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Synthesis of carboxyacyl derivatives of phosphatidylethanolamine and use as an efficient method for conjugation of protein to liposomes

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Carboxyacyl derivatives of phosphatidylethanolamine with different chain length were synthesized. These compounds were generally prepared by conversion of an appropriate dicarboxylic acid to its anhydride with dicyclohexylcarbodiimide, then reaction with phosphatidylethanolamine (PE) and triethylamine, followed by acidification. These derivatives, when incorporated into liposomes, were highly efficient in conjugating protein to liposomes. Liposomes with PE amide derivative incorporated were activated with water-soluble carbodiimide, and subsequently reacted with protein. The protein to lipid coupling efficiency was shown to be dependent on the chain length of the derivative, and the optimum coupling efficiency was achieved with PE amide of 1,12-dodecanedicarboxylic acid. Up to 60% covalent coupling efficiency of mouse IgG to liposomes was demonstrated with little non-covalent binding. This method will be of great importance in the liposome-targeting field.

Introduction

Several methods for coupling protein to liposomes have been reported. Some of these methods involve direct coupling of proteins to unmodified liposomes by water-soluble cross-linking agents such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) [1,2], glutaraldehyde [3], or suberimidate [4]. Cross-linking methods of this type have not been entirely satisfactory in that significant cross-linking of liposomes, or proteins or both may occur. The extent of specific ligand binding achievable is also generally quite low.

One protein coupling technique which allows relatively high levels of protein binding to liposomes has been described by Heath et al. [5]. The method involves periodate oxidation of glycosphingolipids in the liposome outer membranes to form reactive surface aldehyde groups. Proteins are then attached to the aldehyde groups through Schiff-base formation, followed by reduction with $NaBH_4$ or reductive amination with $NaBH_3CN$. Under optimal conditions, up to about 20% of the protein may be coupled to the oxidized vesicles, and coupling ratios between 100–200 μg protein (IgG) per μmol lipid may be achieved. One limitation of the method is the requirement for glycosphingolipids in the liposomes. General oxidative

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Abbreviations: EDCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; PE, phosphatidylethanolamine; MPB, *N*-[4-(*p*-maleimidophenyl)butyryl]-;PDP, *N*-[3-(pyridyl-2-dithiopropionyl]-; PC, phosphatidylcholine; DCDI, dicyclohexylcarbodiimide; SPD, *N*-succinimidyl-3-(2-pyridyldithio)propionate; NHS, *N*-hydroxysuccinimide.

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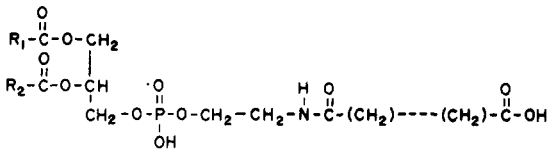

Fig. 1. The structure of carboxyacyl derivatives of PE.

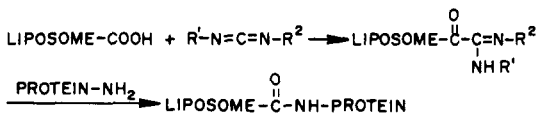

Fig. 2. The conjugation of protein to liposomes containing the carboxyacyl derivative of PE.

damage to liposomes caused by periodate oxidation, and the need to remove periodate before protein coupling, are other limitations.

Other protein coupling methods producing high coupling ratios have been described by Martin et al. [6,7]. The liposomes in this method are formed to incorporate a thio-reactive lipid, such as *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE) or *N*-[3-(pyridyl-2-dithiopropionyl]phosphatidylethanolamine (PDP-PE). The thioreactive liposomes are reacted with either a protein bearing a free sulfhydryl group, or with one thiolated to produce such a group. Coupling efficiencies of between 15 and 30%, and coupling ratios greater than about 200 μg protein (IgG)/μmol lipid have been obtained. The method is limited to proteins which have an available free sulfhydryl group or which can be thiolated without loss of protein activity.

In this paper, we describe the synthesis of different chain length carboxyacyl derivatives of phosphatidylethanolamine (Fig. 1). These compounds may be incorporated into liposomes, activated by water-soluble carbodiimide, and covalently bound to the amino groups of protein (Fig. 2). A chain-length-dependent coupling reaction was demonstrated and up to 60% high protein coupling efficiency was achieved.

Materials and Methods

Materials. Egg phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were purchased from Avanti Polar Lipids (Birmingham, AL) and cholesterol from Nu Check Prep (Elysian, MN). Succinic anhydride, glutaric acid, pimelic acid, sebacic acid, 1,12-dodecanedicarboxylic acid, 1,20-eicosanedicarboxylic and DCDI were obtained from Aldrich Chemical Co. (Milwaukee, WI). Triethylamine was obtained from Pierce Chemical (Rockford, IL), silica gel (E.M. Kieselgel 60, 70–230 mesh) was from Van Waters & Rogers (San Francisco, CA) and thin-layer chromatography (TLC) silica-gel plates from J.T. Baker (Phillipsburg, NJ). EDCI (hydrochloride) and metrizamide were purchased from Sigma Chemical Co. (St. Louis, MO) and IgG from Cappel Labs (Malvern, PA).

Synthesis of carboxyacyl derivatives of phosphatidylethanolamine. These compounds may generally be prepared by starting with an appropriate dicarboxylic acid. The dicarboxylic acid is converted to its anhydride by reaction in chloroform or methylene chloride solution with DCDI (Fig. 3, reaction I). Then, without isolation of free anhydride, phosphatidylethanolamine and triethylamine are added in less than a stochiometric amount (Fig. 3, reaction II). The formation of the triethylammonium salt of the carboxyacyl phosphatidylethanolamine, reaction II, goes rapidly at room temperature. Inasmuch as the product does not hydrolyze rapidly, the salt may be regenerated

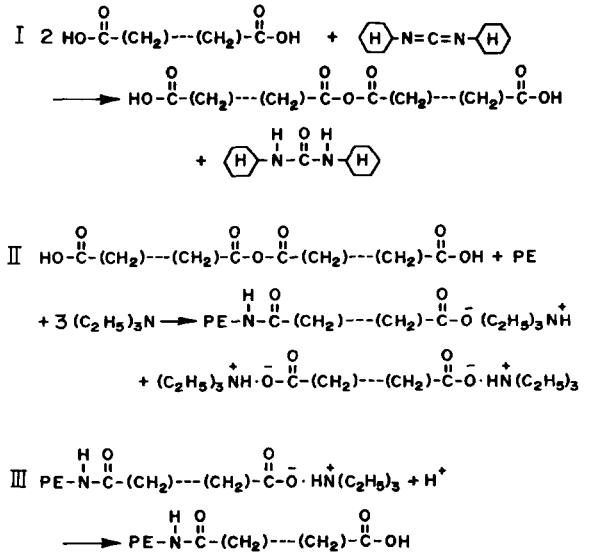

Fig. 3. Synthesis of carboxyacyl derivative of PE.

by shaking its water-immiscible solution with an acidic buffer, as in reaction III of Fig. 3. With those dicarboxylic acids for which anhydrides are readily available, such as succinic anhydride, one may omit reaction I of Fig. 3 and begin the synthesis with the free anhydride.

The synthesis of *N*-glutarylphosphatidylethanolamine is described in detail as an example. To form the anhydride of glutaric acid, 10.6 mg of glutaric acid (0.080 mmol) and 8.7 g DCDI (0.042 mmol) were combined in 2 ml methylene chloride in a screw-cap tube. The tube was capped and the mixture stirred under nitrogen at 23°C for 48 h with a magnetic stirring bar. A solution of PE (0.038 mmol) in 2 ml chloroform and 15 μl of triethylamine (0.108 mmol) were added to the glutaric anhydride/DCDI solution. The reaction mixture was acidified by adding 5 ml chloroform and 4 ml of 0.02 M phosphate/0.02 M citrate buffer (pH 5.5) with vigorous shaking. The aqueous phase was separated by low-speed centrifugation and discarded. The organic phase was dried over anhydrous sodium sulfate. The desired *N*-glutaryl-PE was purified by silica-gel column chromatography. The dried chloroform solution was introduced into 1 × 20 cm silica-gel (Kieselgel 60) column and fractions were eluted by passing through the column 50 ml chloroform effluent solutions containing successively, 0, 10, 20, 30 and 50% methanol. The fractions eluted at each of the five different methanol concentrations were analyzed by TLC on silica gel plates developed with chloroform/ methanol/ water (65 : 25 : 4, v/v). The presence of *N*-glutaryl-PE was detected by I_2 vapor absorption. Most of the product reagent was found in the 30% methanol effluent. *N*-Glutaryl-PE has an R_F value of about 0.3.

Preparation of liposomes. A trace amount of ^{125}I-labeled *p*-hydroxybenzimidyl-PE [8] was included in the liposome preparation for the determination of liposomal lipid concentration. The liposomes were prepared by the reversed-phase evaporation method introduced by Szoka and Papahadjopoulos [9]; briefly, 10 μmol of the lipid mixture (PC/PE derivative/cholesterol/^{125}I-PE = 9 : 1 : 10 : trace) was dissolved in 1 ml of diethyl ether and 325 μl of an aqueous buffer (10 mM $NaPO_4$/0.15 M NaCl (pH 5)) was added. The mixture was sonicated for 30 s to form a water-in-ether emulsion. The liposomes were formed by slowly removing the diethyl ether under reduced pressure in a rotary evaporator.

Conjugation of protein to liposomes. Liposomes were activated by water-soluble (EDCI) at room temperature for 1 h. The liposome concentration was 1 μmol/ml and EDCI was 2.5 mg/ml in a buffer containing 10 mM NaH_2PO_4/0.15 M NaCl (pH 5) during the activation. Then 1.5 cm^3 of the liposome/EDCI mixture was added with 75 μl of 10 mg/ml mouse IgG and 75 μl of 1 M NaCl; the pH was adjusted to 8 with NaOH. The conjugation reaction was carried out overnight at 4°C.

Separation of free protein from liposomes. To the liposome/protein mixture was added dry powdered metrizamide to make a final concentration of 40% (w/v) metrizamide in 1.5 cm^3. The mixture was palced in a Beckman ultra clear centrifuge tube, and carefully layered with 2 cm^3 of 20% (w/v) metrizamide and 0.5 cm^3 buffer. Upon centrifugation at 50000 rpm for 2 h in a Beckman SW 50.1 rotor, the liposomes floated to the buffer/20% metrizamide interface leaving the free protein in the lower metrizamide layer [5,10]. Liposomes were collected and dialyzed overnight against buffer to remove metrizamide.

Measurement of protein-to-lipid coupling ratio. Liposome-bound IgG was measured by using the method of Lowry et al. [11] in the presence of 2% sodium dodecyl sulfate. Lipid concentration in liposomes was determined by the radioactivity level of ^{125}I-PE, based on a known amount of ^{125}I-PE included in the liposomes. The protein-to-lipid coupling ratio was expressed as μg protein/μmol phospholipid.

Results

Preparation of other carboxyacyl derivatives of phosphatidylethanolamine

PE amides of succinic acid, pimelic acid, sebacic acid, 1,12-dodecanedicarboxylic acid and 1,20-eicosanedicarboxylic acid were prepared in a method similar to that as described for *N*-glutarylPE in Methods. The R_F values of the above amides were shown as 0.26, 0.28, 0.42, 0.60 and 0.51, respectively, in the chloroform/methanol/H_2O (65 : 25 : 4) developing solvent. The yield

of each of the above lipids was quantitative, 54, 64, 42 and 40%, respectively.

Attempts to synthesize a shorter spacer chain, the PE amide of malonic acid, were not successful. The yield of malonic anhydride formed by the reaction of malonic acid and DCDI was below 10% and the subsequent synthesis reaction was aborted. Thus, the shortest spacer-arm compound of the PE amide derivatives we have synthesized was *N*-succinyl-PE.

Coupling of IgG to carboxylated-PE liposomes

5 mol% of different chain length of PE amides were included in the lipid mixture of phosphatidylcholine and cholesterol to prepare liposomes. The liposomes were activated with EDCI at pH 5 for 1 h, then mouse IgG was added to the mixture and the reaction was continued overnight at 4°C. Unreacted protein was separated from liposome-conjugated protein by metrizamide density gradient centrifugation. Control coupling reactions were performed by substituting buffer for EDCI. Based on the measured protein and lipid concentration, the protein to lipid coupling ratios and coupling efficiencies are shown in Table I.

The *N*-succinyl-PE amide showed an insignificant amount of protein coupling when included in the liposome preparation. All the other five PE derivatives showed high coupling efficiencies and high levels of protein coupling. The optimal protein coupling occurred at $n = 12$, which gave a protein-to-lipid ratio of 290 μg/μmol for the covalent binding and only 20 μg/μmol for the control non-covalent binding. A high coupling efficiency of 58% was obtained with the $n = 12$ derivative. Although the $n = 20$ reagent gave the highest coupling ratio of 313 μg/μmol, half of the protein binding was due to a non-covalent association of protein to liposomes, which made this reagent less desirable.

TABLE I

EFFECT OF CHAIN LENGTH OF PE AMIDE ON PROTEIN TO LIPID COUPLING RATIO

Sample: PE–N(H)–C(=O)–$(CH_2)_n$–C(=O)–OH

n	Coupling ratio (μg/μmol)		Coupling efficiency (%) [a]
	control	+EDCI	
2	3	4	1
3	6	149	30
5	13	142	28
8	34	197	39
12	20	290	58
20	164	313	63

[a] Calculation based on ratio of liposome-bound protein to total protein added to the reaction mixture.

Discussion

Several different methods for coupling protein to liposomes have been devised. The most efficient coupling ratio (up to 250 μg of protein/μmol of vesicle phospholipid) was obtained by reacting MPB-PE containing liposomes with reduced Fab' fragments of IgG [6]. To use this MPB-PE method, the protein would have to either naturally contain free sulfhydryl groups or be thiolated by a heterobifunctional cross-liking reagent such as SPDP [12] or succinimidyl-*S*-acetylthioacetate [13]. Another method of reacting the amino groups of protein with preformed liposomes which contain the *N*-hydroxysuccinimide ester of *N*-suberyldimyristoylphosphatidylethanolamine (NHS-sub-DMPE) was introduced by Kinsky et al. [14], the protein was bound to liposomes using the *N*-hydroxylsuccinimidyl functional group. Although NHS-sub-DMPE is stable for storage in organic solvents, preformed liposomes lose their protein-binding activity due to the hydrolysis of the *N*-hydroxysuccinimide ester bond [14]. In this paper, we describe an efficient method of conjugating the amino groups of protein to preformed liposomes containing carboxyacyl groups. The carboxyacyl derivatives of PE are stable either in free form or when incorporated into liposomes.

The carboxyacyl derivatives of PE are easy to prepare. We have shown that the length of the spacer which links the functional carboxyacyl group and PE plays an important role in protein coupling. The PE amide of succinic acid was too short to give any significant protein coupling when incorporated into liposomes, probably due to the steric hindrance. The PE amide of glutaric acid gave a good protein coupling efficiency to 30%. Increasing the chain length up to $n = 12$ progres-

sively improves the coupling efficiency to 60%. When $n = 20$, the spacer was too long and a substantial non-covalent association of protein occurred. The optimal coupling was shown with $n = 12$. Although the spacer of PE derivatives is hydrophobic, the hydrophilic carboxyacyl group prevents the spacer chain from bending back to interact with bilayer lipids. Therefore, incorporation of 5% PE derivatives to liposomes is not likely to affect the bilayer permeability.

A number of applications for liposomes containing surface-bound molecules such as small haptens, enzymes, antibodies and protein are reported. The attachment of specific antibodies to liposomes has been suggested as a method of targeting drug-containing liposomes to specific tissues or organ reporters in therapeutic applications [15]. The attachment of protein to liposomes has also been shown to improve latex agglutination for clinical diagnostic assays [16]. The method presented here provides a simple and highly efficient means of conjugating unmodified proteins to liposomes and will likely contribute to an expansion of the use of ligand-bearing liposomes in diagnostics and therapy.

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