2.1 INTRODUCTION

In spite of the enormous amount of efforts on development of new drugs for treatment of diseases, none of the drugs available to this date is free from undesirable biological effects. The latter primarily arises from random toxicity, distribution and nonspecific binding of the drug with undesirable targets in the biophase. In addition, metabolic alterations of drug may also render it ineffective. A logical approach to overcome these problems of chemotherapeutic agents would be to selectively deliver these drugs to the diseased or defective tissue in controlled manner. This may be achieved by using suitable drug delivery systems. Administration of the drug after incorporating them into appropriate delivery system, vehicle, or carrier will make them latent in biophase and would thus protect them from biodegradation or metabolic alterations, inhibit their binding with nonspecific sites and reduce toxicity. The basic requirements of an ideal drug vehicle, thus, are the following:

(a) The carrier should accommodate in its structure sufficient amount of drug either by internalization or by suitable binding of the drug.
(b) The surface properties of the carrier should be such that the release of drugs in biophase can be regulated.
(c) The structure of the carrier should easily accommodate specific molecules (or macromolecules) for transfer to the target sites.
(d) The carrier should be biodegradable.
(e) The chemical constituents of the carrier should not be toxic.

Several possibilities are presently being investigated for their use as drug carriers. Amongst these, microcapsule prepared from polymeric materials, linking of drug with macromolecules (such as, DNA and glycoproteins), cells, sealed erythrocyte, ghosts and liposomes have shown promising results. The biodegradable nature of liposomes along with its ability to associate directly with the target molecules in their structure make them an attractive carrier for use in drug delivery system. Liposomes have been studied as a drug delivery system in the last two decades. Most of its applications have been based on the ability of the liposomes to preferentially migrate to reticuloendothelial systems (RES) or tissues such as, liver and spleen (Vertut-Doi et al., 1996). However, avoiding degradation of liposomes by mononuclear phagocytic system has been a problem (Vertut-Doi et al., 1996). Nonetheless, in principle, liposome as drug carrier for targeted delivery has potentials of applications in chemotherapy of cancer.

2.1.1 Liposome-Composition, Structure And Physical Properties
Liposomes are sphere like structures that possess internal volume and are essentially made up of phospholipids. Their biological properties are to a limited extent controlled by the nature of
their constituents. Most common lipids used for preparation of liposomes are listed in table 2-1.

**TABLE 2-1**

<table>
<thead>
<tr>
<th>NAME</th>
<th>MOL. WEIGHT (Da)</th>
<th>Tc. (°C)</th>
<th>CHARGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (Chol)</td>
<td>387</td>
<td>nk</td>
<td>0</td>
</tr>
<tr>
<td>Phosphatidylcholine (PC)</td>
<td>762</td>
<td>-15</td>
<td>0</td>
</tr>
<tr>
<td>Dipalmitoylphosphatidylcholine (DPPC)</td>
<td>734</td>
<td>+41</td>
<td>0</td>
</tr>
<tr>
<td>Dicetylphosphate (DCP)</td>
<td>547</td>
<td>nk</td>
<td>-</td>
</tr>
<tr>
<td>Phosphatidylserine (PS)</td>
<td>760</td>
<td>+5</td>
<td>-</td>
</tr>
<tr>
<td>Phosphatidic acid (PA)</td>
<td>700</td>
<td>nk</td>
<td>-</td>
</tr>
<tr>
<td>Stearylamine (SA)</td>
<td>269</td>
<td>nk</td>
<td>+</td>
</tr>
</tbody>
</table>

When phospholipids are dispersed in water they form molecular aggregate due to their amphipathic character. The structure of this aggregate may either consist of bilayers or micelles, depending on the nature of phospholipids and degree of hydration. Among the natural phospholipids, lecithin is known to readily form bilayers (Perez-Soler, 1989; Bachhawat, 1991). Thus, dispersion of lecithin in water form closed structures comprising of a series of concentric bilayers alternate with aqueous compartments. Such structures are called multilamellar vesicles (MLV). Unilamellar vesicle (ULV), comprising a single bilayer, is formed upon brief ultrasonic treatments of multilamellar preparations. The size of MLV varies between 1 and 5 µm whereas that of ULV between 0.05 and 0.2 µm.

MLV forms spontaneously upon addition of an aqueous solution to a preformed lipid film or lipid solution. MLV can passively entrap lyophilic molecules with high efficiency. On the other hand, since the hydrophilic compartment is small (< 10 %), MLV are generally not good carrier for water soluble molecules. Very little information is available on the chemical structure characteristics required for a satisfactory incorporation of drugs into MLV. No studies have yet defined how different substituent groups in a given molecule may effect its interaction with the lipids commonly used for preparation of liposomes (Perez Soler, 1989; Mayhew, et al., 1978; Ryman and Tyrell, 1980). However, available evidence suggests that because of the avidity of the lipids, many lipophilic molecules may be efficiently entrapped within the lipid bilayer of MLV at a lipid:drug ratio (w/w) of 15:1 to 25:1 (Perez Soler, 1989; Yatvin and Lelkes, 1982). This rule has, nonetheless, two important exceptions. Some drugs, though lyophilic, do not
interact with the lipid components of the bilayers or cannot be accommodated in the space between the phospholipid molecules and consequently precipitate outside the vesicles. In other cases, liposomes cannot be prepared because the water insoluble drugs are not soluble in the common organic solvents used for the manufacture of the lyophilized mixture that form MLV upon reconstitutions.

ULV are better suited than MLV to carry hydrophilic molecules because their aqueous space is larger than their lipid space. However, the passive entrapment efficiency of the vesicles is always suboptimal unless a positive gradient type of loading is used. Water dispersions of phospholipids possess unique thermal properties below a certain temperature called “crystalline thermal phase transition temperature” or $T_c$. Below $T_c$, the phospholipid structure remains highly ordered whereas at or above $T_c$ the structure becomes greatly disordered. This property of phospholipids is termed as their thermal phase transition behavior. The bilayer possessing phospholipids in disordered form is known as “liquid crystalline phase” (or fluid phase). Liquid crystalline phase of the bilayer is much more permeable to solute as compared to that of the gel crystalline state. However, the permeability is reduced if cholesterol is included in fluid bilayers of lecithin. The cholesterol is known to increase the phospholipid packing in the liquid crystalline state whereas the reverse is true for the gel crystalline phase of the bilayers. Vesicles containing cholesterol have been found more stable and showed a more sustained release of drug.

2.1.2 Liposome As Drug Carriers
Water soluble as well as insoluble substances can be entrapped within the internal space of the liposomes and lipid soluble compounds are accommodated in their membranes. The macromolecules with both hydrophobic and hydrophilic regions occupy accordingly the lipid bilayer and aqueous phases of liposomes. The successful application of liposomes as carriers for drugs and enzymes in therapy is heavily dependent on their stability in circulation, tissue distribution and on their mode of interaction with target cells. These aspects of liposomes have been investigated in great detail (Woodle and Lasic, 1992; Vertut-Doi et al., 1996; Perez Soler, 1989; Kato et al., 1993; Klibanov et al., 1990; Torchilin, 1994).

2.1.2.1 Mode of administration and distribution: Liposomes administered intravenously to humans concentrate mainly in the organs with fenestrated capillaries such as liver, spleen and bone marrow. Liposome entrapped agents might, therefore, be particularly effective in treating tumors that infiltrate these organs (e.g., liver micrometases). For treating large, solid, well established metastases, liposome entrapped drugs may be more effective than free drug depending on the ability of the vesicles to cross tumor capillaries and be taken up by the tumor cell. This ability will depend, among other factors on the characteristics of the tumor endothelium and the size, charge and flexibility of the vesicles. Size and surface charge of the
liposomes appear to control the rate of liposome clearance from blood. Large liposome are cleared more rapidly than the smaller one (Juliono and Stamp, 1975). Liposomal preparation of mixed sizes possess biphasic rates of clearance, whereas liposomes of homogeneous size exhibit a simple exponential clearance kinetics (Gregoriadis et al., 1974). Neutral and positively charged liposomes were cleared less rapidly than were unilamellar negatively charged ones (Kirby et al., 1980). Survival of liposomes in circulation is also increase by increased amount of cholesterol in liposomes (Kirby et al., 1980). Liposome consisting of sphingomyelin and cholesterol have longest known survival times (Gregoriadis et al., 1980; Hwang et al., 1980). The stability may also be enhanced by preparing liposomes from phospholipids that possess Tc greater than 37 °C, from the dialkyl analogs of the phosphatidy cholines or by incorporation of lipids derivatized with hydrophilic polymer polyethylene glycol (PEG) (Kirby et al., 1980; Woodle and Lasic, 1992). The blood clearance and the rate of degradation of liposome can be modified by using different lipid composition. Lipid vesicles that result in a slow drug release can, therefore, be designed and used as carrier of anti tumor agent that are more effective or less toxic when infused continuously (e.g., cytarabine and doxorubicin, respectively).

Major proportion of liposomes from blood circulation is captured by liver and spleen (Gregoriadis et al., 1972; Finkelstein and Weissman et al., 1978). Other tissues such as lung, kidney, skeletal muscle etc. also participate in the uptake of liposomes. The amounts that are taken up by these organs are rather modest and for multilamellar liposome this amount seldom exceeds 2-5% of the dose per gram of tissue (Gregoriadis et al., 1974; 1977; McDongall et al., 1974). When liposomes are administered subcutaneously, they are drained in part by the lymphatic and concentrate in the regional lymph nodes (Perez-Soler, 1989). Small liposomes tend to be cleared more rapidly from the site of injection than large liposomes, but many of small liposomes are cleared by blood vessels, whereas large liposomes are mostly drained by the lymphatics (Perez-Soler et al., 1985). On the basis of these findings, liposome entrapped drugs may be particularly effective for the treatment or prophylaxis of regional lymph node metastatic diseases e.g., malignant melanoma. Because of their ability to act as a 'depot' system, liposomes may also be particularly useful in altering the pharmacology of drugs administered intraperitoneally or intraplurally and thus marked increase the intracavitary drug levels for a significantly prolonged time. The treatment of peritoneal metastases of ovarian carcinoma might be more effective if such an advantage was exploited.

2.1.2.2 Release of the liposome encapsulated drugs: Of the several techniques for the release of the drug includes entrapment of the drug in the polymer matrix, attachment of the drug to the polymeric matrix by covalent bonding and encapsulation of the drug inside the layer of polymer or the liposomes (Williams, 1984).

Release of the drug includes diffusion, i.e., the drug migrates from the initial position to the
outer surface. Diffusion rate can be controlled by two ways: the reservoir and the matrix. In the former case the drug is enclosed in the polymeric film, microcapsules, tubes like hollow fibers and membrane system, liposomes. In the later case, the drug is uniformly distributed throughout the polymer. The matrix system is also known as the monolithic system (Bachhawat, 1991; Bachhawat et al., 1993).

The matrix bound drug is another example of diffusion controlled delivery systems. Drug is dispersed in the polymer matrix. Dispersion may follow three mechanisms. First, it may be that the drug is released at a very slow rate independent of the content of the drug (Gregoriadis and Florence, 1993). In the second case it is possible that the drug is released because of the swelling of the polymer when the environmental fluid is embedded in the system and thus drug diffuses through the swellings. In the third case the polymer that forms the matrix is biodegradable and a slow release of the drug with the surface degradation of the polymer. This process eliminates the unwanted polymer usually left in the body after the drug is consumed. Microcapsules can be undesirable if non biodegradable polymers are used. It is potentially dangerous if the outer layer is burst releasing the incorporated drug at a very high release rate. The main advantages of the matrix system are its low cost of manufacturing and relative safety in case of leakage (Bahadur, 1983). It can also release the leakage of larger molecular weight molecules like insulin, enzymes, antibodies, etc. This method has certain disadvantages as a constant release rate is not attainable as it slows down with the gradual consumption of the drug. The drug and the polymer are linked through covalent bond that undergoes fission releasing the drug at a desired rate. The drug is attached to the polymer by a spacer group. The drug is released when water reacts to break the bond thereby freeing the drug. Release rate in each case changes by changing the hydrophobic character of the polymer backbone (Zaffaroni, 1980). Liposomes can be designed that the enzymes can also break the bond between the drug polymer bond.

Benefits of controlled release system:
(1) Maintenance of drug at its controlled therapeutic level.
(2) Localized drug administration.
(3) Preserving volatile medication.
(4) Decreased expense and wastes.

2.1.2.3 Scope of improvisation: To reduce the uptake of liposomes in liver and spleen and to stop random distribution of liposomal material in biophase much progress has been made in the last few years. Methods have been developed that have decreased uptake by reticuloendothelial system (RES) and increased blood circulation half life (Allen and Chonn, 1987; Allen et al., 1989; Kibanov et al., 1990). Such developments appear to have brought liposome research into a new era. Many reports have appeared in the literature demonstrating...
the increased therapeutic efficacy by using such long circulating liposomes over those with conventional compositions (Allen, 1994; Papahadjopoulos et al., 1991; Gabizon, 1992). The conventional term is used to signify liposomes composed of various phospholipids, cholesterol and possibly other lipids, without additional components that might confer the property of long circulation in blood and diminished recognition by the liver and spleen macrophages. The rapid clearance of conventional liposomes from the circulation has limited their prospects as an in vivo delivery system for transporting drugs to the site of disease beyond the RES. Recent reports have described new liposome formulations that exhibit a prolonged circulation time in blood following intravenous administration in mice and increased accumulation in implanted tumors (Allen and Chonn, 1987; Gabizon, 1990). Because of their ability to evade normally rapid uptake by the resident macrophage cells of the liver and spleen, these sterically stabilized liposomes (Woodle and Lasic, 1992) have been referred to as “stealth”. The term stealth is used to signify liposomes containing specific molecules such as GM1 ganglioside, phosphatidylinositol, PEG derivatized phospholipids etc. Their properties have the potential for significantly expanding the therapeutic utility of liposomes both by a more sustained release of various pharmaceutically active molecules within the circulation and by increasing effective targeting to specific cells and tissue within vasculature. It has been postulated (Gabizon and Papahadjopoulos, 1988) that steric “shielding” of negative charges on the liposome surface may contribute to the long circulation time of “stealth” liposomes, by inhibiting interaction with plasma proteins (Woodle and Lasic, 1992). These plasma proteins, opsonins, are thought to be responsible for the removal of conventional liposomes by the RES (Gregoriadis, 1988). It is also possible that similar steric hindrances may inhibit recognition of surface groups on the liposome by cell surface proteins responsible for their binding (Allen et al., 1991). Unlike conventional liposomes that show dose dependent blood clearance kinetics, the new formulations show clearance kinetics that is completely independent of dosage over a wide range (Papahadjopoulos et al., 1991). Microscopic evidence indicates that accumulation of liposomes in tumors involves extravasation, presumably due to increased permeability of the capillary endothelia (Jain, 1987). The new liposome formulations produce a marked enhancement of the anti tumor activity of encapsulated doxorubicin and epirubicin in mice against intraperitoneal lymphoma and subcutaneous colon carcinoma, with a concomitant decrease in toxicity (Gabizon, 1992). Thus the therapeutic studies with “stealth” liposome mentioned above showed a significant increase in therapeutic index of anti tumor drugs in mice. The evidence obtained indicates the presence of intact extra cellular liposomes within the tumor area is likely to provide a local depot for sustained drug release at a relatively high concentration (Jain and Gerlowski, 1986).

2.1.3 Preparation Of Liposomes

Presently, several methods are available to make liposome of different size and charge. A short review of the various methods available for preparation of liposomes follows:
2.1.3.1 **Sonication method:** This simplest method was originally described by Bangham (1965). The phospholipids are dissolved in a solution of chloroform : methanol (v/v, 2:1). Solvent is evaporated to make a lipid film. The lipid film is then dispersed in water at a temperature above the Tc of the lipid. Encapsulation efficiency of drugs is about 4 % - 7 %. A gentle shaking increases the encapsulation efficiency. Usually MLV are formed. The size of the vesicles may be reduced by disrupting the lamellar structure by sonication.

2.1.3.2 **Ethanol injection method:** This method uses injection of a solution of lipid in ethanol in the buffer. This method was described by Batzri and Korn (1973). Nearly all vesicles formed by this method is unilamellar. The major drawback in this method is that the lipid dispersion is diluted and the encapsulation is poor.

2.1.3.3 **French pressure method:** The lipids are mixed in chloroform:methanol mixture (v/v, 1:1) and dried on the wall of a glass bottle by evaporating the liquid in nitrogen and then evaporated under vacuum in a desiccator. Drug to be entrapped is then added to the film as aqueous solution and the lipid is completely dispersed by vigorous vortexing. In this method large MLV are formed (Barenholz et al., 1979). The white MLV are transferred to a French pressure cell that is held upside down after the shaft is tightly closed and maintained at 4 °C. The pressure cell is inserted in the hydraulic press and the pressure is applied. If the closure of the cell is performed properly the cell will hold the set pressure. Very slow extrusion is a crucial step in the preparation procedure. The size of the resulting liposome depends on the pressure applied. The higher the pressure the smaller the liposome size provided the operations are performed above the Tc of the phospholipids.

2.1.3.4 **Ether infusion method:** This method was first described by Deamer and Bangham (1976). The original procedure used diethyl ether as the solvent and was termed as the “Ether injection method”. The phospholipids are dissolved in diethyl ether or diethyl ether:methanol mixture and an aqueous solution of the material to be entrapped is added either at 55-65 °C or under pressure at 0 °C. The solvent is then removed by evaporation and large ULV of mean size of 1500-2500 Å are formed. This method is suitable for macromolecules. The drawback of this method is the unnecessary exposure of the material to high temperature and organic solvent. Materials like proteins are likely to loose their original structures and will lose its properties. This method has low encapsulation efficiency and produces heterogeneous population of liposomes.

2.1.3.5 **Detergent dialysis (DD) method:** This method involves stabilization of a lipid dispersion with a detergent such as, sodium deoxycholate or sodium cholate followed by its removal with gel filtration or by dialysis (Kagawa and Racker, 1971). Advantage of this
method is that this method produces are homogenous of small ULV. Lipid to detergent ratio plays an important role in the size of small ULV. Complete removal of detergents may not be possible making this method less practicable.

2.1.3.6 **Reverse phase evaporation (RPE) method**: To a mixture of lipids in an organic solvent an aqueous solution is added. The organic solvent is removed under pressure. Large ULV and oligolamellar liposomes are formed (Szoka and Papahadjopoulos, 1978). The entrapment efficiency is as high as about 65%. This method cannot be used for the entrapment of the proteins as they get denatured on exposure to organic solvent.

2.1.3.7 **Rehydration-dehydration (RD) method**: This method is used for the industrial preparation of liposomes. The phospholipids are dissolved in organic solvent and the solvent is removed under reduced pressure and then dispersed into aqueous phase. This procedure is mild requiring the presence of neither solvents nor detergents. The entrapped efficiency ranges from 27-70%. Optimal entrapment efficiency is achieved when the lipid is fully dehydrated. The method produces small ULV. This method was first developed by Kirby and Gregoriadis (1984). The major advantage of this method is that the liposome can be freeze dried and can be stored for more than a year without any major leakage of the entrapped material. Whenever needed the dried lipid can be regenerated into liposome by adding water. The lipid powder could be stored for a long time. The mildness of this condition is particularly important for the encapsulation of labile materials such as enzymes, DNA, cofactor and other biologically active substances.

2.1.3.8 **Freeze and thaw method**: Solutes are added to a concentrated liposome suspension (about 40 mg lipid ml$^{-1}$) prepared by dry film of lipids suspended in aqueous phase. The mixture is rapidly frozen in dry ice/acetone bath and thawed slowly at room temperature. The cycle is repeated at least three times to ensure efficient trapping. The untrapped solutes are removed by the ficoll flotation procedure. About 5% to 6% of the total solute can be encapsulated (Pick, 1981). Although this method is more rapid and equally efficient in solute entrapment, the resulting liposomes are heterogeneous in size. Also many oligolamellar liposomes are produced.

2.1.4 **2-Mercaptopropionylglycine (MPG)**

Sulphydryl compounds, widely distributed in animals, plants and microorganisms, are long known for their efficiency in restoring the activity of protein bound sulphydryl groups (Udupi and Rice-Evans, 1992). Consequently they are able to restore radiation-induced decativation of proteins. The sulphydryl protectors are can be categorized in two types. One is of natural origin...
with low toxicity such as, glutathione while the other type of sulphhydryl protectors are synthetic with high toxicity such as, cystamine. A synthetic aminothiol, 2-mercaptopropionylglycine (MPG), has been one of several such thiol compounds which has generated considerable interest as a radioprotector. The molecular weight of MPG is 163.20 Da and is water soluble white powdery substance. The chemical structure of MPG is shown in Fig.2-1

![Chemical structure of MPG](image)

FIGURE 2-1: Chemical structure of MPG.

MPG is an exceptional synthetic radioprotector *in vivo* and *in vitro*, whose effective dose (20 mg kg\(^{-1}\) body weight) is sufficiently below its toxic dose of (2100 mg kg\(^{-1}\) body weight) (Sugahara & Srivastava, 1976; Ayene & Srivastava, 1985; Garner et al., 1986). The nontoxic nature of MPG at its optimum dose for radioprotection has been the main cause of the interest for using it in chemical radioprotection. In addition, it has some pharmacological properties such as, antiallergic action and detoxificating action against heavy metals and various drugs protecting the liver (Chiba et al., 1973). Two derivative of MPG, namely, 3-MPG, and MPG-amide has been synthesized. Contrary to expectations, the derivatives partially lost the radioprotective ability of MPG (Sugahara and Srivastava, 1976). Consequently, MPG is reported to be one potent radioprotectors among its related compounds (Sugahara and Srivastava, 1976).

MPG is known to chemically “heal” the primary lesion by a donation of an H-atom to a site where it was initially abstracted and thus facilitate DNA single strand break (ssb) rejoining in aerobic system (Revesz et al., 1984). Patt et al. (1949) observed the effect of cystamine in animals for their ability to protect against the ionizing radiations. Many sulphhydryl compounds have been reported to protect the animals though the protection is not universal. MPG and a related compound, WR 2721, show radioprotective effect at subtoxic doses (Nagata et al., 1972). MPG is reported to be an antitoxic agent and an enzyme activator due to the presence of easily available -SH group (Bhanumathy et al., 1986). Therefore, the MPG affordable radioprotection is believed to be mediated by several mechanisms. MPG is reported to mediate release endogenous protectors (Revesz et al., 1972), act as OH and free radical scavanger (Mishra and Srivastava, 1981) or cause elevation of redox potential (Sugahara, 1972) to offer radioprotection to cells. A possibility of MPG induced delay in glutathione metabolism has been suggested (Uma Devi and Prasanna, 1990).
2.1.5 Encapsulation Of MPG In Liposome

MPG is a suitable radioprotective drug which has shown promising radioprotective effects in experimental systems against radiation induced damages (Sugahara and Srivastava, 1976). The radioprotective effect of MPG was reported in vitro for gamma induced radiolysis of catalase (Wary and Sharan, 1988) as well as in vivo (Wary et al., 1989, Sharan, 1990). However, it afforded radiosensitization in cases of γ-induced microsomal lipid peroxidation (Ayene & Srivastava, 1985), catalase radiolysis (Wary & Sharan, 1988) and DNA strand break in human lymphocytes in vitro (Wary et al., 1989; Sharan, 1990). The causes for this undesirable reversal of MPG affordable radioprotection was attributed to (a) the lack of optimal concentration of MPG in tissue of interest for radioprotection and (b) metabolic alterations of MPG to its oxide, disulphide and other derivatives after its administration (Sharan et al., 1995). Since liposomes can, in principle, encapsulate MPG it is possible to overcome the two, essentially undesirable, conditions which reverse the MPG affordable radioprotection. No such report is available in literature where such attempts were made.

2.1.6 Aims And Objectives

Therefore, in this piece of work it was desired to encapsulate MPG in a suitable liposome vesicle. As several methods for encapsulation of the drugs in liposomes are available, it has been attempted to use mild and relatively convenient methods of liposome preparation. The objective of this work was to:

(a) Select and standardise a mild, convenient and reproducible method of liposome preparation.
(b) Encapsulate MPG in such liposomal preparations.
(c) Compare the entrapment efficiency to recommend the best method for MPG encapsulation.
(d) Establish the influence of concentration of MPG on the efficiency of MPG entrapment into liposome by such methods.

2.2 Methods And Materials

2.2.1 Chemicals

2-mercaptopropionylglycine (MPG) was a gift from Prof. T. Sugahara and Santen pharmaceuticals Co., Japan. Dipalmitoylphosphatidylcholine (DPPC), dicetylphosphate (DCP), cholesterol (Chol), dithio-bis-nitrobenzoic acid (DTNB), deoxycholic acid, sodium azide, Triton-X-100, phosphate buffer saline (pH-7.9) and tris base (Trizma) were purchased from Sigma Chemicals Co., USA and Sepharose CL-4B, Sephadex 200 from Pharmacia Fine
Chemicals, Sweden. The other chemicals like ethylenediaminetetraacetic acid (EDTA), chloroform, ethanol, sodium chloride, etc. were of highest purity grades chemicals available from various indigenous sources. Glass double distilled water was used for preparation of all reagents.

2.2.2 Other Materials
The work involved use of ordinary laboratory facilities like, liquid nitrogen, water bath, rotary evaporator, vortex, spectrophotometer, etc.

2.2.3 Preparation Of Liposomes
The following methods were used to prepare liposomes and to encapsulate MPG into it:

2.2.3.1. Preparation of required buffers/solutions: The following reagents were prepared and stored refrigerated for use:

(A) Tris-acetate buffer - It consisted of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>20 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>100 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
</tr>
</tbody>
</table>

The pH of the buffer was adjusted to 8.1 with 1 M acetic acid.

(B) PBS (pH 7.9) - The constituents of this buffer was:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na2HPO4</td>
<td>0.1 M</td>
</tr>
<tr>
<td>NaH2PO4</td>
<td>0.1 M</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.9 M</td>
</tr>
</tbody>
</table>

Appropriate amounts of Na2HPO4 and NaH2PO4 were separately dissolved in water to prepare their respective 0.1 M solutions. The two solutions were mixed until pH of 7.9 was obtained. To this calculated amount of NaCl was added to get its molarity.

(C) MPG solution - Separate solutions of 2.5 mM, 5 mM and 10 mM MPG were prepared by dissolving 0.408 mg, 0.816 mg and 1.632 mg, respectively, of MPG in 1 ml of PBS (pH 7.9). Similarly, another set of MPG solutions were prepared by dissolving MPG in tris-acetate buffer (pH 8.1).

2.2.3.2 Rehydration dehydration (RD) method: The method is essentially based on the method of Kirby and Gregoriadis (1984). DPPC (5.0 mg), DCP (1.0 mg) and cholesterol (2.5 mg) were added in 1 ml of chloroform:ethanol mixture (1:1) in a glass test tube. After thorough vortexing, it was incubated at 37 °C for about 5 min for proper solubilization. The solution was then dried to a thin film on the wall of the tube by flushing the tube with N2 while rotating the tube immersed in a water bath maintained at 37 °C. Typically it took a few min to achieve this.
The dried film was then dispersed in 1 ml of either 2.5 mM, 5 mM or 10 mM MPG solution. For this MPG solution was added drop-wise into the tube with continuous vortexing until the entire lipid film was completely dispersed in the solution. The liposomes thus formed had encapsulated MPG into it. For preparing blank liposomes, PBS was used in place of MPG solution.

2.2.3.3 **Reverse phase evaporation (RPE) method:** The method is based on the method of Szoka and Papahadjopoulos (1978) which was used with minor modifications. A mixture of DPPC (5 mg), DCP (1 mg) and cholesterol (2.5 mg) was dissolved in 1 ml of chloroform:ethanol mixture (1:1) in a round bottom flask at 40 °C. To the organic solution, either 5 mM or 10 mM aqueous solution of MPG was added in small aliquots while the flask was being vortexed continuously. After complete dispersion, the flask was fitted into a rotary evaporator maintained at 40 °C to remove the organic solvents. It usually took about 45 min to complete the process. Blank liposomes were prepared by using PBS instead of MPG solution.

2.2.3.4 **Detergent dialysis (DD) method:** The method is modified from that described earlier by Kagawa and Racker (1971). DPPC (22.0 mg) and cholesterol (5.5 mg) were completely dissolved in 1 ml of chloroform:ethanol mixture (1:1) in a test tube. The solution was then dried at 37 °C by passing a stream of N2 on the walls of the tube which was being continuously rotated. The resulting thin film was dispersed by addition, in small aliquots, of either 5 mM or 10 mM aqueous solution of MPG in 20 mM tris-acetate buffer (pH 8.1). Finally, 1.0 ml of deoxycholic acid (dissolved in 20 mM tris-acetate buffer, pH 8.6) was added into the tube. The tube was continuously vortexed during the entire operation lasting about 5 min. The content of the tube was dialysed for 48 h in tris-acetate buffer with several changes. Blank liposomes were prepared by using tris-acetate buffer in place of MPG solution.

2.2.4 **Separation of free MPG from liposome entrapped MPG (LEM)**

MPG encapsulated in liposome was separated from the free or non-trapped MPG by the following methods:

2.2.4.1 **Dialysis:** A dialysis bag was boiled in water containing 10 mM EDTA and 1mM NaHCO3 for 10 min. After cooling, the bag was washed extensively in water before using it. A known volume of solution containing LEM and free MPG was packaged into the dialysis bag which was dialysed in 250 ml of 20 mM tris-acetate buffer (pH 8.1) with several changes. After 48 h, the liposome preparation was recovered from the bag and its volume measured.

2.2.4.2 **Gel filtration on sepharose:** LEM and free MPG were separated by passing the mixture through sepharose CL 4B column.
18

Packing of column and chromatography - A column (30 x 15 cm) was packed with sepharose containing 0.02% sodium azide. Sepharose was equilibrated with 10 mM PBS, pH 7.4. The column was developed with the same buffer. Gel filtration used to remove trace of chloroform remaining in the liposome and free MPG from the encapsulated MPG. Fractions were collected and read at 412 nm.

2.2.4.3 Centrifugation: To separate free MPG from LEM, the mixture was centrifuged at 46,000 x g in a Beckman centrifuge for 30 min. The supernatant was decanted. The pellet was resuspended in the same buffer. The suspension was recentrifuged and the whole process was repeated thrice. After the final centrifugation, the pellet was recovered and suspended in volume equal to the initial volume.

2.2.5 Assay Of MPG
Since MPG contains a -SH group (Fig. 2-1) the quantification of MPG can be conveniently done by the assay of the -SH group using Ellmans (1959) method using aqueous solution of MPG as a standard. This spectrophotometric method is very convenient and highly reproducible.

2.2.5.1 Preparation of assay reagent: The assay reagent consisted of:
- DTNB 10 mM
- PBS (pH 7.9) 100 mM
- EDTA 0.1 mM
The reagent was stored refrigerated.

2.2.5.2 Methodology: The liposomes encapsulating MPG was first disrupted to ensure complete availability of MPG for the quantification. For this, 0.1 ml of 1 % solution of triton X-100 was added to 0.1 ml of liposome preparation and vortexed. This solution was subjected to the assay.

The assay reagent was first flushed with N2 for 1 min. To 2.9 ml of N2 flushed assay reagent, 0.1 ml of MPG sample was added and vortexed. The absorbance of the solution was read immediately at 412 nm at intervals of 30 sec up to 3 min in a spectrophotometer. The blank contained PBS with 1% solution of triton X-100 in place of MPG sample.

2.2.6 Calculation Of Percent Entrapment Of MPG In Liposomal Preparation
The percent encapsulation of MPG into liposome was calculated by the following equation (Hiroaki Jizomoto et al., 1989):
Total amount of the MPG estimated in the liposome preparation x 100
Total amount of the MPG used for the preparation of liposome

2.3 RESULTS

2.3.1 Liposome
Liposomes were prepared by three standard methods, namely, rehydration dehydration (RD) method, reverse phase evaporation (RPE) and detergent dialysis (DD) method, after suitable modifications. All these methods are known to produce ULV. Fig. 2-2 shows that while RD

FIGURE 2-2 - Photomicrograph of liposomes prepared by rehydration dehydration method (A), reverse phase evaporation method (B) and detergent dialysis method (C) three methods (252 X).
(A) and RPE (B) methods produced small sized ULV liposomes, the liposomes produced by DD method were relatively large (C). The size variability was minimum in liposomes produced by RPE method (Fig. 2-2 C).

2.3.2 Composition Of Liposome
The lipid composition of liposome prepared by the RPE and RD methods and lipid to MPG ratio were calculated and are are shown in table 2-II:

**TABLE 2-II**

<table>
<thead>
<tr>
<th>LIPID COMPOSITION</th>
<th>QUANTITY OF LIPIDS (mg)</th>
<th>MOLAR RATIO</th>
<th>LIPID TO MPG RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC, Chol &amp; DCP</td>
<td>5, 2.5 &amp; 1</td>
<td>1 : 0.9 : 25</td>
<td>5.21</td>
</tr>
</tbody>
</table>

The lipid composition of liposome prepared by the DD method and the lipid to MPG ratio came out to be as shown in table 2-III:

**TABLE 2-III**

<table>
<thead>
<tr>
<th>LIPID COMPOSITION</th>
<th>QUANTITY OF LIPIDS (mg)</th>
<th>MOLAR RATIO</th>
<th>LIPID TO MPG RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC &amp; Chol</td>
<td>22 &amp; 5.5</td>
<td>1 : 0.48</td>
<td>16.85</td>
</tr>
</tbody>
</table>

2.3.3 Effect Of MPG Concentration On Entrapment Efficiency
The effect of starting concentration of MPG on percent of its encapsulation into liposome prepared by the three methods are shown in Figs. 2-3 to 2-5. The RD method yielded very low percent entrapment (19.47±5.49 %) when 2.5 mM MPG was used. The percent of entrapment (69.970±10.650 %) was highest when starting concentration of MPG was 5 mM (Figure 2-3). The entrapment efficiency (37.770±8.740 %) was low when 10 mM MPG was used.
Similarly, the detergent dialysis method did not show (Fig. 2-4) enhancement of percent of MPG entrapment when the starting concentration of MPG was raised from 5 mM (39.990±3.330 %) to 10 mM (31.910±4.09 %). Since percent entrapment was very low (19.47±5.49 %) when 2.5 mM MPG was used (Fig. 2-3), this concentration of MPG was not used in the DD or RPE methods.
On the other hand, RPE method showed progressively increasing efficiency of MPG entrapment (54.54 ±4.95 % to 68.33±6.49 %) when the starting concentration of MPG was raised from 5 mM to 10 mM (Fig. 2-5).
2.3.4 Effect of Method of Liposome Preparation On Entrapment Efficiency

Fraction of MPG entrapment into liposome prepared by different methods is shown in Fig. 2-6. In the RPE method the fraction of MPG entrapped was found to progressively and significantly increased from 0.545±0.050 for 5 mM MPG to 0.683±0.065 for 10 mM MPG (curve ii). The DD method showed relatively poor entrapment. The fraction of MPG entrapped was 0.400±0.033 for 5 mM MPG which showed a marginal decrease to 0.320±0.040 for 10 mM MPG (curve iii). In RD method (curve i) the increase in the fraction of MPG entrapment was from 0.194±0.055 (for 2.5 mM MPG) to 0.699±0.106 (for 5 mM MPG) which was significantly reduced to 0.378±0.87 (for 10 mM MPG).
2.4 DISCUSSION

Ever since the discovery of liposome in 1965, persistent efforts have been made to use them in studies other than the structure and function of biological membranes (Gregoriadis, 1986). During the last two decades, it was discovered that this model cell system may serve a dual role: as a valuable experimental tool for membrane research (Bangham et al., 1974; Papahadjopoulos et al., 1973 and Jost et al., 1982) and in addition, as an in vivo delivery system for enhancing the efficacy of various biologically active molecules (Gregoriadis, 1988).

Animal studies have shown that liposome can decrease the toxicity of several antitumor and antifungal drugs (Papahadjopoulos et al., 1991; Gabizon, 1992; Jones, and Hudson, 1993; Conley, 1993; Thierry et al., 1994, Allen et al., 1995; Nagayasu et al., 1996). The use of liposome as a carrier of antitumor agents has only recently become a clinical reality. Preclinical studies showed that these particulate carriers may improve the delivery of antitumor agents to certain tumors or organs, may decrease drug levels in certain organs particularly sensitize to the drugs toxic effects and may be used as a vehicle of the new lyophilic agents. On the basis of the information available, it is evident that liposome entrapped antitumor agents may offer a clear therapeutic advantage for several specific diseases. Among clinical situations in which liposome
entrapped antitumor agents may result in a higher antitumor activity, the prophylactics of liver metastases of colon carcinoma (treatment of microscopic diseases at the time of reaction of primary tumor) is well supported by the preclinical data (Perez-Solar, 1989). Besides, liposomes have also been found to be efficient carriers of macrophage activators for increasing tumoricidal activity in models of metastasis (Koff et al., 1985; Vertut-Doi et al., 1996; Killon and Fidler, 1994; Fidler, 1994; 1988). No such study has been conducted to test the efficacy of radioprotective drugs, MPG.

MPG is evidently a moderate radioprotective drug in vivo and in vitro experimental animals (Sugahara and Srivastava, 1976; Ayene and Srivastava, 1985; Garner et al., 1986; Wary and Sharan, 1988). In limited chemical studies on humans receiving radiation for cancer treatment, administration of MPG 15 to 30 minutes prior to irradiation afforded significant radioprotection (Sugahara and Srivastava, 1976). This was shown by the studies several biological endpoints. However several studies have shown the radioprotection afforded by MPG could be reversed (Ayene and Srivastava, 1985; Wary and Sharan, 1988). The main cause of this undesirable reversal was attributed to, suboptimal concentration of MPG in the tissue of interest, interaction of MPG with some metabolites or chemical alterations of MPG after its administration to animals. Encapsulation of MPG into liposome may offer several advantages and help maintain the affordable radioprotection.

The major consideration for selecting suitable methods for liposome preparation is to achieve highest efficiency of MPG entrapment as well as to contain the encapsulated MPG into the liposome for a long period. Further, all methods require exposure of the drug to organic solvents and detergent which might alter chemical characteristics of the drug or inactivate it. The third main consideration, therefore, should be that the MPG comes in contact with organic solvents for the shortest period of time so that the probability of its chemical alteration is minimum. The choice of the three methods used in this investigation was based on these considerations. All the three methods, rehydration dehydration method, reverse phase evaporation and detergent dialysis used for encapsulation of MPG meets the criteria at least to a certain extent.

Figs. 2-3 to 2-5 shown that MPG may be encapsulated into liposomes by all the three methods used in this investigation. Depending on the concentration of MPG, the percent entrapment of MPG showed a dose dependence only in the case of RPE method (Fig. 2-5). The percent entrapment did not increase by increasing the concentration of MPG when DD method was employed for liposome preparation (Fig. 2-4). When RD method was used, higher concentration of MPG was inhibitory to its encapsulation efficiency into MPG (Fig. 2-3). These observations are very clearly summarized in Fig 2-6. Since only RPE method of preparation of liposome for MPG encapsulation showed progressively increasing efficiency of
MPG encapsulation, this method seems to be the best of the three methods tried in this work. The entrapment efficiency of MPG into liposome by RPE method was also very reproducible. The method has also been reported to be simple and reproducible for encapsulation of other macromolecules (Alam et al., 1992).

Fig. 2-6 also shows the effect of the starting concentration of MPG on its fractional entrapment into liposome. It is evident that RPE method offers a MPG dose dependent increase in the entrapment of MPG into liposome. This method of preparation of liposome encapsulating MPG did not show any influence of starting concentration of MPG in the range used in this investigation. The other two method that have been used in this investigation were influenced by the concentration of MPG used for encapsulation. It was reported that entrapment of MPG in the liposome is dependent on the pH of the buffer and a major determinant in this is the chemical composition of the buffer (Gregoriadis, 1993). It is possible that the observed inhibition in efficiency of entrapment (RD method) or no increase in efficiency of entrapment (DD method) was due to this.

The major pharmacological problem is the use of cytotoxic agents for treating tumor while reducing or eliminating exposure of susceptible normal tissues. Of many possible unique solutions to this problem, explored over the last few decades, drug encapsulation in synthetic phospholipids vesicles (liposomes) has been found to be promising. The liposome offers infinite possibilities of alteration of its size, charge and chemical structure. This flexibility is a distinct advantage for formulation of tailored liposome for specific pharmacological goals and present unique clinical and experimental opportunities. Drugs that have serious and dose limiting toxic effects on normal organs can be packaged in liposomes to avoid exposure of those organs. High peak drug concentration can be reduced by a liposome formulation resulting in slow drug release. Thus liposomal drug delivery system has great potential for many purposes. Future clinical use is likely provided the preparation of liposome can be reproduced in hospital and pharmacies.

2.5 REFERENCES


Torchilin, V. P. (1994) Immunoliposomes and PEGylated immunoliposomes: possible use for targeted delivery of immunoliposomes. Immunomethod, 4, 244-258.


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