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Inventor Eddie L. Chang

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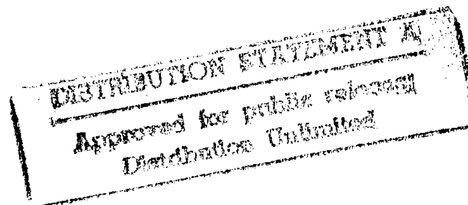

# USE OF LIPID LIPOSOMES AS CARRIERS FOR DELIVERY OF ORAL VACCINES

## FIELD OF THE INVENTION

The present invention relates to the usage of *sn*-2,3 and *sn*-2,3,2',3' ether lipids, particularly bipolar tetraether lipids, as carriers of antigens in oral vaccines.

## BACKGROUND OF THE INVENTION

Immunization is established when the immune system is challenged with a foreign antigen and the system reacts with cellular changes and antibody production that lead to a permanently heightened pattern of chemical and cellular defense (immunity) against the antigen. Although natural immunity comes from exposure to bacteria and viruses introduced through a variety of mechanisms, vaccination is usually done parenterally. Parenteral vaccination confers systemic immunity and is, therefore, the obvious immunization route for diseases that enter parenterally, such as tetanus and malaria. However, there are many diseases, such as cholera and typhoid fever, that enter through the mucosal membranes (oral, nasal, and genital) for which systemic immunity is not efficient or effective in inducing mucosal immunity. (1, 2) For these diseases, the oral route for vaccination is preferable. The mucosal membranes are also a major route of infection for the HIV virus.

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Therefore, a complete HIV vaccine program when available should also include protection of the mucosal system. Oral vaccines can be intrinsically safer, easier to use, and better tolerated. They also would not require the
5 same level of purity and booster doses can be easily administered as often as needed. In addition, diseases that occur in the gut and are poorly controlled by parenteral vaccinations may respond better to oral vaccines.

10 Despite these advantages, oral immunization is not presently commonly used. One reason for the secondary role played by oral immunization is that, until recently, it was thought that mucosal membranes obtained antibodies solely through the serum. It is now
15 recognized, however, that the mucosal system can produce its own antibodies, the secretory immunoglobulin A (sIgA), and that, in fact, there is a common mucosal immune system linked by migrating IgA precursor-cells. Thus, when immunogenic products are introduced through
20 the gastrointestinal route, subsequent sIgA production can be found in various secretory places in the body including intestinal, salivary, bronchial and lachrymal sites. (3) A major inductive site for mucosal immune response is the gut-associated lymphoid tissue (GALT)
25 which occurs along the intestinal tract and is composed mainly of lymphoid aggregates called Peyer's patches. Antigens are currently thought to be absorbed by specialized cells (M cells) in the epithelium of the GALT. The uptake of antigens by these M cells initiates
30 the response by the GALT.

Oral vaccination was first utilized in this century when Bacillle Calmette-Guerin (BCG), a nonvirulent mycobacterium, was used to immunize against tuberculosis in 1921. (4) BCG has been used

extensively since, although oral administration was discontinued in 1976. The major problem with oral BCG was its high pH-sensitivity. Much of the BCG was inactivated by the stomach pH, resulting in a loss of
5 activity by several orders of magnitude. (5) Thus, ten to one-hundred fold more bacilli had to be given orally than intradermally. The situation with BCG is typical of the problems that oral vaccine formulations must overcome. Unprotected vaccines given orally are
10 degraded, poor absorption of antigens occurs, and delivery to the GALT is inefficient.

The potential of using liposomes either as a delivery agent for oral administration of drugs or as an adjuvant to increase absorption of poorly-absorbed drugs
15 in the gastrointestinal tract (6) has been recognized. Patel and Ryman (7) and others (8) demonstrated that, in diabetic rats, orally administered insulin can be as effective in decreasing blood-glucose levels as insulin given intraperitoneally. Liposomes have also been
20 studied as delivery vehicles for both general vaccines,(9) as well as oral vaccines. (7,10,11) For example, it has been shown that cholera toxin (CT) attached to liposomes was effective in both neutralizing the CT toxicity as well as in raising high antibody
25 titer. (12)

Oral liposome vaccines have been introduced by intubation or within capsules with generally encouraging results. In one case, four human volunteers were administered oral doses of encapsulated liposomal antigen
30 from Streptococcus mutans -- a probable agent in the initiation of dental caries -- with subsequent detection

of salivary IgA production. (13) However, it is generally recognized that liposomes may only give partial protection to the antigens during its passage through the stomach (1) and that the limited stability of liposomes leads to short lifetimes with respect to both retention in the gut and shelf life. Furthermore, there is uncertainty as to whether liposomes are taken up by the epithelial cells. But recent positive results from an ultrastructural study of liposome uptake by M cells of rat Peyer's patches (11) give some confidence that liposomes can be taken up by M cells. The intrinsic stability and resistance of tetraether liposomes to degradation can address the first two concerns.

Stability against lipases and bile salt detergents, as well as acid stability, is important in the design of an effective oral carrier. Rowland and Woodley (14) investigated a broad selection of lipids and the stability of the liposomes formed from the various lipid mixtures against pH, bile salts, and pancreatic lipase. It was found that most lipid mixtures were fairly stable against pH as low as pH 2, but only the mixed lipid composition of distearoyl phosphatidylcholine (DSPC)/cholesterol (7:2) was also stable against 10 mM bile salts as well as pancreatic lipase. Chiang and Wiener (10) confirmed these findings but also reported that a combination of lipase and bile salt had a synergistic destabilizing effect on the DSPC/cholesterol mixture. 80% of the internal contents were released within 5 minutes of exposure to the lipase/bile salt combination. Thus, while added cholesterol appears to protect liposomes against bile salt disruption or lipase

degradation alone, detergent and lipases can work in concert to quickly degrade the diacyl liposomes.

The semi-permeable nature of the membranes to protons is not a generally recognized property of lipid membranes. However, as long as membrane stability of liposomes is preserved, the maintenance of a neutral internal pH can be solved. It is known that lipid membranes are highly permeable to protons. Proton-hydroxyl permeability at neutral pH is around $10^{-3}$ cm/s as compared with $10^{-11}$ to $10^{-14}$ cm/s for chloride, potassium, and sodium ions. (15) This large proton permeability would quickly bring the internal pH of vesicles to stomach acidity were it not for the presence of much slower diffusing sodium and chloride ions. Thus, after an initial flux of protons, further permeation of $H^+$ will be slowed considerably by the creation of a counterion gradient. Estimates, based on neutral pH permeability values (16) show that with buffering to ameliorate the effect of the initial proton flux, the liposome interior pH does not have to be adversely affected by $H^+$ penetration.

## SUMMARY OF THE INVENTION

The *sn*-2,3 and *sn*-2,3,2',3' ether lipids used in the invention, with either one or two polar headgroups, are derived from archaebacteria and are distinctly different from the more common monopolar phospholipid. Archaebacterial lipids form very stable, low-permeability

liposomes and are acid- and lipase-resistant. The stability of liposomes formed from archaebacterial lipids makes them ideal for carrying antigens through the stomach and the duodenum into the intestines. The
5 protected antigens can then, either by themselves or in concert with the liposomes and other adjuvants, cross the intestinal epithelium to stimulate gut-associated lymphoid tissues (GALT), such as Peyer's patches, to produce an immune response.

10 The ether liposomes make ideal carriers for oral vaccine delivery because they have low permeability, are thermally stable, and more chemically resistant to normal lipid degradation processes. Adjuvants can also be added to the lipid membranes to enhance the
15 immunogenicity of the encapsulated antigens. Furthermore, the stability characteristics of these liposomes are also advantageous with respect to long shelf life.

Unlike previously described liposomes, the
20 ether lipid liposomes prepared according to the invention are not susceptible to the double-edged attack of lipases and bile salt because they lack the bonds attacked by pancreatic lipase and phospholipases. (17) Pancreatic lipase is specific for catalyzing the hydrolysis of acyl
25 moieties at the 1- and 3-positions of glycerides. Phospholipases A1, and A2 attack the acyl C-O bond at the C1 and C2 positions respectively. Phospholipase B sequentially deacylates C1 and C2. None of these positions is available on tetraether lipids. On the
30 other hand, phospholipases C and D cleave at either side of the phosphorous atom in the phosphate headgroup of C3.

However, the odd stereochemistry of tetraethers (*sn*-2,3 rather than *sn*-1,2) locates the phosphate group of PLFE and P2 lipids at C1. This positional difference substantially prevents hydrolysis of the phosphate
5 headgroup. In any case, even if some hydrolysis were to occur, the presence of ether bonds significantly slows down the rate of hydrolytic action of phospholipases. (17)

An additional desirable feature of tetraether
10 liposomes is that they appear to have intrinsically lower $H^+$ permeability than normal lipid membranes.

The advantages of using tetraether liposomes for oral delivery of vaccines are due to the unique chemical structure of these molecules. These differences
15 give rise to liposomes that are:

- stable in acidic solution
- not susceptible to most phospholipases
- less easily attacked by other kind of lipase
20 - low in membrane permeability

Among the novel features that are significant include the *sn*-2,3 ether linkages of the alkyl chains to the glycerol backbones and the bipolar nature of the molecules. The ether bonds are more stable against
25 general chemical attack as well as against biochemical deacylating lipases. The stereoconfiguration of the ether lipids further provide protection against stereospecific biochemical degradation. The liposome

have low permeability while the bipolar structure of the tetraethers endow the liposomes with high thermal stability. The general effect of all the structural features is to create a liposomal delivery system that is
5 distinctly more rugged than any other lipid system.

Other features and advantages of the present invention will become apparent from the following description of the invention.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

10 Phospholipids are the major structural materials of lipid membranes. These lipids are amphiphilic molecules with two hydrophobic acyl chains linked to a glycerol backbone through ester bonds on carbons 1 and 2 (*sn*-1,2) of the glycerol. The two major
15 classes of phospholipids are phosphodiglycerides and sphingolipids. (17) *Sn*-1,2 phospholipid that contain either one or two ether bonds (as opposed to ester bonds) are also known. (18,19) Their properties are similar to the diesters except the diether lipids readily form
20 interdigitated phases. (20,21) Procedures for formation of liposomes (the term liposomes will be used interchangeably with vesicles, multilamellar vesicles (MLV), large unilamellar vesicles (LUV), or small unilamellar vesicles (SUV) -- all terms essentially
25 denote a lipid superstructure that entraps a definite internal volume) are well-known and can readily be found in the literature (see New) (22) for a comprehensive survey).

Ether lipids that have hydrocarbon chains attached to carbons 2 and 3 (instead of *sn*-1,2) of the glycerol backbone constitute a further distinct category of amphiphiles. These lipids are found exclusively in membranes of archaebacteria. Archaebacterial lipids are iconoclastic molecules that differ in many fundamental respects from the *sn*-1,2 phospholipid. (23) In addition to the *sn*-2,3 ether bonds, the alkyl chains of these lipids are highly branched with methyl groups regularly spaced along the chains (i.e., general form of 2,3-di-O-phytanyl-*sn*-glycerol). Diether *sn*-2,3 lipids with one polar headgroup are mostly associated with halophilic and methanogenic archaebacteria. (23,24) They make up 100% of the halophilic archaebacterial polar amphiphiles and between 50% to 100% of the methanogenic archaebacterial lipids (except for <u>Methanothermus</u> <u>fervidus</u> strain V245 which has only 14% diether lipids). Further, most of the lipids of thermophilic archaebacteria possess two polar headgroups, that is, they have two glycerol (or a glycerol and a nonitol) backbones linked to either ends of two long hydrocarbon chains by ether bonds. These bipolar lipids can be looked on as having a basic structure of 2,3,2'3'-tetra-O-dibiphytanyl-di-*sn*-glycerol and make up close to 100% of the polar lipids of the thermophiles. A summary of the most salient features of these bipolar lipids that distinguish them from "normal" lipids is given below:

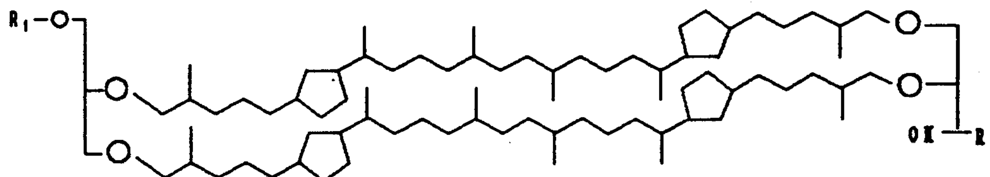
- The linkage between the polar backbones and the hydrocarbon chains are ether rather than ester bonds.

- There are two polar headgroups for each bipolar lipid--making these tetraether lipids.
- The hydrocarbon chains are bidiphytanyls, 32 carbons long with a total of 40 carbons per chain.
- The hydrocarbon chains are linked *sn* 2,3 to the glycerol moieties rather than *sn* 1,2.
- There are no unsaturated bonds but some tetraethers may have several cyclopentane rings along the chains.

The ether linkages, and *sn* 2,3 stereoconfiguration are of special importance for liposomal carriers of oral vaccines and drugs. The bipolarity of the tetraethers is a further stabilizing feature. As is discussed below, these characteristics render the diether and tetraether liposomes uniquely resistant to digestive chemicals in the gastrointestinal tract. For convenience, the remaining discussion will concentrate on describing the tetraethers as they are less well known than the archaebacterial diether lipids. (25) It will be understood, however, that the discussion is equally applicable to the diethers and distinctions between the two will be noted when relevant.

For certain archaebacterial species, such as Sulfolobus acidocaldarius, one of the two headgroups is attached to the more complex nonitol (rather than a glycerol) backbone. Other species (e.g., Thermoplasma acidophilum) only have the symmetric form with a glycerol

on each end. (26) Hydrolysis of the polar headgroups from lipids of S. acidocaldarius yields just two basic forms of tetraether lipid structures. These two forms, the symmetric glycerol dialkyl glycerol tetraether (GDGT) and the asymmetric glycerol dialkyl nonitol tetraether (GDNT), are the basic skeletal frame for the lipids of this microorganism:




| Compound | $R_1$ | $R_2$ |
|---|---|---|
| a | H | H |
| b | H | ß-D-glcp-ß-D-galp |
| c | inositol-P | H |
| d | inositol-P | ß-D-glcp-ß-D-galp (PLE) |

| Compound | $R_1$ | $R_2$ |
|---|---|---|
| e | H | H |
| f | H | ß-glcp |
| g | H | ß-D-glcp-sulfate |
| h | inositol-P | ß-glcp (PLE) |

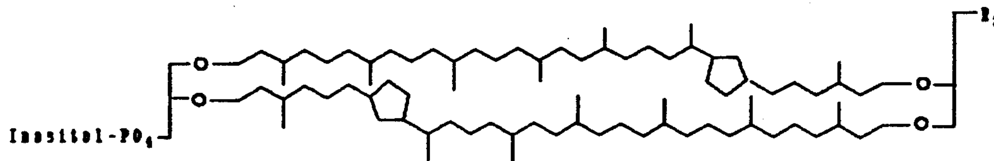
where glcp = glucopyranose, galp = galactopyranose, and P = phosphate. Each canonical structure can have, in turn, zero to eight cyclopentane rings in the hydrocarbon chains. A typical breakdown of the cyclopentane
5 distribution for S. sofataricus grown at 87°C is:

GDGT: (2,2) = 20%, (3,3) = 29%, (2,1) = 6%, (3,2) = 41%, (3,4) = 4%
GDNT: (2,2) = 18%, (3,3) = 30%, (3,2) = 46%, (3,4) = 6%

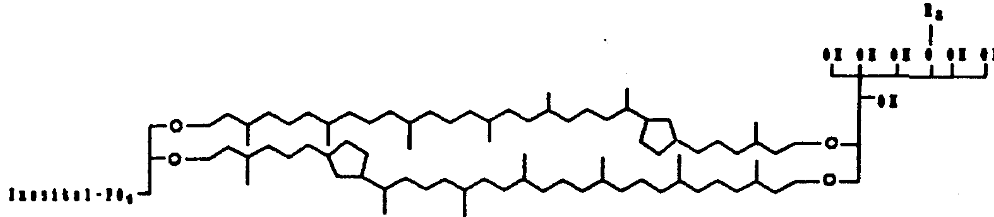
where the numbers in parentheses refer to the number of rings on chain 2 and 3, respectively. (27) Diether
10 archaebacterial lipids do not have cyclopentane rings.

The native unhydrolyzed tetraether lipids are, in addition, different in their headgroups. Lipids found in the biomembrane of S. acidocaldarius include the following: glycolipids, phosphoglycolipids, and
15 sulfolipids. The different lipid classes can be separated by elution with solvents of increasing polarity. At present, there is no consistent nomenclature in the literature. PLFE will therefore be use hereafter to designate the native polar lipid
20 fraction E of S. acidocaldarius (called PLE by Langworthy, (28) and analogous to P2 lipids from S. solfataricus (29)). PLFE contains phosphatidylmyoinositol glycolipids with both GDGT and GDNT structures.

25 GDGT



GDNT



$R_1$ = ß-D-glcp-ß-D-galp and $R_2$ = ß-glcp. The number of cyclopentane rings can vary from none to eight rings per lipid. The phosphatidylmoinositol is linked to a glycerol backbone; the other polar head is either ß-D-glucopyranose attached to a second glycerol backbone (the GDGT form), or ß-D-galactopyranosyl-ß-D-glucopyranose linked to nonitol (the GDNT form). In addition, there can be anywhere from zero to eight cyclopentane rings along the chains for each PLFE lipid. Thus, PLFE is a mixture of similar lipids that differ in structure in terms of either having a GDGT or GDNT form of the tetraether (with a concomitant difference in the nature of the polar groups), or in the number of cyclopentane rings along the hydrocarbon chains.

Liposome formation from diether *sn*-2,3 lipids follow standard procedures (30,31,32), although some problems have been reported when using basal salt solutions. (33) The formation of liposomes from archaebacterial lipids possessing two polar headgroups attached to either each end of the hydrocarbon chains has only recently been demonstrated, first with the main

polar lipids (MPL) of Thermoplasma acidophilum (8) and later with the polar lipid fraction E fraction of S. acidocaldarius. (34) The lag between the discovery of these lipids and successful formation of liposomes from
5 them can be attributed to the inadequacy of the originally published methods for obtaining bipolar lipids that are pure enough for physical characterization (see for example, refs. (24, 35). The archaebacterial lipids obtained by these earlier methods contained a significant
10 amount of impurities that were unresolvable by ordinary TLC. The impure lipids also did not disperse in water. For example, experience from our laboratory showed that extracts of the total lipids, of the total polar lipids, and of the hydrolyzed fractions did disperse
15 spontaneously in aqueous media. The hydrolyzed tetraether glycerol dialky nonitol tetraether (GDNT) formed a suspension only when injected from warm solvent into hot aqueous water. (36) But it was not clear whether the GDNT formed a suspension of liposomes or
20 simply solid droplets of lipids.

Hydrolyzed tetraethers, however, can be mixed with regular lipids to form liposomes. (37, 38) It is interesting that the only two kinds of tetraethers (MPL and PLFE) that form liposomes both contain a negatively
25 charged headgroup on one end and a polar carbohydrate on the other. This type of structure may be a general requirement for liposome formation by tetraethers.

Multilamellar PLFE liposomes can be easily formed by dispersing the lipids in aqueous media.
30 Neither the ionic strength nor the pH appears to affect greatly the lipids' ability to disperse. For making smaller, size-defined PLFE liposomes, extrusion through

defined, submicron, pore-sized membrane filters work best. Though PLFE does not possess a melting transition from a gel to a liquid-crystalline state, the lipid is gel-like in its rigidity below 50°C and extrusion is facilitated when performed above 55°C. Liposomes also can be formed using other tetraether lipids mixtures as long as the geometric constraints for liposome packing are obeyed. (37) Mixtures of bisubstituted and monosubstituted tetraethers (37) or mixtures of tetraethers with monopolar lipids can also disperse into liposomes. (37,38) In the former case, a mixture of the total polar lipid extract with P2 in the ratio of 1:1 produced vesicles with radius of 45 nm, while in the latter example mixtures of phosphatidylcholine/total polar lipid extract or phosphatidylcholine/GDNT produce closed vesicles.

Extraction and purification procedures for diether lipids can be found in the handbook by Kates. (25) Methods for obtaining some bipolar tetraether lipids have been published. (34,37) An example of the steps for obtaining pure GDGT from S. acidocaldarius are described below:

*Growth of Cells: S. acidocaldarius* (ATCC strain 33909, Rockville, MD) was grown at 65-67°C in 20L glass carbuoys containing 15L at ATCC medium 1256 supplemented with 1 g/L glucose. (39) Air was bubbled through the aqueous medium at 1L/min and growth monitored by absorbance at 420 and 540 nm. Cells were harvested by centrifugation at the onset of stationary phase, lyophilized, and stored at -20°C until used. Average yield was 300-400 mg dry cells/L culture.

*Extraction of Crude Lipids for Hydrolyzed Lipids:* The procedure follows the protocol described in Lo et al., 1989. (38)

*Isolation and Purification of GDGT.* Thin layer
5 chromatography was carried out on LK5F or PLK5F plates (Whatman, Clifton, NJ) using the following solvents: A, hexane/isopropanol/chloroform (5:1:0.3, v/v/v); B, toluene/acetone (8:2, v/v). Lipids were detected by exposure to iodine vapors or by phosphomolybdic acid.
10 (25)

The GDGT-containing fraction from the crude lipids was identified by TLC to be the diethyl ether fraction using a standard sample of GDGT, provided by Dr. T. Langworthy. (28) A 12 mg sample of this crude
15 GDGT was suspended in hexane. The hexane suspension was washed with 10% methanol in water solution. After standing overnight, the upper phase was withdrawn and any insoluble material was removed by centrifugation. After drying, the residue was dissolved in 1.5 mL of dried
20 pyridine and 0.5 mL benzoyl chloride. The mixture was warmed at 60°C for 2.5 h and then the pyridine removed under a stream of air until the sample was almost dry. Water was added to the moist residue and the pH adjusted to >13 with 10 N NaOH. The benzoylated GDGT was
25 recovered by three extractions with hexane, then the pooled hexane was washed once with water. The benzoyl derivative was purified by TLC on LK5F plates (10 mg lipids per plate) employing solvent (A) as developing solvent ($R_f$ = 0.5). The lipid bands on the plate were
30 located using UV. A wide band corresponding to benzoyl

GDGT was scraped and eluted with hexane/diethyl ether (1:1, v/v).

The benzoylated intermediate obtained as described above was dissolved in a 3 mL mixture of
5 tetrahydrofuran/1 N NaOH (2:1, v/v) and the solution stirred for 42 h at 70°C. After the evaporation of tetrahydrofuran under a stream of nitrogen, the GDGT was extracted with hexane and purified on LK5F plates employing solvent system B as the developing solvent ($R_f$
10 = 0.6). The bands corresponding to GDGT were scraped and eluted with diethyl ether. The oily residue, after drying, was dissolved in acetone and filtered by a Millex-HV filter. The acetone filtrate was allowed to stand overnight at -10°C to precipitate the GDGT. The
15 precipitate was then collected by centrifugation. TLC of the acetone-precipitated product on LK5F plates developed with solvent B produced a single spot that co-migrated with a standard sample of GDGT.

*Alternative Conversion of GDNT to GDGT*. GDNT (see
20 procedure below) was eluted with the chloroform/methanol (8:2, v/v) fraction described Lo et al. (38) 100 mg of GDNT from this fraction was dried, dissolved in 1 mL tetrahydrofuran along with excess $NaIO_4$ (~ 100 mg), and stirred in the dark for 18 h. After quenching with 0.5
25 mL of 10% glycerol in water, the tetrahydrofuran was removed under a stream of nitrogen and the mixture extracted with chloroform/diethyl ether (1:1, v/v) to obtain the aldehyde. The recovered aldehyde was reduced with 500 mg $NaBH_4$ in 1.5 mL of tetrahydrofuran/0.001N
30 NaOH (1:1, v/v) at room temperature and stirred overnight. Excess $NaBH_4$ was quenched with 1 N HCl and the reduced products, extracted with chloroform/methanol

(2:1, v/v), was isolated by TLC (LK5F) using solvent system B. The GDGT band was scraped and extracted with diethyl ether. Acetone precipitation produced a final product which co-migrated with the standard GDGT and had 5 identical IR spectra.

*Isolation and Purification of GDNT and PLFE*. The procedures used are as described in Lo, et al (38) and Lo and Chang (34).

*Preparation of Liposomes*. Liposome formation from diether sn-2,3 lipids follow standard procedures (30,31,32) although 10 some problems have been reported when using basal salt solutions in the past. (35) These problems may have been due to purity problems similar to that encountered with PLFE lipids before Lo and Chang. (34) They are no longer a probledm. PLFE lipids, prepared as described above, or 15 tetraether-diacyl lipid mixtures, as described below, disperses easily under a variety of conditions. Multilamellar liposomes (MLV) can be formed by the usual means and under a wide variety of conditions. For example, PLFE can be dried down in test tubes with argon 20 and then further dried overnight in a vacuum oven. Distilled water (pH 6.5) or aqueous buffer (for example, HEPES buffer at concentrations of 0.05, 0.1, 0.2, 0.5, and 1 M are commonly used) can then be used to disperse the lipids at whatever concentration required for the 25 application. The aqueous samples should be warmed at 60°C for 1/2 h and then vortexed, or allowed to stand for several hours to overnight at room temperature to form MLV dispersions. In some instances, mild sonication, in addition to the vortexing may be necessary to make all 30 the lipids come off the walls of the test tube.

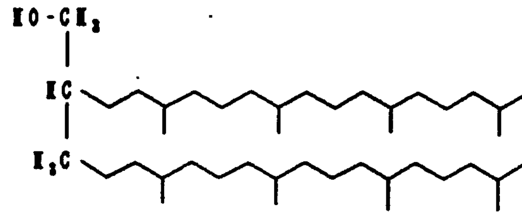
Better size-defined vesicles of fewer lamellae may be made by taking the MLV and freeze-thawing the liposomes several times. This process tends to enhance the encapsulation efficiency. The freeze-thawed MLV can
5 then be extruded, either manually with a syringe, or with a commercially-available high pressure apparatus. Extrusion is facilitated if the temperature of the suspension can be kept close to 60°C. Other standard methods of making vesicles can also be used. (22)

10 Other formulations of tetraether liposomes can also be used. The P2 fraction of polar lipids from S. solfataricus, PLFE (or P2) mixed with GDGT and GDNT, or GDNT mixed with dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPG) are all examples of
15 alternate formulations. General estimates of effective mixture-ratio ranges can be determined by using the relationship between lipid parameters and packing as developed by Israelachvili and co-workers. (40,41) The molecular parameters are taken from the x-ray data of
20 Gulik, et al. (42) Tetraether MLV dispersions can also be produced from mixtures of the polar lipid extract (PLFE) of Sulfolobus solfataricus with the P2 fraction from the same organism. (24) Mixtures of tetraether lipids with regular phospholipid can also form MLV.
25 Procedures for these mixtures can be found in Lelkes, et al. (43) and Cavagnetto, et al. (37)

Other kinds of tetraether lipids may be synthesized from the hydrolyzed substrates GDNT and GDGT to attach different polar headgroups to the OH groups in
30 GDNT and GDGT. Standard chemical coupling strategies can be used to perform the modifications. Diether lipids can also be synthesized to obtain different headgroups or

other characteristics. (44) Examples of diether lipids that have been synthesized include

1,2-di-O-diphytanyl-sn-glycerol



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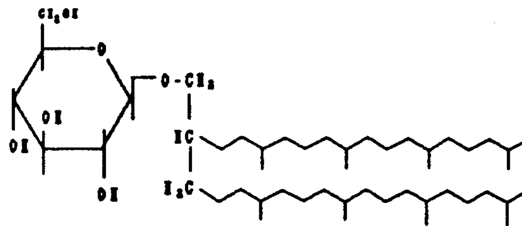
and

1,2-di-O-diphytanyl-3-O-b-D-glucosyl-sn-glycerol. (45)



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Adjuvants can also be added to the liposomes. An adjuvant is considered to be any substance that can enhance the immunogenicity of an antigen, or synergistically enhance the antigen's effect beyond any
15 level expected from the antigen and adjuvant alone. The protection afforded by liposomes to antigens can be considered one form of adjuvancy. As another example, lipid A introduced into liposomal membrane has been shown to be a good adjuvant. Antigenicity of nearly any
20 protein can be enhanced in this manner. (12) Toxicity of the antigen itself can sometimes also be neutralized, without adversely affecting its antigenicity, as in the case where cholera toxin is bound to liposomes through ganglioside $G_{M1}$. Descriptions of various adjuvants can
25 be found in the literature. (12,45,46)

*Encapsulation of Antigens.* Encapsulation of antigens in liposomes is done as follows. Lipid is dried down on the bottom of a flask and the antigen in solution form is introduced. The concentration of antigen will, of
5 course, depend on efficacy of a particular immunogen and whether adjuvants are used. However, it has been shown that concentrations as low as 0.5 to 1.0 mg/mL antigen is enough to raise antibody titer. (11) The solution will have to be buffered as discussed above. Biological
10 buffers are suitable, or a simple solution such as 1.88% sodium bicarbonate can also be used. Lipid concentration is around 2-5 μmol/mL. Testing in animal models can be accomplished with less than 1 micromole lipid per animal, while for adult humans 8 mg/mL lipid is sufficient.

15 Although the present invention has been described in relation to particular embodiments thereof, many other variations and modifications and other uses will become apparent to those skilled in the art.

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# USE OF LIPID LIPOSOMES AS CARRIERS FOR DELIVERY OF ORAL VACCINES


## ABSTRACT OF THE DISCLOSURE

Liposomes composed of sn-2,3 and sn-2,3,2',3' ether lipids, preferably bipolar tetraether lipids, are used to encapsulate antigens for the preparation of oral vaccines. The solution containing the antigen to be encapsulated can be a buffered solution.