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# FUNCTIONALIZED POLYMERIC LIPOSOMES. EFFICIENT IMMOBILIZATION OF ALPHA CHYMOTRYPSIN

Steven L. Regen,* Maninder Singh, and N.K.P. Samuel

Department of Chemistry, Marquette University
Milwaukee, Wisconsin 53233

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A polymerizable phospholipid has been synthesized having a latent aldehyde moiety in the head group ( 1 ). Photopolymerized vesicles which have been prepared from this phosphatidylcholine have been successfully conjugated to alpha chymotrypsin. A high degree of loading was achieved with significant retention of enzymatic function.

Polymerized phospholipid vesicles are of considerable current interest as stable models for biological membranes and as drug carriers (1-7).They are very similar to conventional liposomes in terms of their overall structure, morphology, entrapment ability, and permeability, but are substantially more stable . We have shown that polymerized vesicles derived from 1,2-bis(12-(methacryloyloxy) dodecanoyl)- sn -glycero-3-phosphocholine (DPL) exhibit extraordinary stability toward mechanical and chemical perturbations. They can, for example, retain their overall physical integrity, even after being subjected to ultrasound, organic solvents and detergents (8,9).

Recently we reasoned that polymerized vesicles bearing surface functionality should represent a more versatile and attractive class of liposomes. Their ability to bind proteins (e.g., antibodies) would significanlty increase their viability as drug carriers; i.e., they may be targeted to specific cell types (10,11). "Ultrastable" liposomes conjugated to antibodies could also serve as valuable cytological probes for clarifying endocytotic vs fusion pathways in cellular uptake. Although there has been extensive analysis made of

*To whom correspondence should be addressed.

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liposome-cell interactions over the past several years, the precise mechanisms for cellular incorporation of lipsomes and their contents is still uncertain. A third interesting possibility stems from the potential use of functionalized polymerized liposomes as support material for immobilizing, stabilizing and exploiting enzymes in organic synthesis (12). The biomembrane-like character of these polymers suggests that they would provide an attractive microenvironment for many enzymes. Motivated by these ideas, we have begun to develop synthetic methods for introducing functionality within polymerized vesicles. In this report we describe the synthesis of photopolymerized DPL-liposomes bearing aldehyde moieties located at the vesicle surface. We also demonstrate the feasibility of enzyme conjugation using alpha chymotrypsin as a model protein. These results represent the first successful covalent attachment of a protein to a polymerized vesicle membrane.

## MATERIALS AND METHODS

Unless noted otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. Chloroform used in esterification reactions was freshly distilled. Beta-dimethylaminopropionaldehyde diethyl acetal was obtained using procedures similar to those previously described (13). 1,2-Dipalmitoyl-DL-3-glycero(N-3,3-diethoxypropyl)phosphocholine . A solution of 1,2-dipalmitoyl-DL-3-glycero-3-phosphoric acid bromoethyl ester (0.60 g, 0.754 mmol) was reacted with 4.5 g (25.7 mmol) of beta-dimethylaminopropionaldehyde diethyl acetal, dissolved in 10 mL of methanol (14). The mixture was stirred for 48 h at 40°C, and the solvent removed under reduced pressure. After addition of acetone (40 mL) and cooling (-10°C) for 5 h, the crude product precipitated from solution as a colorless solid. Purification by chromatography over basic alumina (Activity I) using $CHCl_3$, and $CHCl_3/CH_3OH$ (gradient) as eluting solvents afforded 0.250 g (39%) of 1,2-Dipalmitoyl-DL-3-glycero(N-3,3-diethoxypropyl)phosphocholine having the expected $^1H$ NMR and IR spectra, and showed a single spot by TLC (alumina, $CHCl_3/CH_3OH/H_2O$, 65:25:4; Rf=.45).

1,2-Bis(12-(methacryloyloxy)dodecanoyl)-DL-3-glycero-(N-3,3-diethoxypropyl)phosphocholine (1). To a solution of 0.230 g (0.270 mmol) of 1,2-dipalmitoyl-DL-3-glycero(N-3,3-diethoxypropyl)phosphocholine was added 0.5 mL of a 0.1 M methanol solution of tetrabutylammonium hydroxide. The mixture was stirred at 10°C for 18 h, and the solvent then removed under reduced pressure. The residue was washed with 3 X 25 mL of anhydrous ether and dried (18 h, 25°C(0.1 mm)), affording 0.095 g (94%) of 1,2-dihydroxy-DL-3-glycero(N-3,3-diethoxy-propyl)phosphocholine. After dissolving this phosphocholine in freshly distilled chloroform (20 mL), containing 12-methacryloyloxy

dodecanoic acid anhydride (0.512 g, 0.93 mmol), 4-dimethylamino pyridine (0.120 g, 0.980 mmol) was added and the mixture stirred under a nitrogen atmosphere in the dark for 72 h. The solvent was then removed under reduced pressure, and the residue dissolved in 5 mL of $CHCl_3/CH_3OH/H_2O$ (65:25:4) and passed through an AG-501-X8D resin ($1^3$X $17^2$cm) eluting with the same solvent mixture. The eluted fractions were combined and the solvent removed under reduced pressure. Chromatographic purificaion over basic alumina (Activity I) using $CHCl_3$ and $CHCl_3/CH_3OH$ (gradient) afforded 0.081 g (35%) of 1 as a colorless oil, having an Rf of 0.5 (alumina, $CHCl_3/CH_3OH/H_2O$(65:25:4)) and the expected IR and 1H NMR spectra.

Polymerized Vesicles of 1. Using procdures similar to those previously described (6), 6.0 mg of 1 was dissolved in a minimum volume of chloroform and coated onto the walls of a round-bottomed flask by solvent evaporation under reduced pressure. The lipid was then dispersed in 1.2 mL of 10 mM borate buffer (pH 8.5), containing 140 mM NaCl and 2.0 mM $NaN_3$ (Buffer 1), by vortex mixing at $50^oC$. Subsequent sonication at $50^oC$ using a Heat Systems W-375 bath-type sonicator produced a clear dispersion in ca. 1.5 h. The sonicated vesicles were then transferred to a quartz tube, degassed with a stream of nitrogen, and photopolymerized by direct UV irradiation (254 nm, 1 h) in a Rayonet Photoreactor. Thin layer chromatography showed a single spot at the origin indicating complete polymerization.

Coupling of Alpha Chymotrypsin to Polymerized Vesicles. Polymerized vesicles of 1 were dialyzed against HCl overnight in order to liberate surface aldehyde groups. Typically, 1.2 mL of a polymerized dispersion of 1 (6.0 mg of lipid) was placed in a preswollen Spectropor 1 dialysis bag and dialyzed against 100 mL of 1.0 mM HCl at room temperature for 18 h. The dispersion was dialyzed, three times in succession, against 100 mL of Buffer 1 for 1 h at room temperature, and then divided into three 0.40 mL-portions. After adding a solution of 7.0 mg of alpha chymotrypsin in 0.55 mL of Buffer 1 to each of these dispersions, the pH was raised to 9.5 by addition of 0.040 mL of 0.8 M NaOH. The mixtures were then stirred for 2 h at room temperature under a nitrogen atmosphere. To one of the vesicle-enzyme samples was added 0.020 mL of a solution prepared by dissolving 25 mg of $NaBH_4$ in 1.0 mL of Buffer 1; to a second was added 0.020 mL of a solution formed from 25 mg of $NaBH_3CN$ in 1.0 mL of Buffer 1. After stirring for an addiional 2 h at room temperature, each of the three mixtures was placed in a preswollen cellulose bag (Spectrapor 6) and dialyzed against Buffer 1 for 12 h at room temperature. The immobilized enzymes were then separated from uncoupled protein by gel filtration on a Sephadex G-200-120 (1 X 40 cm) with Buffer as the eluent. One milliliter fractions were collected with a flow rate of 15 mL/h. Eluted fractions were monitored by analyzing for phosphorus and protein content (15-17). The yield of the recovered vesicles was typically ca. 80%. In the case of protein analysis, sodium dodecylsulfate was used to reduce the turbidty of the samples. Because polymerized vesicles of 1 were not destroyed by this detergent, corrections in apparent Absorbance (turbidity) at 750 nm were made. Specifically, the apparent Absorbance of each eluted fraction at 750 nm was determined prior to protein analysis, and subtracted from the final value. Control experiments carried out with known quantites of polymerized vesices of 1 plus alpha chymotrypsin, confirmed that the color due to the protein, and the apparent Absorbance due to the turbidity of the vesicles are additive.

Attempted binding (adsorption) of alpha chymotrypsin to polymerized vesicles of 1 (acetal form) using procedures similar to those described above, showed no detectable attachment.

Activity of Immobilized Alpha Chymotrypsin. The activity of immobilized alpha chyotrypsin was determined by kinetic analysis of the hydrolysis of benzoyl-L-tyrosine ethyl ester at 30°C, using literature procedures (18,19).

## RESULTS AND DISCUSSION

The synthetic route used for the preparation of 1,2-Bis(12-(methacryloyloxy)dodecanoyl)-DL-3-glycero-(N-3,3-diethoxypropyl)phosphocholine 1 is outlined in Figure 1. Quaternization of 1,2-dipalmitoyl-DL-3-glycero-3-phosphoric acid bromoethyl ester with beta-dimethylaminopropionaldehyde diethyl acetal followed by saponification with tetrabutylammonium hydroxide and reesterification with 12-methacryloyloxy dodecanoic acid anhydride afforded 1 without difficulty. Photopolymerized vesicles of 1 were formed using conventional methods (6). Dialysis against 1.0 mM HCl liberated surface aldehyde groups which were then used to immobilize alpha chymotrypsin using standard procedures (10). Table I summarizes the loading of the enzyme on the polymerized vesicle surface before and after treatment with $NaBH_4$ and $NaBH_3CN$; also listed are the activities of the immobilized enzyme toward hydrolysis of benzoyl-L-tyrosine ethyl ester. Control experiments carried out with the diethylacetal form of the vesicles showed negligible adsorption of

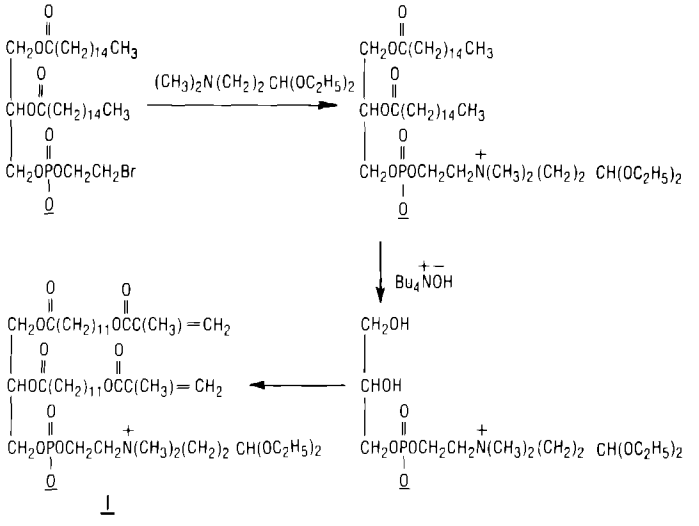


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Figure 1. Synthesis of phospholipid 1 .

Table I. Immobilization of Alpha Chymotrypsin onto Polymerized Vesicles Derived from 1

| | mg of protein/ mmol of lipid | Enzymatic Activity Units/mg of Protein |
|---|---|---|
| Enzyme-Liposome | 286.1 | 6.7 |
| Enzyme-Liposome ($NaBH_4$-treatment) | 409.3 | 5.4 |
| Enzyme-Liposome ($NaBH_3CN$-treatment) | 468.6 | 5.6 |

the protein to the surface. Similar to DPL, photopolymerized vesicles of 1 showed remarkable stability toward sodium dodecylsulfate (8,9).

The efficiency of the immobilization of alpha chymotrypsin onto polymerized vesicles derived from 1 is very high. It compares favorably to the best methods that have been developed thus far for protein conjugation to conventional liposomes (10). Reduction of the Schiff base linkage, used to anchor the enzyme onto the liposomal surface, with $NaBH_4$ and $NaBH_3CN$ results in an even greater loading. In terms of enzymatic function, reduced and nonreduced enzyme-liposome complex retain ca. 15% of their original activity for hydrolyzing benzoyl-L-tyrosine ethyl ester. While enzymatic activity has not yet been optimized, these results clearly demonstrate the feasibility of enzyme conjugation to polymerized liposomes.

Functionalized polymeric liposomes of the type described herein hold considerable promise for practical and mechanistic use in drug delivery. They may also be of considerable value in the design and construction of stable diagnostic devices based on liposome technology. Efforts now underway are aimed at exploring these possibilities.

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