled dipalmitoyl phosphatidylcholine were added to lipids as radioactive markers. For determination of encapsulation efficiency and liposome integrity 0.1 mM calcein was added to the buffer solution used for liposome preparation (10 mM Hepes, 0.145 M NaCl, pH 7.4). The liposome suspension was sequentially extruded through 0.4 μm and 0.2 μm Nuclepore membranes to disrupt larger aggregates.

2.3. *Liposome oxidation and α-chymotrypsin coupling*

Liposomes were coupled with protein by using the method [6] with modifications. Liposomes were developed to acetate buffer (10 mM CH_3COONa, 0.145 M NaCl, pH 5.6) by means of gel-filtration on Sephadex G-50 minicolumns in a centrifuge [11]. A concentrated solution of sodium periodate was added (final concentration, 10^{-2} M) to the liposome sample and the mixture was incubated at 20°C overnight. Samples were dialysed

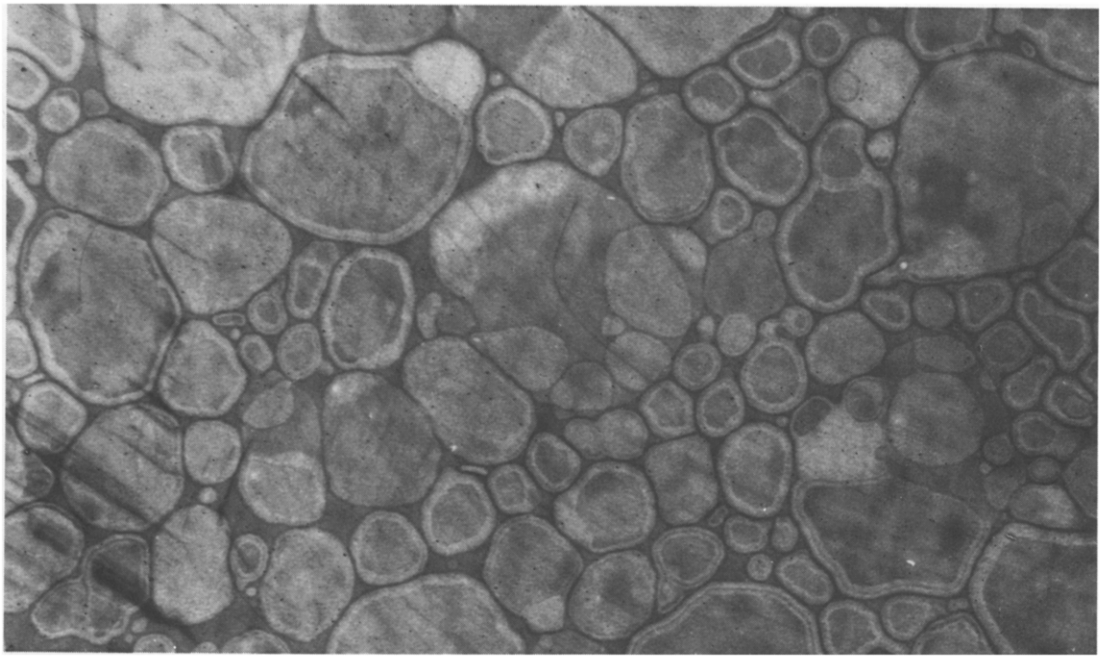

Fig.1. Negatively stained PC:cholesterol:SSP:dicetylphosphate (8:2:2:1) liposomes. (Bar = 100 nm).

against 1000-fold excess of 10 mM BBS, pH 8. 0.25 ml of α-chymotrypsin solution in 10 mM BBS, pH 8, was added to 0.75 ml of liposomes (final concentration of protein varied from $1.5 \cdot 10^{-5}$ M to $2.5 \cdot 10^{-4}$ M). 5 mg of $NaBH_3CN$ were added and incubation proceeded overnight at 4°C. Unbound protein was separated from liposomes by means of Ficoll 400 flotation procedure [12] or by gel chromatography on lipid presaturated Toyopearl HW 75 F (Toyo Soda, Japan). In control experiments non-oxidized liposomes were used.

2.4. *Analytical procedures*

Fluorometrical determination of trapped volume (following calcein fluorescence) was performed similarly to the procedure introduced in [13]. Total lipid was determined by the method in [14]. Catalytic activity measurements were performed as in [15]. The quantity of the protein bound to liposomes was determined following its catalytic activity with ATEE as specific substrate [5].

2.5. *Electron microscopy*

Liposome samples were incubated with 0.1% OsO_4, 0.15 M NaCl, pH 6, for 30 min (1:1 v/v), transferred to carbon coated grids and stained with ammonium molybdate for a few minutes to prepare them for electron microscopy. Electron microscopy was performed on a JEM-100CX (JEOL) instrument.

3. RESULTS AND DISCUSSION

Negatively charged liposomes contained approx. 15 mol% of SSP were found to be suitable for coupling studies because of their relatively high stability against aggregation and ability to retain the captured volume. The increase in SSP molar concentration (25–40 mol%) leads to aggregation of the liposome preparation even in the presence of 10 mol% of dicetylphosphate as the negatively charged liposome component. SSP liposomes retained more than 70% of the initial amount of the entrapped calcein for a 3-day period which is comparable to data obtained with SSP-free liposomes (table 1). The trapped volume was determined fluorometrically and evaluated as equal to 11.4–16.3% of the total amount of the calcein solution. This means that approx. 10.8–15.7 l of aqueous phase are entrapped by liposomes prepared from the 1 mol of the total lipid. Therefore, inclusion of 15 mol% of acylated sucrose into lipid bilayers permits one to obtain stable and non-aggregated liposomes possessing high trapping capacity. SSP-liposomes were examined by means of electron microscopy. Closed monolamellar structures with an average diameter of 140 nm are visualized on micrographs of the negatively stained liposome samples (fig.1).

In order to make liposomes capable of protein binding periodate oxidation of the sucrose residues on the outer surface of liposomes was performed resulting in aldehyde group formation, which allows protein to be coupled with liposomes via the Schiff base (fig.2). Periodate readily oxidizes vicinal glycol bonds of glycosphingolipids [6] in

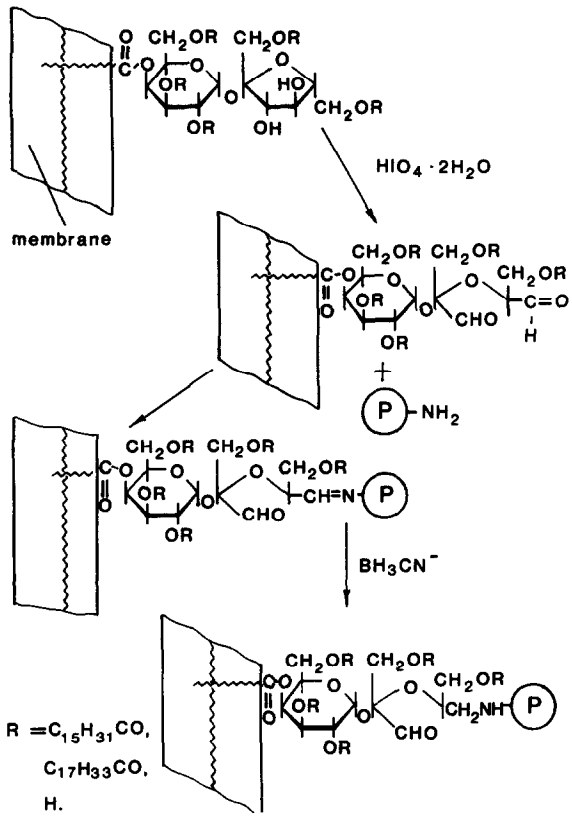

Fig.2. Scheme of the enzyme immobilization on the SSP-containing liposomes. For simplicity only the furanose ring oxidation is presented.

Table 2

Protein binding with SSP-containing liposomes

Lipid (M)	α-Chymotrypsin (M)	Bound protein (mol/mol lipid)
$1.15 \cdot 10^{-3}$ [a]	$1.5 \cdot 10^{-5}$	$2.1 \cdot 10^{-5}$
$1.15 \cdot 10^{-3}$	$1.0 \cdot 10^{-4}$	$7.2 \cdot 10^{-5}$
$1.15 \cdot 10^{-3}$	$2.5 \cdot 10^{-4}$	$1.2 \cdot 10^{-4}$
$2.30 \cdot 10^{-3}$ [b]	$5.0 \cdot 10^{-4}$	$3.1 \cdot 10^{-5}$
$1.15 \cdot 10^{-3}$ [c]	$2.5 \cdot 10^{-4}$	0

[a] Liposomes separated by Ficoll flotation
[b] Liposomes separated by means of gel filtration. Protein/lipid ratio was determined for each lipid-containing fraction, mean value is presented
[c] Liposomes were not oxidized

acidic or neutral media. Using the method of aldehyde determination [16] we found that 15% of SSP was oxidized when liposomes were incubated overnight at pH 5.6 in the presence of 10^{-2} M $NaIO_4 \cdot 2H_2O$. Assuming acylated sucrose molecules are arranged symmetrically on both sides of the lipid bilayer the theoretical yield of the immobilization could be $2 \cdot 10^{-3}$ mol of protein/mol of the total lipid (immobilization proceeds on the outer liposome surface only). In our experiments values as high as $1.2 \cdot 10^{-4}$ mol of active enzyme/mol of lipid were achieved for liposomes separated from unbound enzyme by Ficoll flotation (table 2). Alternatively, liposomes were separated from unbound α-chymotrypsin by gel filtration on Toyopearl HW 75 F and protein/lipid ratios were calculated for each lipid containing fraction. A typical gel-filtration profile is presented in fig.3. In control experiments non-oxidized liposomes were incubated with enzyme. We had not been able to detect any active enzyme on liposome in this case, hence non-specific electrostatic interactions between protein and negatively charged liposomes were minimized. Changes in permeability for oxidized liposomes were monitored fluorometrically. It was found that the total loss of the entrapped volume had not exceeded 9% after incubation of both SSP-containing and SSP-free liposomes in the presence of periodate. Thus, it is likely that periodate oxidation does not affect the integrity of liposome membrane.

In the present work, we have demonstrated that

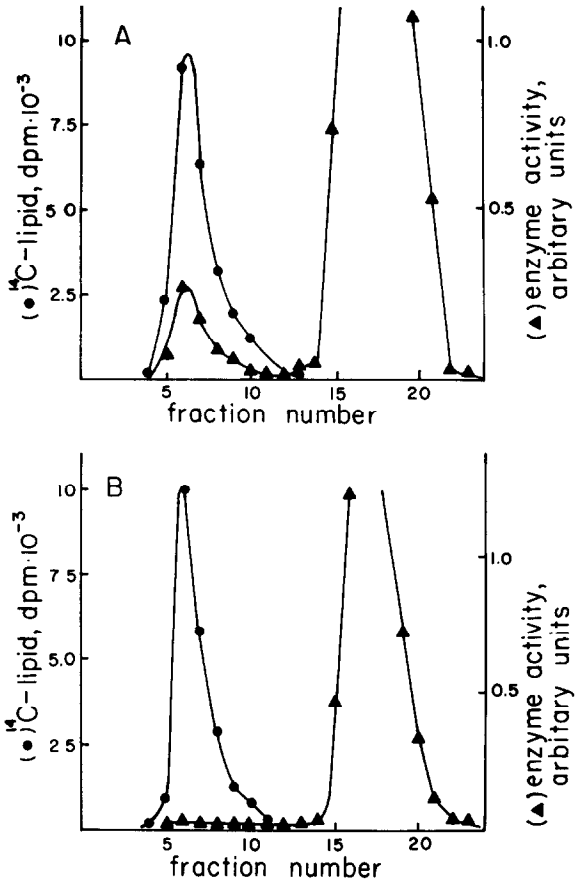

Fig.3. Separation of liposomes from unbound α-chymotrypsin by gel filtration on Toyopearl HW 75 F. Experiments with (A) oxidized liposomes; (B) control (non-oxidized) liposomes.

liposomes prepared from SSP-containing lipid mixtures can be used successfully for protein coupling with high protein/lipid ratios and with preservation of liposome stability and integrity.

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