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An improved method for the covalent coupling of proteins to liposomes

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A method is described for the preparation of liposomes carrying covalently attached protein at the outer surface. It is based upon the reaction between proteins, thiolated with the heterobifunctional reagent succinimidyl-*S*-acetylthioacetate (SATA), and liposomal maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine. Advantages of the procedure are that it is not restricted to proteins carrying native SH-groups and that it is substantially more convenient and less time-consuming than previously published methods. Applications for these liposomes are to be found in the fields of liposome targeting and the production of monoclonal antibodies.

Introduction

In the past few years several methods for the covalent coupling of proteins to lipid vesicles (liposomes) have been described (for a review, see Ref. 1). However, all of these have more or less serious drawbacks or are not generally applicable. An elegant method for coupling immunoglobulin G-Fab fragments to liposomes was presented by Martin and Papahadjopoulos [2]. Their method makes use of the availability of a highly reactive sulfhydryl group in reduced Fab fragments to form a thioether linkage with the maleimide residues incorporated in liposomes. This reaction proceeds spontaneously under mild conditions, without causing cross-linking between liposomes. Obviously, not all proteins possess free sulfhydryl groups; the ability to introduce exogenous sulfhydryl groups into proteins will, therefore, greatly improve the applicability of the method. Several methods have been described for the introduction of sulfhydryl groups; the one that is most widely applied involves the use of the heterobifunctional reagent *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) [3]. Recently, the synthesis of succinimidyl-*S*-acetylthioacetate (SATA), a new reagent to introduce sulfhydryl groups in proteins, has been described by Duncan et al. [4]. The use of SATA offers several advantages over SPDP; most importantly, it eliminates the need of a reducing agent, such as dithiothreitol, to deprotect the thiol: traces of dithiothreitol interfere with the coupling reaction. With SATA deprotection is accomplished by hydroxylamine, which creates an additional advantage of the use of SATA over SPDP, in that the half-life of the free sulfhydryl is drastically lengthened.

In this paper we describe the use of SATA to couple proteins to liposomes containing maleimide residues. The coupling efficiency under various reaction circumstances has been investigated.

Abbreviations: SATA, succinimidyl-*S*-acetylthioacetate; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; DOPE, dioleoylphosphatidylethanolamine; MPB-PE, maleimido-4-(*p*-phenylbutyrate)phosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Materials and Methods

Bovine γ-globulin (Cohn fraction II), hydroxylamine-HCl, succinimidylmaleimido-4-(*p*-phenylbutyrate), egg-yolk phosphatidylcholine (type V-E), cholesterol (CH-S) and dicetyl phosphate were obtained from Sigma. Dioleoylphosphatidylethanolamine (DOPE) was from Avanti and Dextran T-40 from Pharmacia.

Synthesis of MPB-PE

Maleimido-4-(*p*-phenylbutyrate)phosphatidylethanolamine (MPB-PE) was synthesized as described by Martin and Papahadjopoulos [2], except that purification of the product was achieved by chromatography on Merck PLC Silicagel 60 F-254 + 366, 2-mm plates under argon, with chloroform/methanol/water, 65/25/4, as a solvent. Three bands appeared (R_F, 0.29, 0.42 and 0.74); only band 2 (R_F 0.42) contained phospholipid, as determined by phosphate assay, as well as sulfhydryl binding capacity, as demonstrated by reaction with excess 2-mercaptoethanol and subsequent determination of remaining sulfhydryl groups. This band, thus identified as MPB-PE, was scraped off and eluted from the silicagel with chloroform/methanol (4 : 1). The MPB-PE solutions were stored in sealed ampoules under argon at −80°C.

Preparation of thiolated protein

SATA was synthesized and used to acetylthioacetylate bovine γ-globulin as described by Duncan et al. [4], resulting in an introduction of 4–6 mol of sulfhydryl groups per mol γ-globulins (assuming a molecular weight of 150000 for IgG and neglecting the IgM and IgA). Acetylthioacetyl–γ-globulin was deacetylated by addition of 10 μl of a freshly prepared solution of 0.5 M hydroxylamine-HCl, 0.5 M Hepes, 25 mM EDTA and adjusted to pH 7.0 by addition of a few drops 10 M NaOH to every 100 μl of protein solution to be deacetylated. After about 30 min at room temperature the total amount of free sulfhydryl groups showed no further increase. Immediately after deacetylation the resulting thioacetyl–γ-globulins were coupled to MPB-PE-containing liposomes.

Preparation of liposomes

Large negatively charged unilamellar vesicles, consisting of phosphatidylcholine, cholesterol, dicetyl phosphate and MPB-PE in a molar ratio of 19 : 16 : 4 : 1, unless otherwise indicated, were prepared by the reverse-phase evaporation method as described by Szoka and Papahadjopoulos [5] and sized by extrusion through 0.4 μm polycarbonate filters (Unipore, Bio-Rad). Liposomes were prepared in buffer 1 (10 mM Hepes, 135 mM NaCl (pH 7.5)).

Coupling of thiolated protein to liposomes

To the liposomes varying amounts of thioacetyl–γ-globulin (details are given in the legends to figures) were added and allowed to react for 2 h, unless otherwise indicated, at room temperature, after which 50 μl of *N*-ethylmaleimide solution (8 mM in buffer 1) was added to cap unreacted sulfhydryl groups. 15 min after addition of the *N*-ethylmaleimide, liposomes were isolated from the reaction mixture by flotation on a Dextran gradient, essentially as described by Heath et al. [6]. Briefly, 1.0 ml liposome suspension was mixed with 0.8 ml of a 30% solution of Dextran T-40 in buffer 1 in a 5 ml nitrocellulose centrifuge tube (Beckman). This solution was gently overlaid with about 3.5 ml of a 10% solution of Dextran T-40 in buffer 1. The tube was topped off with buffer 1. After 15 min of centrifugation at 100000 × *g* in a Beckman SW 50.1 rotor the liposomes were pipetted off the gradient in a minimal volume, usually less than 0.5 ml, and resuspended in buffer 1.

Other methods

Protein coupled to liposomes was determined according to the microprotein assay modification by Peterson [7] of the method described by Lowry et al. [8] and expressed as micrograms of protein per micromole of lipid. Bovine γ-globulin was used as a standard. Phospholipid was assessed by phosphate assay after perchloric acid destruction [9]. Free sulfhydryl groups were assayed with 5,5′-dithiobis(nitrobenzoic acid) according to Ellman [10].

Results

The coupling of thioacetyl–γ-globulin to maleimide vesicles is a relatively rapid reaction:

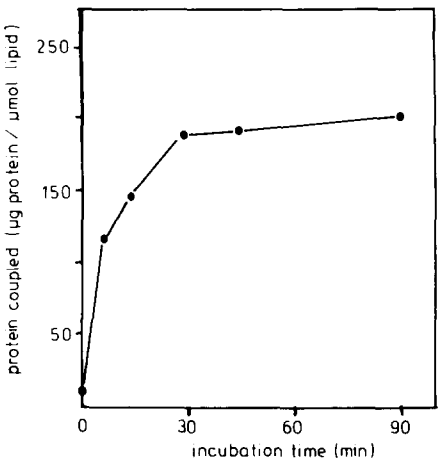

Fig. 1. Protein coupling and incubation time. Thiolated bovine γ-globulin (0.6 mg) and MPB-liposomes (2 µmol total lipid) were allowed to react in 0.3 ml buffer 1 at room temperature. After the time indicated the reaction was stopped and γ-globulin-liposomes were isolated as described.

the amount of protein coupled to liposomes reaches a plateau value in less than 1 h at room temperature (Fig. 1). One of the factors that determine the ultimate ratio of protein to lipid is the mole fraction of MPB-PE in the liposomes. Fig. 2 shows the increase in the amount of coupled protein with an increasing mole fraction of MPB-PE. For practical reasons we have chosen 2.5% MPB-PE, on a total lipid basis, as a standard maleimide content in the liposomes.

Other factors that determine the protein to lipid ratio in the reaction product are the protein and liposome concentrations in the reaction mixtures. To obtain a satisfactory extent of protein binding to the liposomes it is advisory to maintain a protein concentration in the reaction mixture above 2 mg/ml, as shown in Fig. 3. The coupling efficiency appears to be inversely correlated with the total liposome concentration in the reaction mixture (Fig. 4). The coupling reaction does not appear to be very sensitive to pH variation between pH 6.5 and pH 8.0 (results not shown). Standard physiological buffers are therefore quite suitable for use in the coupling reaction.

Fig. 5 shows the effect of addition of a competitive substrate, *N*-ethylmaleimide, to the reaction mixture. As can be expected, an excess of *N*-ethylmaleimide suppresses the coupling of the thioacetyl–γ-globulin to the liposomes, probably through lack of available sulfhydryl groups. This also rules

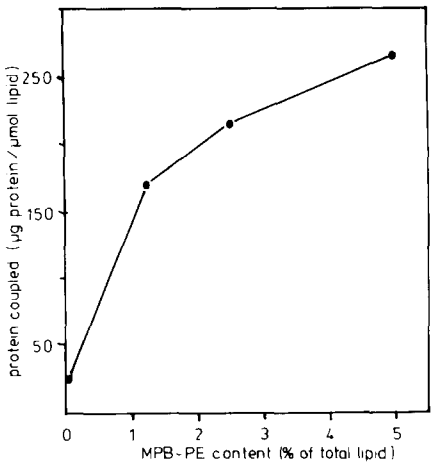

Fig. 2. Dependence of protein coupling on MPB-PE content in liposomes. The percentage of MPB-PE was corrected for by an equivalent change in the percentage of egg PC, in order to keep the total phospholipid fraction at 60%. The percentages dicetylphosphate and cholesterol were kept constant. From these liposome preparations 2 µmol was pipetted to 2 mg/ml of thiolated bovine γ-globulin in buffer 1 in a total volume of 0.3 ml and incubated for 2 h at room temperature. The reaction product was isolated as described.

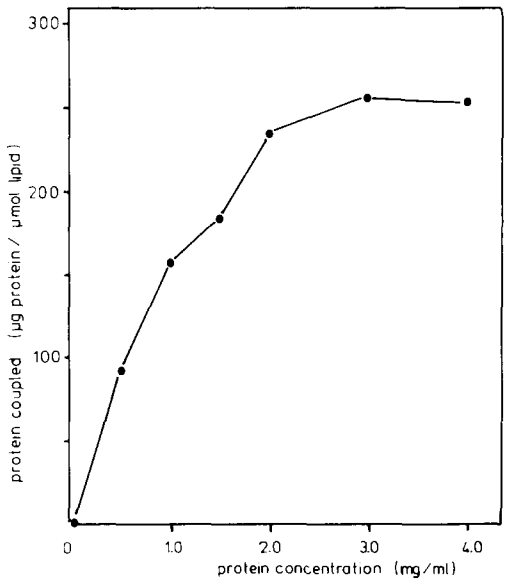

Fig. 3. Coupling efficiency dependence on protein concentration. MPB-liposomes (2 µmol total lipid) and thiolated bovine γ-globulin in various concentrations were allowed to react in 0.3 ml buffer 1 for 2 h at room temperature.

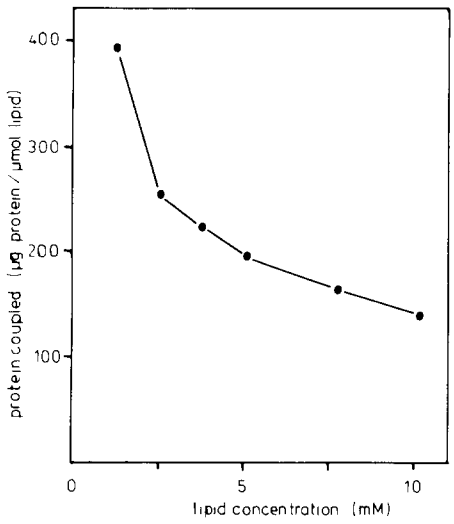

Fig. 4. Effect of liposomal lipid concentration on protein coupling. Thiolated bovine γ-globulin (2 mg/ml in buffer 1), and increasing amounts of MPB-liposomes were allowed to react for 2 h at room temperature. Liposomes were subsequently isolated as described.

out the possibility that the protein is only associated with the liposomes by non-covalent attachment, since in that case the addition of *N*-ethylmaleimide to the reaction mixture should have no effect on the protein to lipid ratio obtained in the reaction product.

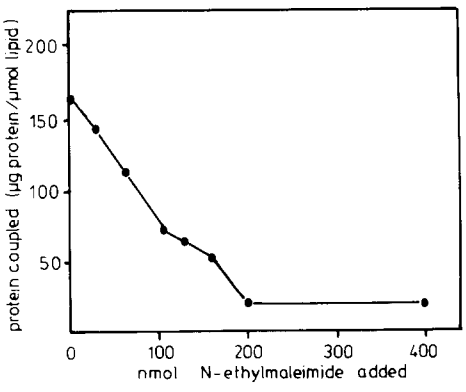

Fig. 5. Effect of *N*-ethylmaleimide on protein-liposome coupling. To 0.6 mg thiolated bovine γ-globulin and 2 µmol MPB-liposomes in 0.3 ml buffer 1 various amounts of *N*-ethylmaleimide were added. After 2 h of incubation protein-coupled liposomes were isolated by Dextran gradient flotation as described.

Discussion

Several different types of reaction leading to attachment of proteins to liposomes have been devised in the last 5 years. One of the first techniques described made use of glutaraldehyde to couple amino groups of proteins to phosphatidylethanolamine-containing liposomes [11]. Soon other methods, not producing cross-linked liposomes, were published. Amongst these are detergent dialysis of fatty acid-coupled proteins [12–14], cross-linking via Schiff's base formation between proteins and periodate-oxidized glycosphingolipid-containing liposomes [6,15], coupling of protein to diazotized *N*-(*p*-aminophenyl) stearylamide-containing liposomes [16] and coupling of proteins to *N*-hydroxysuccinimidyl-PE containing liposomes [17].

The most efficient coupling ratio obtained (up to 500 µg protein/µmol liposomes) was achieved by reacting MPB-PE-containing liposomes under very mild conditions with free sulfhydryl-containing proteins, such as reduced immunoglobulin G-Fab fragments [2]. To use this method for non-sulfhydryl-containing proteins it is obviously necessary to thiolate these proteins. The most widely used reagent to introduce sulfhydryl groups is the heterobifunctional cross-linking reagent SPDP. Several investigators have used this reagent to couple proteins to maleimide-containing liposomes [18–20]. To prevent oxidation of sulfhydryl groups during storage, it is necessary to shield them with protective groups. The protective group in SPDP, 2-thiopyridone, can be removed by a strong reducing agent, such as dithiothreitol. Since dithiothreitol also reacts with maleimide groups, it has to be removed thoroughly before the thiolated protein can be added to the maleimide liposomes. Recently the synthesis of a new sulfhydryl-introducing reagent has been described that lacks this disadvantage [4]. Deprotection of this reagent, SATA, is accomplished by reaction with hydroxylamine at neutral pH, which does not interfere with the sulfhydryl-maleimide conjugation. The presence of hydroxylamine also prevents the oxidation of the liberated sulfhydryl, which constitutes a second advantage over the use of SPDP. By making use of the SATA-maleimide technique we are able to achieve a coupling ratio of about 300 µg

immunoglobulin per μmol of liposomal lipid, under optimal conditions. This is appreciably higher than the ratio obtained by the SPDP-maleimide technique, reported to be about 170 μg/μmol lipid [19,20]. This difference in coupling efficiency may be caused by the untimely oxidation of the SPDP-derived thiol. The half-life of thiols at neutral pH is known to be as short as about 4 h at pH 6.5 [2].

The achieved attachment ratio of protein to lipid of 300 μg/μmol lipid corresponds to roughly 3000 molecules IgG per liposome, assuming a uniform vesicle diameter of 400 nm [21] and a molecular weight for IgG of 150000 (disregarding the presence of IgM and IgA in the bovine γ-globulin preparation, which amounts to approx. 10% [22]).

We have found the SATA-maleimide coupling technique a very convenient method to produce protein-coupled liposomes, in which the amount of protein bound to the vesicles can easily be controlled by varying the lipid or protein concentration in the reaction mixture. Since the SATA-protein conjugate and the MPB-PE are stable for several months at −20°C or lower they can be made in advance in large quantities. Thus, the coupling reaction itself only consists of the preparation of liposomes, the addition of thioacetyl-protein and subsequent isolation of the liposomes from the reaction mixture. The whole procedure can be performed within 3 h and is therefore substantially faster than the SPDP-maleimide coupling procedure, since two column chromatography steps can be omitted.

We are currently applying the technique described in this paper in a study of receptor-mediated endocytosis in Kupffer cells in monolayer culture. The Fc, mannose and galactose receptors of these cells are addressed with liposomes containing covalently coupled IgG, horse radish peroxidase and asialofetuin, respectively. In preliminary experiments we observed up to 40-fold stimulation of liposome uptake as a result of covalent immunoglobulin coupling.

Because SATA can bind to any amino group, the technique described in this article is not restricted to protein attachment to liposomes. In fact, we have used this technique also to attach the carbohydrate moiety of pronase-digested membrane glycoproteins from transformed cells to liposomes, in order to produce monoclonal antibodies against these carbohydrate structures (unpublished data). There are probably numerous other applications for which the technique here presented will prove to be fruitful.

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