

## Phospholipids and their Applications in Medicine and Diagnosis

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Glycerol containing phospholipids have been put to extremely wide range of applications. Their applications in medical devices, liposomal drug delivery systems, artificial blood and in diagnostic testing are reviewed in this article.

**P**HOSPHOLIPIDS are compound lipids derived from glycerol (phosphoglycerides) or sphingosine (sphingomyelin)<sup>1</sup>. They occur naturally in cell membranes and serve to maintain its integrity, act as precursor for synthesis of Platelet Activating Factor and arachidonic acid and second messengers in signal transduction. They also occur in egg yolk and soya bean and can be easily extracted from them.

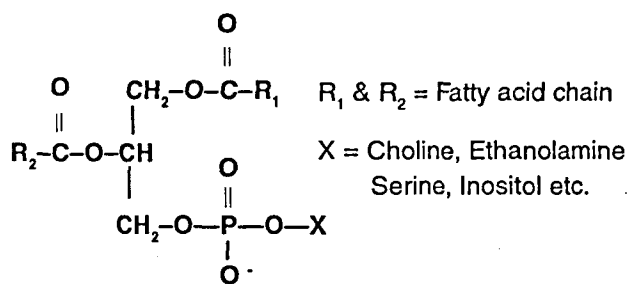


Fig. General structure of phospholipids

Being amphipathic molecules, phospholipids in aqueous medium form minute, closed, bilayered vesicles called **Liposomes**, which serve as permeability barriers. Phospholipids have many important uses in medicine and diagnosis. In this article, a few of the applications of phospholipids in medicine and diagnosis have been highlighted.

## Biotechnological applications:

Conventional medical devices like intravenous 'giving-in' sets, urinary catheters, and contact lenses, which are made of synthetic polymers like Polyvinylchloride (PVC), acrylates, polyethylene and others, when come in contact with body fluids, start adsorbing proteins and platelets onto them, majority of which is fibrinogen<sup>2</sup>. This may lead to blockade due to clot formation or bacterial adsorption leading to infection and limiting their lifetime of use in patients. Coating of these medical devices by phospholipids is found to considerably decrease fibrinogen and platelet adsorption, which is proportional to the concentration of the phospholipid used.

Biocompatibles UK<sup>3</sup>, use Diacetylenyl phosphatidylcholine (DAPC) as the phospholipid for coating medical devices, which can be further crosslinked (after coating) by gamma irradiation to give a polymeric layer, highly stable to aqueous environment, compared to the unpolymerised layer. Alternatively, the phospholipid and the polymer (PVC) can be coextruded. Recently, copolymers of polyacrylic acid and phospholipids called Coatable Phosphorylcholine Copolymers (CPC) consisting of a polyacrylic acid backbone, to the carboxylate moieties of which are randomly attached phosphorylcholine and hydrocarbon chains, are used for coating and are more efficient (3-4 folds) than DAPC.

Rapid and sensitive *in-vitro* immunoassay methods<sup>4</sup> for measuring fibrinogen and platelet adhesion are used

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to compare performance of conventional devices with that of coated devices.

#### Medical applications:

The major use of phospholipids in medicine is perhaps their use in liposomal drug delivery systems<sup>5</sup>. Liposomes are microscopic vesicles composed of one or more phospholipid bilayers separated by an equal number of aqueous spaces.

The major advantage<sup>5</sup> of liposomally encapsulated drugs is a) targeted drug release to the liver, spleen, bone marrow, lymph nodes, and tumors and b) apparent increase in the therapeutic efficacy of the encapsulated drug due to a significant decrease in its toxicity compared to the free drug. Some examples of drugs which are successfully administered in liposomal form are amphotericin-B, doxorubicin, cisplatin and AZT.

**Amphotericin B :** Free Amphotericin B (Amp-B) is the first drug of choice for most systemic fungal infections, but its acute and chronic toxicity often limits its practical use. Amp-B is also often ineffective as antifungal drug for treatment of liver abscess in neutropenic and immunodeficient patients<sup>6</sup> on anticancer chemotherapy or suffering from AIDS and may give rise to adverse reactions like severe

fever and chills. Interestingly, patients treated with liposomal amphotericin B (L-Amp-B), improved considerably with negligible side effects and no adverse reactions<sup>5,7</sup> as summarized in the following tables.

A ten fold increase in LD<sub>50</sub> values of L-Amp-B and no significant change in BUN levels with almost ten times the dose compared to free Amp-B is observed. This is due to the selective uptake of L-Amp-B by the reticuloendothelial system of the liver. Similar effects were observed for liposomal nystatin<sup>8,9</sup>.

#### Liposomal AZT for Retroviral Infections :

3'-Azido-3'-deoxythymidine (AZT) is the drug of choice in the treatment of Acquired Immunodeficiency Syndrome (AIDS) caused by Human Immunodeficiency Virus (HIV)<sup>10</sup>. The initial treatment period is of benefit to the patient but longer treatment periods exhibit significant AZT - induced bone marrow toxicity<sup>10-13</sup>. Also, AZT resistant strains of HIV are found to develop on longer treatment<sup>14</sup>. Interruption of AZT therapy to reduce these side effects may lead to enhanced viral production and infectivity<sup>15</sup>. Liposomal AZT (L-AZT) exhibits cell specific drug targeting and thus has following specific advantages over free AZT<sup>16</sup>.

Table 1 : Comparison of hepatotoxic nature of Amp-B and L-Amp-B.

Drug	Dosage (mg/kg)	*BUN (mg/dl)	ED <sub>50</sub> (mg/kg)	LD <sub>50</sub> (mg/kg)
Amp-B	0.8	28±2.9	0.8	1.0
	0.8 daily x 5 days	35±1.0		
L-Amp-B	9.6	30.7±3.0	0.8	10.0
	8.0 daily x 5 days	31.8±3.0		

\*BUN: Blood Urea Nitrogen Levels.

Table 2 : Comparison of dose and toxic reactions of Amp-B and L-Amp-B

Drug	Dosage (mg/kg)	Infection cure	Toxic effects
Amp-B	0.8 daily x 2 days	Incomplete	Fever and chills
L-Amp-B	3.0 as single dose	Complete	Negligible

**Table 3 : Comparative bone marrow concentrations of AZT and L-AZT**

Drug	Dose (mg/kg)	Conc. In bone marrow (% inj. Dose / 10 <sup>7</sup> cells)	V <sub>d</sub> (L <sup>-1</sup> )
AZT	10	0.018	0.595
L-AZT	10	<0.00001	0.047

**Table 4: Lymphocyte Stimulation Index of AZT and L-AZT**

Treatment	Lymphocyte Stimulation Index (7 weeks post infection)		
	Con A	PHA	LPS
Normal (uninfected)	38±6	15±6	22±5
Saline	16±8*	04±2*	03±1*
AZT (2 mg/kg)	13±9*	03±2*	04±3*
L-AZT (2 mg/kg)	36±8	14±4	19±5
Blank liposomes	14±6*	03±1*	04±2*

\* : significant difference from normal.

**a) Bone marrow localization of free and liposomal AZT :**

A ten fold decrease in the volume of distribution (bone marrow) of L-AZT compared to free AZT is observed due to selective liposomal uptake of L-AZT.

**b) Activity of free and liposomal AZT in AIDS :**

One of the consequences of AIDS is a rapid suppression of the ability of the lymphocytes to respond to mitogenic stimulation. The splenocyte mitogenic responses in mice to the lymphocyte mitogens, Concanavalin A (ConA), lipopolysaccharide (LPS) and phytohemagglutinin (PHA) are significantly reduced compared to control, seven weeks post infection as shown in the following table.

**Liposomes As Vaccine Carriers<sup>17</sup> :**

Since macrophages are important for the processing of liposomal antigens, the property of liposomes to target to macrophages is utilised in the use of liposomes as vaccine carriers.

**Advantages of liposomes as vaccine carriers:**

1. Small amounts of antigen may be suitable as immunogen.
2. Multiple antigens can be incorporated into a single liposome.
3. Higher titre and longer duration of functional antibody activity may be achieved.

**Liposomal Malaria Vaccine :**

Various sequences of the tetrapeptide repeats of the antigenic region of the circumsporozoite (CS) protein (the major sporozoite antigen) can be conjugated to Bovine serum albumin (BSA) as carrier and used as immunogen along with Freund's adjuvant.<sup>18,19</sup> Single injection of liposome encapsulated peptide-BSA conjugate with Lipid-A adjuvant induced a greatly increased antibody titre, which is maintained for over 20 weeks after immunization<sup>20</sup>. The vaccine produces very high titre of antibodies acting on the sporozoites even before they can attack the liver cells and this protect the patient.

**Table 5 : Effect of immunoadjuvants on anti-TAA antibody isotypes in mice**

Immunization with	% Response					
	IgM	IgG	1	2a	2b	3
CEA alone	90	10	95	05	05	-
CEA-FCA	80	20	90	04	04	02
CEA/MDP	78	22	96	02	02	-
Liposomal CEA	37	63	50	30	18	02
Liposomal CEA/MDP	42	58	44	28	20	08

**Table 6 : Comparison of NRC v/s natural RBC**

Properties	NRC	RBC
Mean particle size ( $\mu\text{m}$ )	0.2	5-8
Total lipid concentration (g/dl)	9.3	0.5
Packed volume ratio (%)	51.0	45.0
Viscosity at 37° (cps)	4.3	3.8
Oxygen partial pressure for half saturation of hemoglobin (mm of Hg)	40.0	28.0
Hill coefficient (n)	1.9	2.8
A-V oxygen delivery (ml/dl)	7.7	5.6

**Subcutaneous liposomal tumor vaccine<sup>21</sup> :**

Immunization with the tumor associated antigens (TAA), immediately after surgical removal of primary tumor has been found to be successful in decreasing metastatic tumor growth<sup>22</sup>. Administration of liposomal TAA like carcinoembryonic antigen (CEA) with adjuvant like muramyl dipeptide (MDP) is highly effective in eliciting immune response in animal models<sup>23-25</sup>.

Liposomal TAA/MDP resulted in significant increase (4 to 6 fold) in induction of IgG-2a and 2b antibody isotypes<sup>29-32</sup>, which are of crucial importance in eliciting efficient antitumor immunity, compared to the unencapsulated TAA/MDP.

There was a highly significant ( $p < 0.001$ ), positive correlation ( $r = 0.975$ ) between liposomal TAA induced Lymphocyte Stimulation Index, Tumor stasis activity and the final clinical response.

**Artificial Blood:**

Adequate supplies of blood are not always available due to shortage of blood, specific blood group required and risk of transmission of infectious diseases. Hence, efforts are being made to develop artificial red blood cells.

**Requirements of an artificial RBC :**

1. No toxicity and antigenicity.
2. Easy passage through capillaries.

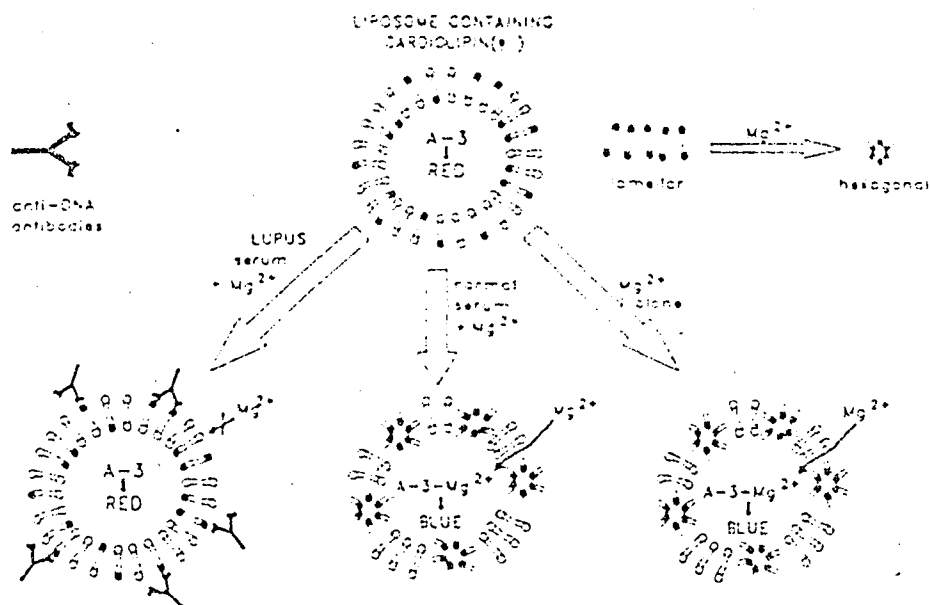


Fig. 2: Principle of divalent cation mediated lysis immunoassay

- Oxygen carrying characteristics similar to that of natural RBC.
- Compatibility, stability and suitable half life in circulation.
- Possibility of large scale production.

Liposome entrapped hemoglobin (Hb) called Neo Red Cells (NRC) are prepared<sup>33</sup> from lipid mixture of Soya lecithin : Cholesterol : Myristic acid : Tocopherol :: 7 : 7 : 2 : 0.28 and Stroma free hemoglobin (SFH) with Inositol hexaphosphate (IHP) as an allosteric effector (IHP:Hb::1:1).

Table 6 gives the comparative properties of NRC v/s RBC.

NRC exhibited no toxicity with respect to blood lactate levels, pH, body temperature, number of leukocytes and platelets. However, NRC tend to interact with plasma proteins leading to aggregation. This can be prevented by surface modification with a polyethylene glycol derivative.

UV polymerised diacetylinic, dienate and eleostearate phospholipids are also used to make another kind of artificial RBC's called 'hemosomes'<sup>34</sup> which remain uniformly dispersed for 8 hours at room temperature and stable for 3-4 months at 4°.

#### Diagnostic Applications :

##### Phospholipids used as liposomes in immunoassays<sup>35</sup>:

Principle : It relies on some kind of a cryptic or suppressed marker in liposomes, which is released into the surrounding medium by membrane lysis to generate a signal. The extent of antigen-antibody binding is proportional to extent of liposome lysis.

##### Recent assay method for Digoxin :

It uses Flavin Adenine Dinucleotide (FAD) as the encapsulated marker. The external medium used contains Apo-Glucose oxidase (Apo-GOx) with a microperoxidase/ isoluminol chemiluminescent system. Interaction of the antigen and antibody causes liposome lysis and the released FAD converts inactive Apo-Go-x to active Gox and luminescence occurs, the amount of emitted light being proportional to the extent of lysis. This method is 30-300 fold more sensitive than usual test methods, with a sensitivity of 0.39 pg./ml. It is called the Apoenzyme Reactivation Immunoassay version of Liposome Immunoassay System (ARIS-LIS).

##### Divalent cation mediated lysis:

It is a specialised technique for the assay of

antibodies binding to Cardiolipin and is diagnostic of Systemic Lupus Erythematosus (SLE) in humans. SLE is one of the adverse reactions occurring on administration of drugs like isoniazid, Penicillins, Phenytoin, Phenobarbitone, Practolol and others<sup>36</sup>.

Cardiolipin, alone does not form lamellar liposomes because it reverts to the hexagonal phase. However when mixed at a rate of 40 mol % with liposome forming lipids, it forms stable vesicles which when contain the dye Arsenazo III are red in color. If Mg<sup>++</sup> ions are added to a suspension of such liposomes, the cardiolipin is induced to rearrange to hexagonal phase and the liposomal membrane is disrupted. Simultaneously the Mg<sup>++</sup> ions interact Arsenazo III which turns blue.

Normal human serum has no effect on this process but if serum from an SLE patient is added, the anti DNA antibodies in it, bind to the cardiolipin and prevent the Mg<sup>++</sup> ions from inducing the cardiolipin rearrangement and thus prevent membrane lysis. Hence with SLE serum the vesicles remain red in color.

## CONCLUSION

The applications to which man has put phospholipids over the years are extremely ranging. These applications make use of physical, chemical and biological aspects of behaviour of these molecules. As the time progresses, the design and use of phospholipid vesicles in areas such as drug targeting, diagnostic testing will become increasingly more routine and in the true sense, phospholipids could be called as the "wonder molecules" of the 21st century.

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