

bis p-nitrophenyl esters of dicarboxylic acid, aromatic disulfonyl chlorides and bifunctional arylhalides such as 1,5 difluoro-2,4-dinitrobenzene and p,p-difluoro -m,m-dinitro-diphenylsufone. Also diimide esters, aromatic and aliphatic diisocyanates, metamaleimidobenzoyl n-hydroxysuccinimide, (MBS), or n-gamma-maleimidobutyryl-oxysuccinimide (GMBS).

Endogenous complement activity can be prevented by the addition of the sulfonic acid azo dye "chlorazol fast pink" which inhibits human complement, or the serum can be heated before use for 30 to 60 minutes at 58° C.

There are a number of approaches to binding antibody to liposomes as illustrated by the following references which are expressly incorporated herein:

Magee and Miller, *Nature* 235:339-341 (1972), describe rehydrating dried lipid films in the presence of antibody preparations. The antibody becomes liposome associated through a combination of hydrophobic and ionic interactions.

Huang and Kennel, *Biochemistry* 18:1702-1707 (1979), describe the co-sonication of multilamellar vesicles with antibodies. The extent of binding increases by the inclusion in the phosphatidylcholine liposomes of anionic phospholipids and depends on the time and power intensity of sonication.

Published reports by Heath et al *Biochim. Biophys. Acta* 599:42-62 (1980); Martin et al *Biochemistry* 20:4229-4238 (1981); and Dunnick et al *J. Nucl. Med.* 16:483-487 (1975), describe methods for covalently coupling proteins to functional groups on the liposome surface or attaching a hydrophobic residue covalently to the proteins and allowing them to intercalate non-covalently into the bilayer during or after the liposome formation. See also Huang et al, *J. Biol. Chem.* 255:8015-8018, (1980).

Heath et al, *Biochim. Biophys. Acta*, 599:42-62 (1980); and 640:66-81 (1981) used periodate to oxidize bilayer situated glycosphingolipids, thereby generating a reactive aldehyde moiety in the liposome capable of binding to free amino groups of protein. Others have used SPDP derivatized phosphatidyl ethanolamine which can be inserted in the bilayer of the liposome and incubated with rabbit Fab' fragments at pH 8.0 under inert atmosphere.

Coupling of antibodies can also be accomplished by use of water soluble cross-linking reagent 1-ethyl-3, 3-dimethylaminopropyl carbodiimide (EDCI), as reported by Endoh et al, *J. Immunol. Meth.* 44:79-85, (1981). The fixation of antibody, either by EDCI mediated coupling or by adsorption, on the surface of the liposomal membrane reduces the activity of complement dependent cytotoxicity, probably because of inactivation of the Fc part of the molecule.

Shen et al *Biochim. Biophys. Acta* 689:31-37, (1982) describe a method of coupling monoclonal antibody to palmitic acid and incorporating this conjugate into liposomes by reverse phase evaporation methods. Palmitoyl antibody in 0.15% deoxycholate is added to a liposome suspension after the majority of the organic solvent has been removed by evaporation. Efficient incorporation, over 80%, of palmitoyl antibody occurs without leakage of the encapsulated drug.

EXAMPLE 1

Synthesis of PDP-DPPE

N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) 75 mg, was dissolved in 9.0 millimeters of anhy-

drous methanol under nitrogen. Twenty one microliters of triethylamine was added. L alpha-dipalmitoylphosphatidylethanolamine, 104 mg, dissolved in 14.2 milliliters of chloroform was added to the SPDP solution and reacted for 18 hours at room temperature. The reactants were placed in a rotoevaporator and the chloroform-methanol was evaporated at 44 degrees centigrade. Nine milliliters of fresh chloroform was added to the flask, followed by the addition of 9.0 milliliters of 0.01M phosphate buffered saline, pH 7.3, and mixed. The mixture was transferred to a separatory funnel and the aqueous phase was removed and discarded. The organic phase was washed again with phosphate buffered saline followed by distilled water. The organic phase (20 milliliters) was dried with calcium chloride, filtered and placed on a 100 ml silica gel column equilibrated in chloroform. The column was washed with 300 ml chloroform:methanol (10:1) and eluted with 300 ml. chloroform:methanol (5:1). The eluted fraction was concentrated under vacuum to 10.0 milliliters and found to contain 3.3×10^{-3} moles/liter N-3-(2-pyridyldithio-propionyl)-dipalmitoylphosphatidylethanolamine (PDP-DPPE).

Preparation of ATP Marker

Two hundred milligrams of adenosine 5'-triphosphate disodium salt (ATP), and 7.4 milligrams of ethylenedinitrotetraacetate disodium salt (EDTA) were dissolved in water and the pH adjusted to 8.0 with IN NaOH. The solution was then adjusted to a final volume of 20.0 milliliters with water with a final concentration of ATP at 1.0% (w/v).

Preparation of ATP Loaded Liposomes

L alpha-dipalmitoylphosphatidylcholine (DPPC), 48.4 milligrams, 6.0 milligrams of PDP-DPPE, and 255.0 mg cholesterol were combined, followed by the addition of 1.1 milliliters chloroform, 3.0 milliliters diethyl ether, 0.5 milliliters anhydrous methanol and 2.0 milliliters ATP marker solution. The reactants were vigorously shaken under nitrogen and an additional 10.0 milliliters of ATP marker solution was added. The mixture was rotated on a rotoevaporator at 44° C. The liposomes were then dialyzed extensively against 1 liter of 0.50M TRIS buffer containing 0.001M EDTA, and 0.340M sodium chloride, pH 8.0, with four changes of dialyzing buffer.

Preparation of Fab' Fragments

Affinity purified rabbit anti Group A streptococcal antibody was digested with pepsin at pH 4.5 for 18 hours at room temperature. The Fab'₂ fragments were separated on a G-25 sephadex column. Fab'₂ fragments (1.7 mg) were reacted with 0.02 mM dithiothreitol (DTT) and separated on a G-25 column equilibrated with 50 mM TRIS containing 340 mM sodium chloride, 1 mM EDTA, pH 6.0. The protein peak in the void volume of the column contained 850 ug/ml protein.

Coupling of Fab' to PDP-Liposomes

Fab' (170 micrograms) was reacted for 18 hours at 4° under nitrogen with 1.5 ml of the dialyzed PDP-liposomes. Without any further treatment, the Fab'-coupled liposomes were diluted in 0.1% BSA, 50 mM TRIS, 340 mM sodium chloride, 1 mM EDTA, 0.02% sodium azide, pH 8.0.