Biochimica et Biophysica Acta, 769 (1984) 543–550
Elsevier

BBA 71981

SYNTHESIS OF *N*-HYDROXYSUCCINIMIDE ESTERS OF PHOSPHATIDYLETHANOLAMINE AND SOME PROPERTIES OF LIPOSOMES CONTAINING THESE DERIVATIVES

STEPHEN C. KINSKY, KEIICHIRO HASHIMOTO, JOAN E. LOADER and AMY L. BENSON

Department of Pediatrics, National Jewish Hospital and Research Center, Denver, CO 80206 (U.S.A.)

(Received August 17th, 1983)

Key words: N-Hydroxysuccinimide ester; Phosphatidylethanolamine; Liposome

The *N*-hydroxysuccinimide (NHS) ester of *N*-suberyl-dimyristoylphosphatidylethanolamine (sub-DMPE) was synthesized by reaction of DMPE with disuccinimidyl suberate, and isolated by preparative plate chromatography. Liposomes, which contain NHS-sub-DMPE, can covalently bind compounds that possess a free amino group such as ε-dinitrophenyl-lysine. The extent of DNP-lysine binding is influenced by the time and temperature of incubation, the amount of NHS-sub-DMPE incorporated into the liposomes, and the initial concentration of DNP-lysine. Binding occurs as a consequence of the formation of a new dinitrophenylated compound which has been characterized. Although NHS-sub-DMPE is stable to storage in organic solvents, preformed liposomes rapidly lose their ability to bind DNP-lysine due to hydrolysis of the *N*-hydroxysuccinimide ester bond. These findings bear on the future applicability of liposomes, containing *N*-hydroxysuccinimide esters of PE, as illustrated by the preparation of immunogenic liposomes.

Introduction

We have recently described an alternative procedure for the preparation of immunogenic liposomal model membranes which circumvents the need to synthesize the *N*-(hapten)-substituted derivatives of phosphatidylethanolamine (PE) that were originally employed for this purpose [1,2]. This new procedure is based on the generation of liposomes containing the *N*-hydroxysuccinimide (NHS) esters of palmitic acid (Pal), cholesteryl-hemisuccinate (succ-Chol), or *N*-succinyl-dipalmitoylphosphatidylethanolamine (succ-DPPE). The reactive NHS function, which is anchored in the lipid bilayers, enables these preformed liposomes to covalently bind haptens that possess a free, substitutable, amino group (for example, ε-DNP-lysine). As a consequence of this binding, the liposomes can elicit hapten-specific plaque-forming cells in mice.

The preceding investigation revealed a significant difference in the ability of liposomes to induce a DNP response depending on whether DNP-lysine was covalently attached to the model membranes via either NHS-succ-DPPE, NHS-succ-Chol or NHS-Pal [2]. When administered to mice at a constant dose and epitope density, liposomes prepared with NHS-succ-DPPE elicited approx. 25-fold and 5-fold more TNP-specific plaque-forming cells than did liposomes prepared,

Abbreviations: NHS, *N*-hydroxysuccinimide; DSS, disuccinimidyl suberate; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; Chol, cholesterol; DCP, dicetylphosphate; Pal, palmitic acid; succ-Chol, cholesterylhemisuccinate; succ-DPPE, *N*-succinyl-dipalmitoylphosphatidylethanolamine; sub-DMPE, *N*-suberyl-dimyristoylphosphatidylethanolamine; NHS-Pal, NHS-succ-Chol, NHS-succ-DPPE, and NHS-sub-DMPE, the *N*-hydroxysuccinimide esters of Pal, succ-Chol, succ-DPPE, and sub-DMPE, respectively; DNP-lysine, ε-(2,4-dinitrophenyl)lysine; DNP-Cap-PE, *N*-(2,4-dinitrophenyl-6-aminocaproyl)phosphatidylethanolamine; PFC, plaque-forming cells.

respectively, with NHS-Pal and NHS-succ-Chol. In anticipation that liposomes prepared with NHS esters of PE would also prove to be more effective in raising antibodies to protein/peptide determinants, as well as binding to cells (see Discussion), the necessity for adequate quantities of such derivatives became obvious.

Unfortunately, the method by which NHS-succ-DPPE was previously synthesized suffered from several deficiencies. This procedure involved two steps: reaction of DPPE with succinic anhydride to give succ-DPPE, followed by reaction of the isolated succ-DPPE with NHS in the presence of dicyclohexylcarbodiimide to give NHS-succ-DPPE. However, conversion of succ-DPPE to the NHS ester in the second step was incomplete and variable, frequently resulting in low yield (20%) of the desired product. In contrast, Pal and succ-Chol were completely converted to the corresponding NHS derivatives by the same reaction, and pure NHS-Pal and NHS-succ-Chol could be isolated in high yield (90%) [2].

Accordingly, the present investigation was undertaken to devise a new method for the synthesis of NHS esters of PE. This procedure is described herein in addition to some properties of liposomes, containing these derivatives, that relates to their use in the preparation of immunogens.

Materials and Methods

Chemicals

Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) were obtained from Avanti Polar Lipids, Birmingham, AL. Cholesterol (Chol), dicetylphosphate (DCP), ε-DNP-lysine, and NHS were purchased from Sigma Chemical Co., St. Louis, MO. Disuccinimidyl suberate (DSS) came from Pierce Chemical Co., Rockford, IL. *N*-Succinyl-DMPE (succ-DMPE) was synthesized by the same procedure previously described for succ-DPPE [2].

Preparation and purification of NHS-sub-DMPE

The *N*-hydroxysuccinimide ester of *N*-suberyl-dimyristoylphosphatidylethanolamine (NHS-sub-DMPE) was synthesized by reaction of DMPE with DSS (Fig. 1). DMPE (100 μmol) was dissolved in 5 ml of chloroform containing triethylamine (Et_3N, 200 μmol) that had been redistilled over ninhydrin. The DMPE was added drop-wise to a stirred solution of DSS (500 μmol/10 ml chloroform), and the mixture was incubated for 5 h at room temperature. The mixture was then taken to dryness by rotary evaporation under reduced pressure at 40°C, and the residue was redissolved in approx. 3 ml of chloroform. This fraction was subjected to preparative plate chromatography, as described in the following section, to separate NHS-sub-DMPE from NHS, Et_3N, and DSS.

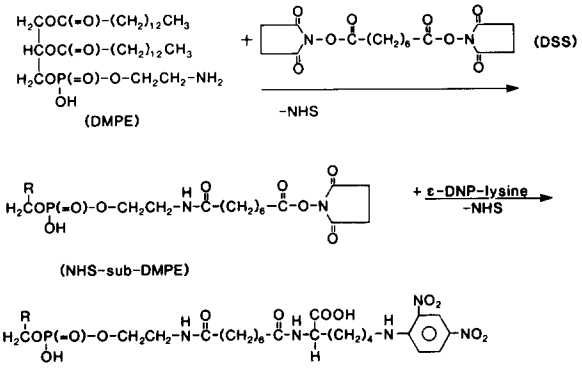

Fig. 1. Synthesis of NHS-sub-DMPE and reaction of NHS-sub-DMPE with DNP-lysine.

The band (R_F value 0.53), which contained the desired product as indicated by a positive test for both phosphate and NHS groups (see below), was scraped off the plate and layered over a 30 mm high bed of Unisil (Clarkson Chemical Co., Williamsport, PA) at the bottom of a 1.5 × 20 cm column. The Unisil had been previously washed with chloroform, followed by chloroform/methanol (1 : 1, v/v). NHS-sub-DMPE was subsequently eluted by passage of 150 ml of the latter solvent mixture. The eluate was taken to dryness, the residue was redissolved in 10 ml of chloroform/methanol (1 : 1, v/v), and analyzed for organic phosphate by a minor modification of the procedure of Gerlach and Deuticke [3].

Chromatography and detection

Analytical thin-layer chromatography was carried out on silica gel GHL plates, 0.25 mm thick,

TABLE I

REAGENTS EMPLOYED FOR COMPOUND DETECTION AFTER ANALYTICAL CHROMATOGRAPHY

+, positive test; −, negative test. ANS, 8-anilinonaphthalene sulfonate.

Detection reagent	Compound				
	Et_3N	DSS	DMPE	NHS	NHS-sub-DMPE
I_2 vapor	+	+	+	+	+
ANS	−	−	+	−	+
$NH_2OH/FeCl_3$	−	+	−	±	+
Molybdenum blue	−	−	+	−	+
Ninhydrin	−	−	+	−	−

(Analtech, Newark, DE) in two solvent systems. These were chloroform/methanol/water (70 : 30 : 5, v/v) (system I) and chloroform/methanol/acetic acid (60 : 20 : 3, v/v) (system II). Preparative chromatography was performed on silica gel 60 plates, 2 mm thick, (Brinkmann Instruments Inc., Westbury, NY) in system I.

Compounds were detected as indicated in Table I by iodine vapor, 8-anilinonaphthalene sulfonate (ANS) [4], hydroxylamine-ferric chloride reagents ($NH_2OH/FeCl_3$) [5], and phosphate (molybdenum blue) and ninhydrin sprays (Applied Science, Deerfield, IL).

Liposome preparation

Multilamellar liposomes were generated from dried lipid films containing DMPC, Chol, and DCP in molar ratios of 2 : 1.5 : 0.2, respectively. Succ-DMPE (control liposomes) and/or NHS-sub-DMPE (experimental liposomes) were added to give the densities (expressed as mol%) indicated in the appropriate table and figure legends. The films were dispersed by vortexing in Dulbecco's phosphate-buffered saline (DPBS, pH 8, without Mg^{2+} and Ca^{2+} (buffer 1)) to yield a 10 mM liposomal phospholipid (DMPC) suspension.

DNP-lysine binding and assay

Except where noted, binding of DNP-lysine to the liposomes was determined as previously described in detail [2]. Briefly, this method involved the addition of one volume (usually 500 μl) of 10 mM DNP-lysine in DPBS (buffer 1), pH 8, to an equal volume of liposomes as soon as possible after liposome generation. The mixture was incubated at 37°C with gentle shaking. At various times thereafter, the reaction was stopped by the addition of acetic anhydride (15 μl, 160 μmol) to block the amino group of DNP-lysine. Free DNP-lysine was subsequently removed by dialysis of the mixture at room temperature for 24 h against two changes (1 liter each) of DPBS (buffer 1), pH 8. Aliquots of the dialysed liposomes were then diluted, if necessary [2], to a volume of 250 μl with DPBS (buffer 1) and solubilized by the sequential addition of 625 μl of methanol and 312 μl of chloroform. The absorbance of this solution was measured at 345 nm. After correction for the absorbance in identical aliquots of the final dialysate, which had been treated in the same manner, the amount of DNP bound to the liposomes was calculated using a molar extinction coefficient of 17 000.

Immunization and hemolytic plaque assay

Reference 2 (and papers cited therein) should also be consulted for details of these procedures, and the preparation of immunogenic liposomes that had been sensitized by the incorporation of *N*-(2,4-dinitrophenyl-6-aminocaproyl)phosphatidylethanolamine (DNP-Cap-PE), without and with lipid A. Experimental groups (consisting of a minimum of three BDF_1 mice per group) were immunized intraperitoneally with 200 μl of liposomes that had been diluted to contain 25 nmol of bound DNP-lysine or 25 nmol of DNP-Cap-PE. Control groups received 200 μl of diluted liposomes, prepared without NHS-sub-DMPE, to which 25 nmol of free DNP-lysine had been added. Spleen cell suspensions were prepared four days later, and assayed for the frequency of direct plaque-forming cells against trinitrophenylated sheep erythrocytes (TNP-SRBC) as targets. These values were corrected for plaque-forming cells detected with non-haptenated sheep erythrocytes, and the results expressed as the arithmetic mean ± standard error (S.E.) of the number of hapten-specific plaque-forming cells (TNP-PFC) per 10^6 spleen cells.

Results

Synthesis of NHS-sub-DMPE

Chromatographic analysis revealed that, after 5 h incubation with DSS, the amino group of DMPE was completely substituted. Loss of reactivity with ninhydrin was paralleled by the liberation of NHS in the reaction mixture. Theoretically, this result could occur with the formation of two products: NHS-sub-DMPE and 'bis-DMPE' in which 2 mol of DMPE are linked by a suberyl bridge. However, under the synthetic conditions employed (5-fold molar excess of DSS over DMPE; see Materials and Methods), we only observed formation of a new phospholipid that behaved as anticipated for NHS-sub-DMPE (Table I, Fig. 2). Thus, in addition to containing phosphate, the product could be detected by iodine vapor and ANS, and a NHS group was present as indicated by a positive reaction with $NH_2OH/FeCl_3$ (n.b. NHS would be absent in bis-DMPE).

After purification as described in Materials and Methods, NHS-sub-DMPE was obtained as a chromatographically pure compound (Fig. 2). NHS-sub-DMPE could be readily distinguished from either DMPE or succ-DMPE, particularly in solvent system II. On the basis of phosphate recovery, the yield of pure NHS-sub-DMPE averaged 65% (five preparations).

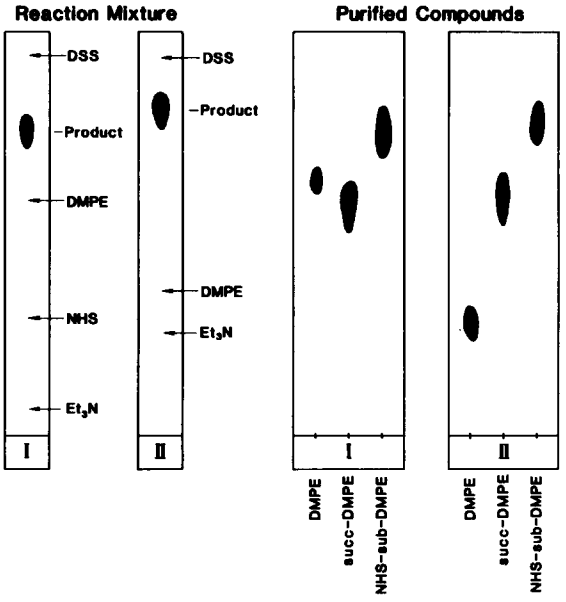

Fig. 2. Analytical chromatography of NHS-sub-DMPE (product) before and after purification. Composite diagrams obtained by developing five plates each in solvent systems I and II. Amounts applied were 80 nmol phospholipid (reaction mixture), reference substances (positions indicated by arrows) in approximately the same ratio to DMPE as initially present in the reaction mixture, and 70 nmol of the purified compounds. Materials were visualized by treating individual plates with the detection reagents listed in Table I (n.b. DMPE could not be located by ninhydrin spray after development in system II unless the plates were heated).

Characteristics of DNP-lysine binding to liposomes

The successful synthesis of NHS-sub-DMPE was further substantiated by the fact that liposomes, which contained this derivative, could bind significantly more DNP-lysine than liposomes lacking NHS-sub-DMPE. The extent of binding was dependent not only on the density of NHS-sub-DMPE incorporated into the liposomes (Fig. 3), but also on incubation time (Fig. 3) and temperature (Table II). At 37°C, binding was maximal after 5 h. In contrast, binding was less at 4°C or 23°C and, at these temperatures, was not complete after 5 h incubation. The amount of DNP bound was also a function of the initial concentration of DNP-lysine (Fig. 4).

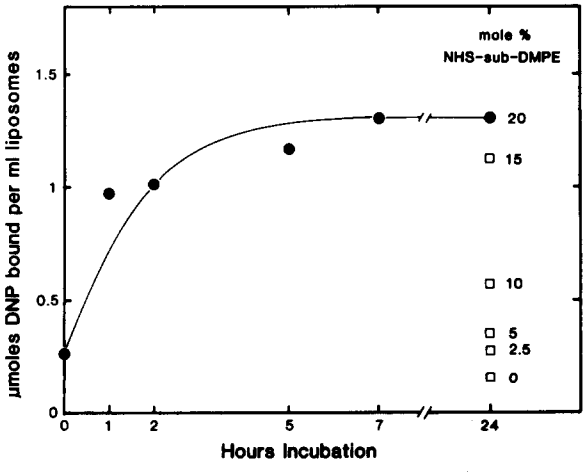

Fig. 3. Effect of incubation time and density of NHS-sub-DMPE on binding of DNP-lysine to liposomes. The abscissa indicates the time between the addition of DNP-lysine (to start the reaction) and acetic anhydride (to stop it). Total incubation time (before plus after acetic anhydride) was 24 h, prior to dialysis and determination of bound DNP.

TABLE II

EFFECT OF INCUBATION TEMPERATURE ON BINDING OF DNP-LYSINE TO LIPOSOMES

Liposomes were prepared with 10 mol% NHS-sub-DMPE, and incubated with DNP-lysine for various times at the temperatures indicated. Results have been corrected for the amount of DNP absorbed to control liposomes prepared with 10 mol% succ-DMPE.

Incubation temperature (°C)	Incubation time (h)	μmol DNP bound per ml liposomes
4	5	0.201
4	24	0.311
23	5	0.480
23	24	0.660
37	5	0.809
37	24	0.811

Product formed upon binding of DNP-lysine to liposomes prepared with NHS-sub-DMPE

The preceding discussion refers to bound DNP-lysine present in the dialysed liposomes. However, the actual form in which DNP-lysine was bound varied depending on whether or not

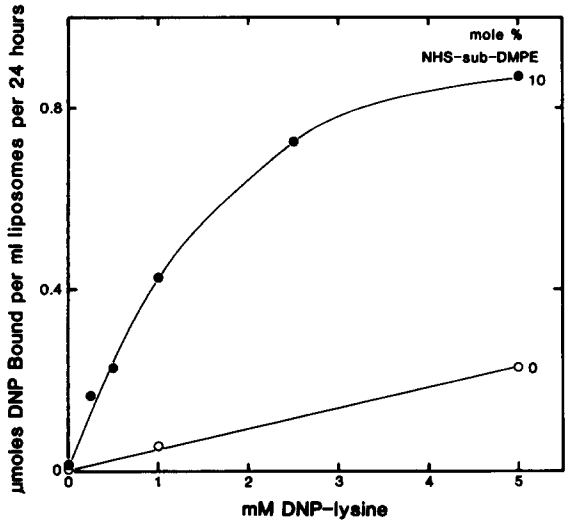

Fig. 4. Effect of DNP-lysine concentration on binding to liposomes. Liposomes were prepared with 10 mol% NHS-sub-DMPE or 10 mol% succ-DMPE (0 mol% NHS-sub-DMPE), and incubated for 24 h with DNP-lysine at the concentrations indicated on the abscissa.

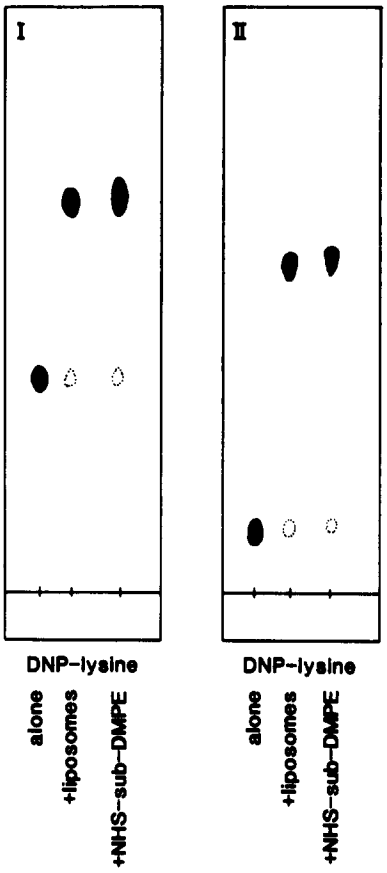

Fig. 5. Identity of dinitrophenylated product formed by reaction between DNP-lysine and either liposomes prepared with NHS-sub-DMPE or the free NHS derivative. 0.5 ml of 10 mM DNP-lysine (5 μmol) was added to a tube that contained 0.5 ml of liposomes (which were sensitized with 2 μmol of NHS-sub-DMPE (= 18 mol%)), or to a tube that contained 2 μmol of NHS-sub-DMPE (initially suspended in 0.5 ml of DPBS (buffer 1), pH 8). In the latter case, CH_3OH (2.5 ml) and $CHCl_3$ (1.25 ml) were added to solubilize the free NHS derivative. These volumes were also added to the tube with liposomes after 5 h incubation at room temperature. The dinitrophenylated product was then extracted by the addition of $CHCl_3$ (1.25 ml) and H_2O (1.25 ml) to each of the tubes. Following centrifugation (10000 × *g*; 15 min), the lower $CHCl_3$ phase was recovered, and taken to dryness under a N_2 stream. The residue was redissolved in $CHCl_3$ (2.5 ml) and mixed with CH_3OH (2.5 ml) and H_2O (2.25 ml). The lower $CHCl_3$ phase was again isolated and dried as above, and the residue dissolved in 1 ml of chloroform/methanol (1:1, v/v). Aliquots of each of these fractions (containing 10 nmol of DNP as determined spectrophotometrically) and DNP-lysine (10 nmol) were spotted on analytical thin-layer plates which were developed in either solvent systems I or II. The dinitrophenylated compounds were visualized by their yellow color.

NHS-sub-DMPE had been incorporated into the liposomes. Thus, the small amount of DNP, which was associated after 24 h incubation with liposomes that did not contain NHS-sub-DMPE (Figs. 3 and 4), was identified chromatographically as DNP-lysine (results not shown). Therefore, it may be more appropriate to designate this as 'absorbed' DNP-lysine.

In contrast, binding of DNP-lysine to liposomes, which were prepared with NHS-sub-DMPE, occurrred primarily (> 90%) as a consequence of the formation of a new dinitrophenylated compound. The same yellow product was formed upon reaction of DNP-lysine with free NHS-sub-DMPE that had not been incorporated into liposomes (Fig. 5). This compound was isolated by preparative plate chromatography, and analysis indicated that it contained 0.98 μmol of DNP per μmol of phosphate. Therefore, in the strictest sense, the term 'bound' DNP should be reserved for this compound which represents DNP-lysine that has become covalently attached to the liposomal bilayers via NHS-sub-DMPE. It need be added that the observed stoichiometry is consistent with the reaction scheme shown in Fig. 1, and thus provides indirect support for the structure of NHS-sub-DMPE.

Quantitative aspects of DNP-lysine binding to liposomes

In view of the above results, the experiment described in Fig. 3 may be analyzed further. The data indicate that 1.302 μmol of DNP-lysine had become associated after 24 h with liposomes that contained 2.312 μmol of NHS-sub-DMPE (the amount added to obtain a density of 20 mol%). If this value is reduced by 10% to correct for absorbed DNP-lysine, the quantity of covalently bound DNP is 1.17 μmol. Accordingly, approx. 50% of the NHS-sub-DMPE (i.e., $100 \times (1.17/2.312)$) has reacted with DNP-lysine to form the new dinitrophenylated compound. This value agrees favorably with similar calculations previously applied to liposomes prepared with NHS-succ-Chol, and is also consistent with the earlier conclusion that a significant portion of the DNP-lysine has become covalently bound to inner liposomal bilayers [2]. This conclusion was based on the assumption that 50% of the NHS derivative could not be located in the external half of the outermost lipid bilayer, and raises the possibility that the kinetics (Fig. 3) and temperature dependence (Table II) of binding may be at least partially determined by the permeability of liposomes to DNP-lysine.

Retention of liposomal capacity to bind DNP-lysine

Complete conversion of NHS-sub-DMPE to the new dinitrophenylated compound would not be expected because of the sensitivity of NHS esters to hydrolysis. This bears on the observation that preformed liposomes lost binding capacity if they were stored for only 24 h before the addition of DNP-lysine. This phenomenon was studied in some detail because of its relevance to the potential applications of these liposomes. Table III shows that binding capacity was rapidly reduced when the liposomes were stored at pH 8 at temperatures of 4°C or above. Even frozen liposomes suffered a slight diminution in their ability to bind DNP-lysine. Because hydrolysis of NHS esters is favored

TABLE III

DNP-LYSINE BINDING TO LIPOSOMES AFTER STORAGE

In Expt. 1, liposomes with 10 mol% NHS-sub-DMPE were prepared by dispersing the dried lipid film in DPBS (buffer 1), pH 8 (the standard procedure). In Expt. 2, the liposomes were prepared in 50 mM sodium acetate-150 mM NaCl, pH 4.5. After storage for 24 h at the indicated temperatures, an equivalent volume of 10 mM DNP-lysine (in DPBS, pH 8) was added to the liposomes in Expt. 1. An equivalent volume of 10 mM DNP-lysine (in 10X DPBS, pH 11) was added to the liposomes in Expt. 2 so that the final pH was also 8. In both experiments, DNP-lysine bound was determined after 24 h incubation at 37°C, and corrected for the amount of DNP associated with control liposomes containing 10 mol% succ-DMPE. This nonspecific binding was unaffected by storage (results not shown). Values in parentheses indicate the percentage of DNP-lysine bound compared to liposomes that were not stored (i.e., incubated with DNP-lysine immediately after preparation).

Storage temperature (°C)	μmol DNP bound/ml liposomes; storage pH	
	8.0 (Expt. 1)	4.5 (Expt. 2)
Not stored	0.635(100)	0.788(100)
−20	0.586 (92)	0.713 (90)
+4	0.245 (38)	0.522 (66)
+23	0.061 (10)	0.322 (41)
+37	0 (0)	0.262 (33)

TABLE IV

IMMUNOGENICITY OF LIPOSOMES

Liposomes were prepared with the indicated derivative at a density of 5 mol%, without and with 0.4% lipid A. Mice were immunized with either 25 nmol of free DNP-lysine (liposomes made without any derivative), 25 nmol of bound DNP-lysine (liposomes made with NHS-sub-DMPE), or 25 nmol of DNP-Cap-PE (incorporated into liposomes). n.d., not determined.

Derivative incorp. into liposomes	Lipid A added	TNP-PFC per 10^6 spleen cells		
		Expt. 1	Expt. 2	Expt. 3
None	−	13 ± 9	n.d.	6 ± 4
	+	17 ± 4	38 ± 7	15 ± 5
NHS-sub-DMPE	−	84 ± 23	n.d.	75 ± 8
	+	1440 ± 314	858 ± 99	635 ± 136
DNP-Cap-PE	−	n.d.	n.d.	108 ± 44
	+	n.d.	988 ± 93	537 ± 103

under alkaline conditions, it is also noteworthy that the reduction of binding capacity could be retarded (but not eliminated) by lowering the pH of the liposomal suspension from 8 to 4.5.

In contrast to the above results, periodic chromatographic analysis revealed that NHS-sub-DMPE was stable to storage at −20°C in chloroform/methanol (1 : 1, v/v) for at least one month. Stability was confirmed by the fact that liposomes, which were freshly prepared with the stored compound, retained fully their ability to bind DNP-lysine if they were incubated with the latter immediately after generation (data not shown).

Immunogenicity of liposomes with bound DNP-lysine

As noted in the Introduction, our interest in the NHS esters of phosphatidylethanolamine evolved from the observation that liposomes, which had bound DNP-lysine via NHS-succ-DPPE, were very effective immunogens [2]. However, NHS-sub-DMPE contains four more methylene units in the 'spacer' than NHS-succ-DPPE. Therefore, it was considered essential to determine if liposomes, to which DNP-lysine was covalently attached via NHS-sub-DMPE, were also immunogenic. Table IV shows that these liposomes can not only induce hapten-specific plaque-forming cells, but that they do so at frequencies comparable to those elicited by liposomes which had been sensitized by DNP-Cap-PE. This was observed regardless of whether or not the B cell mitogen, lipid A, had been incorporated into the liposomes to enhance the response.

Discussion

Using the synthetic procedure described in this paper, pure NHS-sub-DMPE has been obtained in 3-fold higher yield than NHS-succ-DPPE prepared as previously described (see Introduction). This difference most likely reflects the fact that NHS-sub-DMPE was synthesized under homogeneous conditions, whereas the synthesis of NHS-succ-DPPE was carried out in a solvent mixture in which all of the reactants did not remain in solution [2]. It should be noted that NHS-sub-DPPE has also been prepared by the new procedure. For the purposes of this investigation, these NHS derivatives were made, in part, because of the commercial availability of DSS. However, other disuccinimidyl esters can also be purchased or synthesized by the method of Hill et al. [6]. Thus, the present method could be employed to synthesize numerous NHS esters of PE that differ in the length of the spacer between the NHS function and the PE amino group. The importance of spacer length is discussed below.

In the preceding paper [2], it was shown that liposomes, which contain NHS-succ-Chol, can covalently bind DNP-lysine in a reaction that is dependent on incubation time and the amount of NHS-succ-Chol incorporated into the liposomes.

With the availability of adequate quantities of NHS-sub-DMPE, we have been able to confirm these observations and to characterize the product formed in the reaction between DNP-lysine and the NHS derivative. The present investigation also has revealed two other aspects that bear on the applicability of liposomes prepared with such NHS derivatives: (a) DNP-lysine binding is markedly influenced by incubation temperature, and (b) the capacity to bind DNP-lysine is rapidly lost upon storage of the liposomes. Thus, these liposomes should be used immediately after preparation.

A number of potential uses for liposomes containing lipophilic NHS derivatives have been previously mentioned [2]. These include the preparation of immunogenic liposomes that bear, not only covalently attached haptens (such as DNP-lysine), but also peptide and protein determinants. In this regard, reference need be made to a previous investigation [7] in which liposomes were rendered immunogenic by the incorporation of various synthetic dinitrophenylated phosphatidylethanolamine derivatives prepared with spacers of different length. These experiments indicated that liposomal immunogenicity was dependent on an optimum distance separating the determinant (DNP) from the hydrophobic anchor (PE). Thus, we found that liposomes, which had been sensitized with either DNP-PE (no spacer) or DNP-lauryl-PE (C_{12} spacer) were nonimmunogenic, in contrast to the maximum response induced by liposomes sensitized with DNP-Cap-PE (C_6-spacer). It is therefore significant that liposomes, which contain DNP-Cap-PE, and liposomes, which contain DNP-lysine bound via NHS-sub-DMPE, are equally immunogenic.

Preliminary experiments have also revealed no difference in the immunogenicity of liposomes in which DNP-lysine was attached to the bilayers with either NHS-sub-DMPE or NHS-sub-DPPE. A difference might be expected if the acyl chain length of the anchor has a significant effect on the distance between the hapten and the liposomal surface. However, it remains to be determined whether an effect could be demonstrated using NHS esters of PE that contain fatty acids shorter (or longer) than myristate and palmitate, and/or shorter spacers.

Additional experiments (manuscript in preparation) have shown that NHS-sub-DMPE can be used to make liposomes that are covalently bound in vitro to various cells (e.g., mouse B and T lymphocytes, fibroblasts). Studies are now underway to determine whether such attachment may facilitate the transfer of materials from the liposomes to the cells.

Acknowledgements

This research was supported by National Instituted of Health (USA) grant AI-15796. We thank Beverly Polt for preparation of the manuscript, and Barry Silverstein and Kurt Tidmore for the illustrations. One of us (S.C.K.) is a Catherine Kramer Foundation Scientist in Pediatrics.

References

1 Kinsky, S.C., Loader, J.E. and Benson, A.L. (1983) Fed. Proc. 42, 931
2 Kinsky, S.C., Loader, J.E. and Benson, A.L. (1983) J. Immunol. Methods 65, 295–306
3 Gerlach, E. and Deuticke, B. (1963) Biochem. Z. 337, 477–480
4 Gitler, C. (1972) Anal. Biochem. 50, 324–325
5 Lapidot, Y., Rappoport, S. and Wolman, Y. (1967) J. Lipid Res. 8, 142–145
6 Hill, M., Bechet, J. and d'Albis, A. (1979) FEBS Lett. 102, 282–286
7 Dancey, G.F., Isakson, P.C. and Kinsky, S.C. (1979) J. Immunol. 122, 638–642