Preparation and Characterization of Deferoxamine loaded liposomes for targeted delivery to the heart and liver

THESIS PRESENTED

BY

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Abstract

The aim of this study was to prepare and characterize Deferoxamine mesylate (DFO) loaded liposomal formulations for targeted delivery to specific organs such as the liver and heart and also to increase the circulation time of DFO in the body for enhanced delivery for potential treatment of iron toxicity. Liposomes which have been extensively studied in the last few decades are spherical lipid vesicles having one or more phospholipid bilayers encapsulating an aqueous core containing the active drug. The preliminary study of this research focuses on optimization of the method for preparation of small unilamellar vesicles and physical characterization of six different DFO encapsulated liposomal formulations. Three different surface modifications of the liposomes employed in this research are grafting of a polyethylene glycol moiety on conventional liposomes to increase the blood circulation time of the encapsulated drug, addition of mannose moieties to the lipid bilayer to target the mannose receptors present on the Kupffer cells in the liver and use of DOTAP which is a cationic lipid to facilitate drug targeting to the heart and the liver via ionic interaction. Focus was also given to the characterization of size, surface charge and drug incorporation capacity of these liposomes. The final step in the proposed study was focused on in vitro and in vivo targeting of the mannosylated liposomes to the mannose receptors in the liver. As a part of this study the liposomes formulated with rhodamine labeled lipid were used for qualitative and quantitative experiments. The quantitative and qualitative studies were undertaken via FACS analysis and fluorescence microscopy respectively.

Successful completion of this set of experiments confirm that Deferoxamine loaded mannosylated liposomes can be used to increase the accumulation of the drug in the kupffer cells of the liver and that cationic liposomes may suffice in targeting to hepatic cells in general.
Thesis title: Optimizing the method of preparation and characterization of deferoxamine loaded liposomes for targeted delivery to heart and liver.

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A. Introduction

1.1 Objectives and Hypothesis:

The main objectives of this study are to design, optimize the method of preparation and characterize plain as well as surface modified Deferoxamine mesylate loaded liposomes for longer blood circulation as well as targeted delivery to the heart and liver.

The hypothesis of this project is that novel surface modified liposomes can be used for delivering Deferoxamine to specific organs in the body for potential use in iron chelation therapy.

1.2 Statement of Problem:

1.2.1 The need for liposomal delivery of Deferoxamine mesylate:

Deferoxamine mesylate is an iron chelator used in the treatment of acute iron intoxication resulting from transfusion requiring anemias like thalassemia and chronic iron overload. This drug is administered as a slow subcutaneous or intravenous infusion due to its poor bioavailability and short plasma half life (1). DFO is hydrophilic, has a short half life and is rapidly eliminated from the body. Thus it would be beneficial if DFO is delivered to the affected areas of the body via a delivery vehicle which increases the circulation period of DFO in the body. Cardiotoxicity and hepatoxicity due to iron overload are lethal. Therefore drug carriers like liposomes loaded with DFO which can target the liver and heart can be beneficial to the sub-population of patients with cardiotoxicity and liver toxicity due to iron overload.

1.2.2 Problem with conventional liposomes:

After intravenous administration, conventional liposomes bind to serum proteins such as immunoglobulins, fibronectin, C-reactive protein, etc., which function as opsonins (2). The process of attaching foreign particles to promote clearance by phagocytosis is called opsonization. Mononuclear Phagocyte System (MPS) recognizes these opsonins attached to the surface of liposomes and removes the liposomes from the blood circulation (2). This decreases the residence time of the liposomes in the blood thus reducing the time available for drug delivery.
1.3 Specific Aims:

**Aim 1:** Preparation of DFO loaded Multilamellar vesicles and optimization of the method of preparation of small unilamellar vesicles.

- Preparation of plain, cationic, mannosylated, pegylated, pegylated cationic and pegylated mannosylated DFO loaded multilamellar vesicles using reverse phase evaporation technique.
- ‘Sonication only’, ‘sonication and extrusion’ and ‘extrusion only’ techniques will be optimized and used for the preparation of small unilamellar vesicles.

**Aim 2:** Characterization of six liposomal preparations:

- Characterization of particle size, zeta potential and incorporation capacity of the above prepared liposomes.

**Aim 3:** Evaluation of mannosylated liposome binding to mannose receptors:

- J774A.1, a mouse macrophage cell line will be used for this experiment.
- Cells will be treated with fluorescent liposomes prepared with rhodamine labeled lipid.
- Fluorescence microscopy and FACS analysis of the cells will be done.

**Aim 4:** Targeting of mannosylated and Cationic Liposomes to the rat liver and heart:

- Sprague-Dawley rats will be used for this experiment.
- Injection of Lissamine rhodamine-PE lipid labeled plain, cationic and mannosylated liposomes.
- Fluorescence microscopy of the cryosections of rat liver and heart.
B. Background and Significance

Iron acts as a catalytic agent in many reactions that generate reactive oxygen species which are the cause of peroxidative damage to vital cells in the body (3). In individuals with normal iron load, iron usually complexes with the transport protein transferrin which binds iron reversibly for transportation between absorption, storage and utilization sites and is thus not available in free form to catalyze free radical production. Human body has a limited capacity to excrete excess iron due to extensive intake of dietary iron and/or frequent blood transfusion (3). In individuals with a heavy iron load, transferrin reaches a level of saturation and a lot of free unbound iron can be detected in the plasma (3, 4).

2.1 Acute Iron Intoxication and Chronic Iron Overload:

Acute and chronic iron overload lead to toxicity because of an increase in iron concentration in the body either due to multiple blood transfusions in patients who are suffering from thalassemia or other transfusion dependent anemias. Thus iron overload is a treatment of thalassemia. Acute iron intoxication affects multiple organs and occurs in 5 stages. In the first stage there is a direct effect on the mucous membrane of the GI tract, thus affecting the endocrine system. The second stage is the lag phase where absorption of iron continues. Metabolic acidosis due to the release of hydrogen ion by the conversion of ferric iron to ferrous iron develops in the third stage. Iron also has a direct effect on circulation by causing direct depression of myocardial contractility and reduction in the cardiac output which together may lead to shock. The fourth stage is the stage of hepatotoxicity. Iron is taken up by kupffer cells which are the liver macrophages present in the reticuloendothelial system thus causing hepatic injury which may lead to hepatic fibrosis. Stage five is the stage of acute toxicity. (5)

Heart and liver are the major sites of iron intoxication where chelatable iron is detected. In the heart, the cardiac myocytes become iron overloaded, whereas in the liver both the kupffer cells and hepatocytes are iron overloaded. Iron plays a significant role in myocardial injury and iron overload cardiomyopathy is a well known cardiac disease. Age related myocardial infarction due to iron overload has been hypothesized by Sullivan in 1981 (6). In the absence of a therapy, iron toxicity induced myocardial disease prevails as the life limiting condition and the main cause of mortality in thalassemia major patients (4). The liver being the major storage site for iron, hepatic fibrosis is observed in a significant percentage of patients. Liver damage remains the major cause of death in young adults suffering from thalassemia (4).
2.2 Iron chelation therapy:

The aim of iron chelation therapy is to chelate and eliminate free unbound excess iron and also to prevent the formation of deleterious reactive oxygen species causing injury to cells (3, 7). Iron chelating agents bind to the available unbound iron to form an iron chelate which is readily excreted thus causing a reduction in iron stores. Some of the clinically used iron chelating agents are deferoxamine, deferiprone and deferasirox (1). Deferoxamine has been used as a standard drug for iron chelation therapy for many years (1).

2.2.1 Deferoxamine as an iron chelating agent:

Deferoxamine B produced by *Streptomyces pilosus* is the most widely used strong iron chelator (4). It is a specific chelator of ferric ions with high affinity (\(K_d = 10^{-31}\) M) and has more affinity for iron over other metal ions (8). DFO chelates the ferric ions from ferritin and hemosiderin but not from cytochrome and hemoglobin (9). It binds to all six coordination sites of iron and therefore is categorized as a 1:1 hexidentate chelating agent (3). As shown in the structure below, deferoxamine has many carbonyl and hydroxyl groups which help in binding with ferric ions. The main problem which hinders the utility of DFO is its poor oral bioavailability due to which it has to be administered parenterally thus increasing the cost of the treatment leading to poor patient compliance (1). 100 mg of Deferoxamine is capable of binding about 9.35 mg of elemental iron. Deferoxamine interacts with iron and forms a stable, metabolically inactive and non toxic chelate called ferrioxamine which is excreted in the urine (3, 10). On intravenous bolus injection, DFO is metabolized by plasma enzymes and its plasma clearance is rapid with a half life of 5-10 minutes (11, 9). Due to those reasons extended infusion of this drug is administered in order to ensure efficient chelation. DFO chelation therapy has been shown to be very effective in reducing organ damage by reducing iron stores, improving hepatocellular function and avoiding cardiac injuries in thalessemia patients (3).
Despite being one of the most effective iron chelators, DFO has patient dependent toxicities such as vision impairment, neurotoxicity, growth retardation in thalassemic children, which may limit its treatment in these patients (3).

2.3 Liposomes:

Since their discovery in 1965, liposomes have been extensively investigated and have emerged as excellent drug carriers. Liposomes are spherical structures made of one or more bilayers of phospholipid molecules enclosing an aqueous core. On the basis of their size they are classified into small unilamellar vesicles (25 – 100nm), large unilamellar vesicles (100 – 400 nm), and multilamellar vesicles (200 nm – several microns). Their stability appears to be the best in the range of 100 – 300 nm (13). Conventional liposomes are made of natural, neutral, cationic or anionic lipids. Because of the lipid membrane, poorly soluble drugs can be incorporated into the liposomal membrane allowing delivery. On the other hand hydrophilic drugs that are susceptible to degradation in the blood may be encapsulated in the intra-liposomal cavity for delivery. By encapsulating the drug in liposomes the non specific activity of the drug can be reduced, the bioavailability increased and therefore the amount of drug required for activity is reduced and modulate drug toxicity. The aim of this study is to develop a liposomal preparation of DFO in order to avoid prolonged infusion and increase bioavailability of the drug.

2.3.1 Poly ethylene glycol coated liposomes:

By grafting a hydrophilic polymer, Polyethylene glycol (PEG) on the surface of the liposomes the increased clearance from blood due to opsonization effect of plain liposomes can be overcome to generate long circulating liposomes. In the 1970s Davis, Abuchowski and colleagues (14) revolutionized the field of advanced drug delivery systems by the
introduction of pegylation to liposome preparation. Pegylation increases the size of the liposomes, alters their physicochemical properties thereby helping them to avoid being taken up by the reticuloendothelial system. Pegylated liposomes, also known as stealth liposomes have an increased half life, decreased volume of distribution and decreased plasma clearance (2). Pegylation also increases the hydrophilicity of the liposomes thus masking them from plasma proteins and lipoproteins leading to reduced opsonization effect (14). Opsonization of the liposomes is hindered by forming a hydrophilic cloud around the liposomal surface. It also improves the stability of the preparation by providing repulsion to overpower the attractive forces thus lowering vesicle aggregation (2). PEG is biocompatible, non-toxic polymer and is therefore used widely. Reduced uptake by the MPS and longer residence time in the blood makes pegylated liposomes an appealing drug delivery system.

2.3.2 Targeted liposomes:

Attachment of surface ligands to liposomes capable of recognizing and binding to cells of interest can target the drug to sites of action. Cationic liposomes targeting the heart and liver and mannosylated liposomes which target the kupffer cells of the liver are examples of targeted liposomes.

A. Cationic Liposomes:

Cationic liposomes can be used to target drugs both to the heart and the liver. Liposomes can be taken up by the heart by various mechanisms such as fusion with sarcolemma, endocytosis, by adsorption of liposomes and by exchange of lipid molecules between the liposomes and the sarcolemma (15). Mueller, et al, have shown that positively charged liposomes accumulate to a greater extent in the ischemic dog heart compared to neutral and negatively charged counterparts (16). Caride and Zaret have also shown that cationic liposomes accumulate largely in the myocardial infarct or ischemic regions of dog heart (17). This may be due to the interference of cationic charges with the electrical conduction of the heart. Kayawake and Kako have also shown that positively charged liposomes concentrate more in the perfused non ischemic rabbit heart compared to the negatively charged and neutral liposomes (15). It has also been shown that serum enhances the liver uptake of positively charged liposomes in contrast to the negatively charged and neutral liposomes. This could be due to the negative charge of the cell surface. The possible reasoning proposed by the authors was that since the serum proteins are negatively charged in physiological conditions, they may induce liposome aggregation which may get entrapped when passing through the liver (18). Thus cationic liposomes can be developed to preferentially treat sub-populations of patients with iron overload induced cardiac dysfunctions.
B. Mannosylated liposomes:

Macrophages:

Macrophages which play a crucial role in the host defense system constitute a major portion of the mononuclear phagocyte system (MPS) or the reticuloendothelial system (RES). Monoblasts differentiate into monocytes which enter into the peripheral blood circulation and further differentiate into macrophages in the extravascular tissue (19). Macrophages which are located in the liver are called kupffer cells. Macrophages are the major source of plasma transferrin iron. Kupffer cells phagocytize the red blood cells and process hemoglobin iron and transfer it back to plasma transferrin. Thus iron is released from the macrophages in a chelatable form and is believed to be a major source of urinary iron with DFO therapy (3). Patients suffering from chronic inflammatory disease have a defect in iron metabolism and its release from the macrophages and can thus develop hepatic fibrosis. Drugs administered directly in a soluble form reach the macrophages only in small amounts (19). Therefore lipid carriers such as liposomes have been investigated for delivering drug in larger amounts to the macrophages. Liposomes are taken up by the macrophages by a process comprising of three steps. The first step involves binding of the liposomes to the macrophage cell surface after which the liposomes are taken up by the cells and degraded in the lysosomes which causes them to release their contents into the cells (19). Liposomal uptake by macrophages can be enhanced by altering its physicochemical properties. Small size (0.05 – 0.1 μm diameter) of liposomes can improve delivery to macrophages. Surface charge of liposome plays an important role. Anionic and cationic liposomes have been shown to have higher uptake by macrophages compared to neutral liposomes (19).

Targeting the macrophage mannose receptors:

Liposomes are rapidly taken up by macrophages of the RES in the liver thus making macrophages their natural targets (20). Despite being a natural target of liposomes, liver targeting is necessary as sometimes weak binding of liposomes to the macrophages results in inefficient liposome uptake by macrophages. As discussed above, in the process of uptake of liposomes by macrophages, the binding of the liposomes to the macrophages seems to be the rate limiting step (19). Earlier, the best approach to increase the rate of uptake by macrophages seemed to be the incorporation of negatively charged phospholipids into the liposomes but this is not macrophage specific as similar results were obtained with other cells like fibroblasts, lymphoma and myeloma (21). This problem can be overcome by incorporating ligands on the surface of these liposomes which are specific for receptors present on macrophages and can undergo rapid receptor
mediated endocytosis. There are various kinds of receptors on macrophages such as Fc receptors, carbohydrate receptors, complement receptors, fibronectin receptors, lipoprotein receptors and many others (19). There are two types of carbohydrate receptors. Galactose receptors which are also found on hepatocytes, bind to galactose-terminated ligands. Mannose receptors which are C-type animal lectins have been extensively studied and identified on many macrophage types (alveolar, peritoneal, spleen and kuper cells), hepatic and lymphatic endothelia, mesengial cells in the kidney, tracheal smooth muscle cells and retinal pigment epithelium (22) and seem to be the best receptors for targeting drugs to macrophages (20). This 175 kD receptor possesses eight carbohydrate recognition domains (CRDs) which recognize glycoconjugates having terminal fucose, D-mannose and N-acetylglucosamine with affinities in the order of L-fucose > D-mannose > D-N-acetyl-glucosamine >>> D-galactose (23) and facilitate binding of multivalent ligands in a calcium ion dependent manner. Binding to these receptors leads to internalization of the liposomes by endocytosis, followed by dissociation of the mannose receptors from the liposomes and the receptors are recycled back at the rate of 15 mins to the surface after internalization. The ligand is delivered to the lysosomes thus providing efficient cellular uptake of the ligand (21, 24). Thus inclusion of α-D-mannose residues on the surface of phospholipid containing liposomes can ensure their efficient cellular uptake by macrophages. Previous studies have shown that mannosylated liposomes are mainly taken up liver non parenchymal cells which include kupffer cells (24). Mannose can be incorporated in the surface of the liposomes by either inclusion of a mannose containing phospholipid into the lipid mixture or by coupling mannose to preformed liposomes (20). Naturally occurring glycolipids are the most convenient to use but the problems of purity and antigenecity exist. Mannosylated liposomes have an added advantage of not involving chemical modification of the drug to be incorporated, thus proving to be suitable targeted drug carriers. Several studies have shown increased uptake of mannosylated liposomes by macrophages compared to unconjugated liposomes suggesting increased binding of liposomes with D-mannose residues on their surface to macrophages. *In vivo* studies have shown maximal liver and spleen uptake after injecting p-amino-phenol sugars (25). The above studies suggest that mannosylated liposomes can be used to increase the accumulation of DFO in kuper cells.
C. Materials and Methods

3.1 Materials:

**Lipids and other materials:** Cholesterol (ovine wool), L-α-phosphatidylcholine, hydrogenated (soy) (HSPC), 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethyleneglycol)-2000](Ammonium salt) (mPEG-DSPE) 1,2-Dioleoyl-3-Trimethylmethylammonium-Propane (chloride salt) (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) were purchased from Avanti Polar Lipids, Inc., Alabama, USA. 4-Aminophenyl-α-D-Mannopyranoside was purchased from Medical Isotopes, Inc., Pelham, New Hampshire. Deferoxamine mesylate (DFO), L-Histidine, ammonium sulfate, sucrose, sodium chloride and chloroform were purchased from Sigma Aldrich St. Louis, MO, USA. Methanol (99% +) was purchased from Fisher Scientific, Pittsburgh, PA, USA. Chelex 100 was obtained from Biorad laboratories, Richmond, CA. All other reagents used were of analytical grade. The desalting columns (PD-10) were obtained from GE healthcare UK Limited, Buckinghamshire, UK. The dialysis membranes, Spectra/Por® dialysis membrane (MW cut off 3,500) were purchased from Spectrum Laboratories, Inc., CA.

**Equipment for Liposomal preparation and characterization:** Branson 450 digital sonifier was used for sonication of liposomes. Pressure Driven Extruder, Lipex™ and Whatman Nucleopore Track-Etch polycarbonate filters (0.8, 0.4, 0.2, 0.1 µm) are used for extrusion of liposomes and were purchased from Northern Lipids, Burnaby, BC Canada. Rotavapor R-215 advanced was purchased from Buchi Corporation, New Castle, DE, USA.
3.2 Methods:

Six different liposomal preparations were prepared for this investigation. DFO encapsulated - plain liposomes, pegylated liposomes, mannosylated liposomes, pegylated mannosylated liposomes, cationic liposomes, pegylated cationic liposomes were made.

3.2.1 Preparation of Liposomes:

3.2.1.1 Preparation of Multilamellar vesicles (MLVs):

Reverse Phase Evaporation Technique: The MLVs were prepared by reverse phase evaporation method described by Szoka and Papahadjopoulos with some modifications (26). Lipids (Molar ratios in Table 1) were dissolved in 50 ml of chloroform and the solution was added to a round bottom flask with a long extension neck. A DFO solution was prepared in normal saline by dissolving 300 mg of DFO 12.5 ml of saline (0.9% saline prepared in deionized water and filtered through 0.2µm filter). L-histidine (0.0155g) (buffer), ammonium sulfate (0.02g) (to hold the drug in the aqueous core of liposomes) and sucrose (0.94g) (for isotonicity) were added to the aqueous DFO solution. In case of mannosylated liposomes and pegylated mannosylated liposomes 4-Aminophenyl-α-D-mannopyranoside (0.008g) was added to the aqueous solution of DFO.

The aqueous solution was added to the lipid chloroform solution in the round bottom flask and the final mixture was vortexed vigorously for 6-7 minutes. Liposomes were then obtained by evaporating the organic solvent under reduced pressure by allowing the round bottom flask to rotate in the buchi rotavapor at a preset speed for 30 – 45 minutes. The round bottom flask was immersed in a water bath maintained at a preset temperature (Table 2) while evaporation was continued. Traces of chloroform were removed by additional rotaevaporation. 10ml of liposomal solution was obtained after rotaevaporation.
Table 1: Types of Liposomes and molar ratios of lipids used in each preparation.

<table>
<thead>
<tr>
<th>Type of liposome</th>
<th>Lipids used</th>
<th>Molar ratio</th>
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<tbody>
<tr>
<td>Plain Liposomes</td>
<td>HSPC, Cholesterol</td>
<td>60.3:39.65</td>
</tr>
<tr>
<td>Pegylated Liposomes</td>
<td>HSPC, Cholesterol, mPEG-DSPE</td>
<td>57.22:37.59:5.18</td>
</tr>
<tr>
<td>Cationic Liposomes</td>
<td>HSPC, Cholesterol, DOTAP</td>
<td>57.63:37.86:4.5</td>
</tr>
<tr>
<td>Pegylated cationic</td>
<td>HSPC, Cholesterol, mPEG-DSPE, DOTAP</td>
<td>54.77:35.99:4.95:4.28</td>
</tr>
<tr>
<td>Mannosylated Liposomes</td>
<td>HSPC, Cholesterol</td>
<td>60.3:39.65</td>
</tr>
<tr>
<td>Pegylated mannosylated</td>
<td>HSPC, Cholesterol, DSPE-mPEG</td>
<td>57.22:37.59:5.18</td>
</tr>
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</table>

3.2.1.2 Optimization of Large Unilamellar Vesicles (LUVs) preparation:

Since sonication and extrusion are the best available techniques for the preparation of LUVs, both these techniques were tried and tested for the preparation of LUVs. LUVs were prepared by three methods: sonication, sonication + extrusion and extrusion. These LUVs were then tested for their particle size and intraliposomal DFO concentration and based on these results; one technique was selected for the preparation of DFO loaded liposomes. Pegylated MLVs were randomly selected for this purpose.

**Sonication:** MLVs prepared by reverse phase evaporation were sonicated using a Branson 450 digital sonifier equipped with a microtip. MLVs were subjected to sonication at 10% amplitude and the time duration of sonication required for obtaining optimum particle size was optimized. MLVs were sonicated for 5, 10, 15, 20, 25 and 30 minutes on ice for this purpose and the particle size was analyzed for every time period.

**Extrusion:** The MLVs thus formed were made of the desired size (~ 100nm) by extruding them through a pressure driven extruder, Lipex which is maintained at a specific temperature based on the transition phase of the type of liposomal preparation (Table 2). For optimal results pegylated liposomes were extruded at 74°C. The liposomes are passed 2 – 3 times through 0.2µm Whatman Nucleopore Track-Etch polycarbonate filters and then eight times through 0.1µm filters in
order to obtain liposomes of size in the range 100 – 150 nm. The successive passage through a series of filters makes the extrusion of liposomes easier and faster.

The temperatures for rotaevaporation and extrusion were chosen according to the phase transition temperature (Tm) of the phospholipids used in each preparation.

**Table 2:** Reverse Phase evaporation and extrusion temperatures used for each type of liposomal preparation.

<table>
<thead>
<tr>
<th>Type of Liposome</th>
<th>Phospholipids</th>
<th>Phase Transition Temperature (Tm) (°C)</th>
<th>Reverse Phase Evaporation Temperature (°C)</th>
<th>Extrusion Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain</td>
<td>HSPC</td>
<td>55</td>
<td>42</td>
<td>60</td>
</tr>
<tr>
<td>Pegylated</td>
<td>HSPC</td>
<td>55</td>
<td>60</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>mPEG</td>
<td>74</td>
<td></td>
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</tr>
<tr>
<td>Mannosylated</td>
<td>HSPC</td>
<td>55</td>
<td>42</td>
<td>60</td>
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<tr>
<td>Pegylated mannosylated</td>
<td>HSPC</td>
<td>55</td>
<td>60</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>mPEG</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cationic</td>
<td>HSPC</td>
<td>55</td>
<td>42</td>
<td>60</td>
</tr>
<tr>
<td>Pegylated cationic</td>
<td>HSPC</td>
<td>55</td>
<td>60</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>mPEG</td>
<td>74</td>
<td></td>
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</tr>
</tbody>
</table>

Three new batches of pegylated MLVs were prepared and for the preparation of LUVs one batch was subjected to sonication (at 10% amplitude for 5 min). The second batch was sonicated (at 10% amplitude for 5 min) and extruded (twice through 0.1µm polycarbonate filter). The third batch was subjected to extrusion (twice through 0.2µm and eight times through 0.1µm polycarbonate filters) only. The liposomes were then chromatographed in sephadex G-25 columns (PD-10 columns) and extensively dialysed as described below (section 3.2.1.3). Intraliposomal DFO concentration of the liposomes (Table 5) prepared by the three techniques was measured after and before dialysis by the method described in section 3.2.2.2.

### 3.2.1.3 Removal of non-liposomal DFO:

Principle: Encapsulation of the entire amount of added DFO cannot be achieved. Thus the non-encapsulated DFO was removed by column chromatography. PD-10 desalting columns were used to desalt the liposome samples by column centrifugation method for the liposomal preparations. These columns contain *Sephadex G-25* medium, which allows rapid
separation of high molecular weight substances (>5000 M_r) from low molecular weight substances (24). Thus large molecules which are larger than the column pores will be eluted first and the small molecules which are smaller than the column pores will get entrapped in the pores. Thus DFO which has a molecular size of 656.79 will get entrapped in the column and liposomes will be eluted in the void volume of the PD-10 columns.

Method: The columns are first equilibrated with the equilibration buffer (saline in this case) by allowing the buffer (25 ml) to flow through the packed column bed. This is repeated three times. Then the columns were centrifuged at 1000 X g for 4 minutes at room temperature and the flow – through volume sample was discarded. The columns then placed in fresh falcon tubes and the liposomal preparations (1.75 – 2.5 ml per column) were then loaded on the packed columns. Elution was obtained by centrifugation at 1000 X g for 4 minutes at room temperature. The eluate containing liposomes minus free DFO was collected in the eluate. All centrifugation was undertaken at 4°C in a refrigerated desktop Beckmann centrifuge.

In order to further purify the preparation and also to make them ion free all liposome preparations in this research were dialyzed against 4 litres of 0.1M Phosphate buffered saline containing 0.5 g of Chelex 100. The liposomes were placed in a 3500 MW cut off dialysis bag and dialyzed overnight at 4ºC. Further dialysis of liposomes against large volumes (4 litres) of 0.1M Phosphate Buffered Saline was carried out for 24 hours (change of buffer every 4 hours for the first 12 hours) to ensure complete removal of the remaining non-liposomal DFO. The liposomes were then stored at 4ºC.

3.2.2 Characterization of DFO-loaded Liposomes:

3.2.2.1 Determination of maximal absorbance wavelength (λ_max) of DFO:

In order to determine the spectrophotometric absorbance of DFO, DFO was dissolved in methanol at the concentration of 0.01mg/ml and a full spectral scan was performed using the Spectronic Genesys 5 instrument.

3.2.2.2 Intraliosomal DFO concentration:

Optical density was used to measure the intraliposomal DFO concentration. For this purpose, the maximal absorbance wavelength of DFO determined previously was used and a standard DFO calibration curve was generated.
a. Preparation of Standard Calibration curve for DFO:

A standard calibration curve for DFO was prepared using increasing concentrations of DFO in methanol. A 0.1mg/ml stock solution of DFO in methanol was prepared by dissolving 10mg of DFO in 100ml of methanol. In order to generate a calibration curve, dilutions of the stock solution were made and the optical density of each dilution was read at 204nm (Tables 3 and 6). This was repeated three times.

**Table 3:** Concentrations of DFO in methanol prepared for calibration curve.

<table>
<thead>
<tr>
<th>0.1mg/ml DFO stock solution (ml)</th>
<th>Methanol (ml)</th>
<th>[DFO] mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>9.9</td>
<td>0.001</td>
</tr>
<tr>
<td>0.25</td>
<td>9.75</td>
<td>0.0025</td>
</tr>
<tr>
<td>0.5</td>
<td>9.5</td>
<td>0.005</td>
</tr>
<tr>
<td>0.75</td>
<td>9.25</td>
<td>0.0075</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>0.01</td>
</tr>
<tr>
<td>1.5</td>
<td>8.5</td>
<td>0.015</td>
</tr>
<tr>
<td>2.0</td>
<td>8</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The mean values of the optical densities at 204nm obtained for each DFO concentration was used to generate a scatter plot with regression line using Microsoft excel.

b. Determination of Concentration of intraliposomal DFO:

When liposome membranes are dissolved, they release their intraliposomal content of DFO. Liposomes were treated with methanol and the DFO that is released into the solution was determined by measuring the optical density of the solution at 204nm using methanol as blank. 12.5μl of each type of liposomal preparation was transferred to a glass vial. The liposomes were then diluted 400 times with methanol. The diluted samples were vortexed to dissolve the lipids in methanol leading to release of DFO. The optical density of the dilutions was determined at 204nm and the value (mg/ml) was extrapolated from the standard calibration curve of DFO previously prepared. The DFO concentration was then multiplied by the dilution factor (400) to obtain the final
concentration of intraliposomal DFO. The concentration of DFO was determined before and after complete
dialysis of liposomes.

c. Encapsulation efficiency of liposomes:

The intraliposomal DFO concentration value gives the maximum drug encapsulation. The total amount of DFO
per preparation of liposomes was calculated by multiplying the intraliposomal DFO concentration (after dialysis)
obtained above by the volume of liposomes recovered after rotaevaporation. The encapsulation efficiency of
liposomes or the % Incorporation of the DFO in liposomes was calculated by using the formula:

\[
\text{% incorporation} = \left(\frac{\text{total amount of DFO per preparation}}{\text{mg of DFO added to the preparation}}\right) \times 100
\]

3.2.2.3 Measurement of the particle size of liposomes:

As mentioned above, the stability of liposomes seems to be best in the range of 100 – 300nm (13). Particle size was
measured using dynamic light scattering also known as quasi elastic light scattering which is one of the most popular
techniques to determine particle size (28, 29). The principle behind this technique is that when a light beam hits a particle
which is in random motion or ‘Brownian’ motion, it causes a Doppler shift in the wavelength of the incoming light and
this shift relates to the size of the particle (28). This technique requires a very short time to measure the particle size.

![Dynamic Light Scattering Diagram](image)

**Figure 2:** Diagram illustrating Dynamic Light Scattering method\(^{(30)}\).
Brookhaven 90 Plus Nanoparticle size analyzer (Brookhaven Instruments Corporation, NY, USA) was used to measure the particle size of the liposomes. The particle size was measured at an angle of 90 degrees using a 35mW solid state standard laser. All measurements were made at 25 °C and 657nm wavelength of light. Briefly, 5µl of liposomal solution was placed in a cuvette and diluted to 3ml with saline. After capping the cuvette the sample was shaken well and placed in the sample holder. The sample size was measured in 5 runs of 30 seconds each.

3.2.2.4 Measurement of Zeta Potential of liposomes:

There exists an electrical double layer at the interface between solid particle surface and the surrounding liquid medium. The inner electrical layer is tightly bound to the surface whereas the outer layer where balanced charges due to electrostatic forces and Brownian motion develop is relatively loosely attached (31). The liposomal particle moves in the liquid medium along with its associated charges as a unit. The ionic potential at the surface between this unit and the surrounding medium is called the zeta potential and it is measured in millivolts (31). Measurement of zeta potential is necessary as the stability of a liposomal suspension greatly depends on the electrical double layer at the interface.

For the measurement of zeta potential of the DFO loaded liposomes, ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation) equipped with a palladium electrode with acrylic support was used. All the measurements were taken at 25°C before and after the dialysis of the liposomes.

3.2.3 In vitro Studies:

Rhodamine labeled mannosylated, cationic and plain liposomes were used for in vitro experiments. The liposomes were prepared as mentioned above using HSPC, Cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) in the ratio 60:40:0.5. In case of cationic liposomes 2% DOTAP was used. Plain, cationic and mannosylated fluorescent liposomes were characterized using zeta potential, particle size for this experiment.

Cell Culture:

To check the binding of mannosylated liposomes to macrophages in comparison to plain and cationic liposomes, a primary mouse macrophage cell line (J774A.1) was used. Briefly, the macrophage cell line was maintained in high glucose concentration (4.5 g/l) supplemented by Dulbecco’s Modified Eagle Medium (DMEM) and 10 % fetal clone with appropriate amounts of antibiotics (penicillin/streptomycin). Cells were grown in T-25 flask until confluent at 37 °C and
were sparged with 5% CO₂ in a sterile incubator set up (Thermo Scientific Inc.) Adherent macrophages on reaching confluency were then removed using cell scraper. In case of BT-20, a human mammary adenocarcinoma adherent cell line maintained under same conditions as macrophages, cells were removed using 0.25% trypsin on reaching confluency. BT-20 was used as a negative control for mannosylated liposomes. Approximately 40,000 cells were plated in a 6 well plate and incubated at 37°C. On reaching confluency the cells were treated with liposomes (0.5mg/ml) (the volume in the well was made up to 1ml with culture media) and incubated at 4°C for 45 minutes. After 45 minutes, liposomal solution in the wells was replaced by cold 0.1M PBS and the cells were removed by scraping (J774A.1) or by adding 0.25% trypsin (BT-20). The cell suspension in cold PBS was then centrifuged at 2000rpm for 4 minutes and then re-suspended in new PBS, rewashed and centrifuged again. The cells were then suspended in about 200µl of cold PBS and the sample was used for fluorescent microscopy using Bioquant camera – microscope and FACS analysis (using Becton-Dickinson FACSCalibur) studies.

3.2.4 In Vivo experiments:

Targeting of mannosylated and cationic liposomes to rat liver and heart:

For this experiment rhodamine labeled plain, mannosylated and cationic liposomes were prepared and characterized. The formulations were assessed by injecting them into 12 Sprague-Dawley rats (4 for each formulation). The rats were anaesthetized using ketamine/ xylazine (80:20) following which the liposomal formulations were injected intravenously. The rats were then sacrificed after 3 hours. Liver and heart of all the rats were collected. The organs were stored in Tissue-Tek® O.C.T. compound at -80°C until further use. Cryosections (20 micron thickness) of each organ were prepared using a cryostat (Dr. Waszczak’s lab). The sections were then evaluated using fluorescence microscopy and the intensity of fluorescence was evaluated by computer planimetry using ImageJ software.

D. Results
4.1 Optimization of Large Unilamellar Vesicles (LUVs) preparation:

Sonication: After sonicating the pegylated liposomes at 10% amplitude for 5, 10, 15, 20, 25 and 30 minutes on ice the following particle sizes obtained are as shown in Table 4 and Figure 3.

**Table 4:** Particle size distribution of pegylated liposomes after different durations of sonication.

<table>
<thead>
<tr>
<th>Sonication time (minutes)</th>
<th>Particle size(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>111.2</td>
</tr>
<tr>
<td></td>
<td>99.6</td>
</tr>
<tr>
<td></td>
<td>119.4</td>
</tr>
<tr>
<td>10</td>
<td>66.4</td>
</tr>
<tr>
<td></td>
<td>63.9</td>
</tr>
<tr>
<td></td>
<td>74.2</td>
</tr>
<tr>
<td>15</td>
<td>30.3</td>
</tr>
<tr>
<td></td>
<td>35.9</td>
</tr>
<tr>
<td></td>
<td>28.8</td>
</tr>
<tr>
<td>20</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td>61.1</td>
</tr>
<tr>
<td></td>
<td>54.4</td>
</tr>
<tr>
<td>25</td>
<td>73.2</td>
</tr>
<tr>
<td></td>
<td>68.8</td>
</tr>
<tr>
<td></td>
<td>81.3</td>
</tr>
<tr>
<td>30</td>
<td>87.4</td>
</tr>
<tr>
<td></td>
<td>82.7</td>
</tr>
<tr>
<td></td>
<td>93.6</td>
</tr>
</tbody>
</table>
Figure 3: Particle sizes of pegylated liposomes (n=3) sonicated at 10% amplitude for different time periods. The error bars represent ± standard deviation.

As shown in figure 3 the particle size of pegylated liposomes decreased by almost half when the time was increased from 5 to 10 minutes and again by half from 10 to 15 minutes. After 15 minutes there was an increase in the size observed until 30 minutes. From the above results 5 minutes was chosen as the optimum time required for sonication at 10% amplitude in order to obtain maximum number of LUVs having size near 100nm.

Extrusion: The particle sizes of the liposomes prepared by ‘sonication only’ and ‘extrusion only’ techniques were measured before and after dialysis and are shown in figure 4. Liposomes prepared by ‘sonication and extrusion’ technique had results similar to ‘extrusion only’ technique.

Table 5: Intraliposomal DFO concentration of liposomes prepared by three techniques.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Intraliposomal DFO concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before dialysis</td>
</tr>
<tr>
<td>Sonication only</td>
<td>15.4</td>
</tr>
<tr>
<td>Sonication and extrusion</td>
<td>14</td>
</tr>
<tr>
<td>Extrusion only</td>
<td>9.8</td>
</tr>
</tbody>
</table>
It was found that the decrease in the intraliposomal DFO concentration after dialysis was the least in liposomes prepared by ‘extrusion only’ technique.

**Figure 4:** Particle size distribution of liposomes prepared by ‘sonication only’ and ‘extrusion only’ techniques before and after dialysis. (A) ‘Sonication only’ before dialysis (78nm to 128.6nm) (B) ‘Sonication only’ after dialysis (395nm to 625 nm) (C) ‘Extrusion only’ before dialysis (137.3nm to 141.9nm) (D) ‘Extrusion only’ after dialysis (143.5nm to 145.4nm).

As per the results obtained in table 5, the intraliposomal DFO concentration was maximum only when extrusion was used. As seen in figure 4 the size of the LUVs which were prepared by the ‘sonication only’ technique increased by almost 5 fold after dialysis while the size of ‘extrusion only’ and ‘sonication and extrusion’ LUVs remained the same. On the basis of these results ‘extrusion only’ technique was used for further preparation of DFO loaded liposomes.
4.2 Determination of maximal absorbance wavelength ($\lambda_{\text{max}}$) of DFO:

The maximal absorbance wavelength of DFO was found to be 204nm as shown in figure 5.

![Graph showing the maximal absorbance wavelength of DFO ($\lambda_{\text{max}}$) which was determined to be 204nm.]

**Figure 5:** Spectrum showing the maximal absorbance wavelength of DFO ($\lambda_{\text{max}}$) which was determined to be 204nm.

4.3 DFO calibration curve, intraliposomal DFO concentration and encapsulation efficiency:

The optical densities of the dilutions, prepared from the stock solution of DFO as shown in Table 3, measured at 204nm using spectrophotometer were as shown below in Table 6.
Table 6: Mean optical density values of various DFO solutions at 204nm

<table>
<thead>
<tr>
<th>[DFO] mg/ml</th>
<th>OD at 204nm</th>
<th>Mean OD at 204nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>0.046</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>0.070</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.080</td>
<td></td>
</tr>
<tr>
<td>0.0025</td>
<td>0.126</td>
<td>0.139</td>
</tr>
<tr>
<td></td>
<td>0.163</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.127</td>
<td></td>
</tr>
<tr>
<td>0.005</td>
<td>0.269</td>
<td>0.262</td>
</tr>
<tr>
<td></td>
<td>0.246</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.271</td>
<td></td>
</tr>
<tr>
<td>0.0075</td>
<td>0.368</td>
<td>0.355</td>
</tr>
<tr>
<td></td>
<td>0.338</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.360</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>0.476</td>
<td>0.487</td>
</tr>
<tr>
<td></td>
<td>0.504</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.481</td>
<td></td>
</tr>
<tr>
<td>0.015</td>
<td>0.700</td>
<td>0.708</td>
</tr>
<tr>
<td></td>
<td>0.718</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.705</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>0.927</td>
<td>0.923</td>
</tr>
</tbody>
</table>

The mean values of the optical densities at 204nm obtained for each DFO concentration were used to generate a scatter plot with regression line r using Microsoft excel (Figure 6).

![Figure 6: Calibration Curve for Deferoxamine mesylate.](image-url)
Intraliposomal concentration and encapsulation efficiency calculated as shown above are shown in Table 7 below.

**Table 7:** Intraliposomal DFO concentration and encapsulation efficiency of liposomes.

<table>
<thead>
<tr>
<th>Type of liposome</th>
<th>Intraliposomal DFO concentration (mg/ml)</th>
<th>Encapsulation efficiency (%) after complete dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before dialysis</td>
<td>After Dialysis</td>
</tr>
<tr>
<td>Plain Liposome</td>
<td>8.6mg/ml ± 1.74</td>
<td>2.2mg/ml ± 0.18</td>
</tr>
<tr>
<td>Pegylated Liposome</td>
<td>6.8mg/ml ± 1.1</td>
<td>2.6mg/ml ± 0.2</td>
</tr>
<tr>
<td>Mannosylated Liposome</td>
<td>4.6mg/ml ± 0.4</td>
<td>1.9mg/ml ± 0.13</td>
</tr>
<tr>
<td>Pegylated Mannosylated Liposome</td>
<td>9.1mg/ml ± 4.2</td>
<td>1.7mg/ml ± 0.2</td>
</tr>
<tr>
<td>Cationic Liposome</td>
<td>10.3mg/ml ± 3.4</td>
<td>2mg/ml ± 0.19</td>
</tr>
<tr>
<td>Pegylated Cationic Liposome</td>
<td>9.4mg/ml ± 2.6</td>
<td>2mg/ml ± 0.16</td>
</tr>
</tbody>
</table>

**4.4 Measurement of Particle Size:**

Particle size and polydispersity were measured using dynamic light scattering. The distribution width or the polydispersity index of all the samples was determined to be in the range of 0.035 – 0.16. The particle sizes measured before and after dialysis were as shown in Table 8 below.

**Table 8:** Particle size measurements of liposomes before and after dialysis.

<table>
<thead>
<tr>
<th>Type of Liposome</th>
<th>Particle size before dialysis (nm)</th>
<th>Particle size after dialysis (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain Liposome</td>
<td>102.55 ± 6.75</td>
<td>109 ± 1.9</td>
</tr>
<tr>
<td>Pegylated Liposome</td>
<td>139.6 ± 2.3</td>
<td>144.45 ± 0.95</td>
</tr>
<tr>
<td>Mannosylated Liposome</td>
<td>143.6 ± 33.9</td>
<td>150 ± 35.4</td>
</tr>
<tr>
<td>Pegylated Mannosylated Liposome</td>
<td>127.05 ± 10.15</td>
<td>118.4 ± 18.6</td>
</tr>
<tr>
<td>Cationic Liposome</td>
<td>114.2 ± 14.4</td>
<td>129 ± 20.2</td>
</tr>
<tr>
<td>Pegylated Cationic Liposome</td>
<td>134.9 ± 41.8</td>
<td>122.8 ± 20.1</td>
</tr>
</tbody>
</table>

Figure 7 shows the multi modal size distribution of the liposomes.
Figure 7: Multimodal size distribution of DFO loaded liposomes after dialysis. (A) Plain liposomes (B) Pegylated liposomes (C) Mannosylated liposomes (D) Pegylated Mannosylated liposomes (E) Cationic liposomes (F) Pegylated Cationic liposomes.
4.5 Measurement of Zeta Potential:

The zeta potential of the liposomes measured before and after dialysis was as shown in Table 9 and Figure 8:

Table 9: Mean zeta potential measurements before and after dialysis of liposomes with standard error.

<table>
<thead>
<tr>
<th>Type of liposome</th>
<th>Zeta potential (millivolts)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before dialysis</td>
</tr>
<tr>
<td>Plain liposomes</td>
<td>-20.76 ± 1.20</td>
</tr>
<tr>
<td>Pegylated liposomes</td>
<td>-41.08 ± 1.51</td>
</tr>
<tr>
<td>Mannosylated liposomes</td>
<td>-29.37 ± 4.88</td>
</tr>
<tr>
<td>Pegylated mannosylated liposomes</td>
<td>-31.06 ± 10.72</td>
</tr>
<tr>
<td>Cationic liposomes</td>
<td>29.66 ± 0.84</td>
</tr>
<tr>
<td>Pegylated Cationic liposomes</td>
<td>-22.01 ± 2.45</td>
</tr>
</tbody>
</table>

As seen in table 8 the zeta potential measurements after dialysis were less positive for cationic liposomes and more negative for the remaining liposomes compared to the zeta potential before dialysis.
Figure 8: Zeta potential measurements after dialysis. (A) Plain liposomes (B) Pegylated liposomes (C) Mannosylated liposomes (D) Pegylated Mannosylated liposomes (E) Cationic liposomes (F) Pegylated Cationic liposomes.
4.6 *In vitro studies:*

**FACS Analysis:**

FACS analysis or cell sorting which is an application of flow cytometry is used to separate cells according to subtype or epitope expression for biological studies. Here, the cells after being treated with fluorescent labeled liposomes are subjected to a filter which identifies cells according to their size and fluorescent intensity. The higher the fluorescent intensity, higher the amount of liposomes bound to the cells. This experiment distinguishes the binding efficiency of mannosylated liposomes to macrophages which have mannosylated receptors, from cationic and plain liposomes. As mentioned previously, J774A.1 a murine macrophage cell line was used to test the binding efficiency of mannosylated liposomes and BT 20, a tumor cell line was used as a negative control for mannosylated liposomes. The results obtained after FACS analysis of the 2 cell lines are as shown in Figure 9.

(A) J774A.1 (Macrophages)  
Untreated cells:

(B) BT 20 (Tumor cell line – negative control)  
Untreated cells:

Plain Liposomes:

Plain Liposomes:
Figure 9: FACS Analysis of J774A.1 and BT 20 cells treated with Rhodamine labeled liposomes. Panel A shows forward scatter and side scatter plot and fluorescent intensity plot of J774A.1 cells untreated and treated with plain, cationic and mannosylated liposomes from top to bottom. Panel B shows forward scatter and side scatter plot and fluorescent intensity plots of BT 20 cells untreated and treated with plain, cationic and mannosylated liposomes from top to bottom.
Fluorescence Microscopy:

In vitro fluorescence microscopy was performed to demonstrate binding of mannosylated liposomes to J774A.1 murine macrophage cell line. The results obtained in this study were as shown in Figure 10 (A) and (B). Figure 10(A) shows 10 X bright fields (BF), differential interference contrast (DIC) and fluorescent images (FL) of cells alone, cells treated with mannosylated, cationic and plain liposomes. Figure 10 (B) shows the corresponding 20X images.
Panel (A): J774A.1 cells alone

Panel (B): Cells treated with mannosylated liposomes

Panel (C): Cells treated with cationic liposomes

Panel (D): Cells treated with plain liposomes

Figure 10(A): Panels A, B, C and D show BF, DIC and FL images of cells alone and cells treated with liposomes at 10X.
Panel (A): J774A.1 cells alone:

(BF)  (DIC)  (FL)

Panel (B): cells treated with mannosylated liposomes:

(BF)  (DIC)  (FL)

Panel (C): cells treated with cationic liposomes:

(BF)  (DIC)  (FL)

Panel (D): cells treated with plain liposomes:

(BF)  (DIC)  (FL)

Figure 10(B): Panels A, B, C and D show BF, DIC and FL images of cells alone and cells treated with liposomes at 20X.
4.7 In vivo Studies:

After preparing the cryosections and evaluating them by fluorescence microscopy the results obtained were as shown in Figures (11) and (12):

Cationic Liposomes                Mannosylated Liposomes          Plain Liposomes

**Figure 11:** Fluorescent and bright field images of cryosections of rat heart. The above images are cryosections of rat heart observed under 10X. Shown in the first panel are fluorescent images in the order cationic liposomes, mannosylated liposomes, plain liposomes and the second panel shows the corresponding bright field images of the cryosections in the same order.

To calculate the fluorescent intensities of the images above, intensities of four different areas (0.490 pixel units) of one image were measured using the ImageJ software. The intensity of the background fluorescence of the image was measured in a similar manner. The intensity of the background was then subtracted from the intensity of the fluorescent area and the mean intensity of the four areas of the image was calculated along with the standard deviation. Finally, the mean intensity of all images was calculated along with the standard deviation and the measurements were as shown in Table 10.
Table 10: Fluorescent intensities of cryosections of rat heart treated with Rhodamine labeled liposomes

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Mean Area (pixel units)</th>
<th>Mean Fluorescent intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic Liposomes</td>
<td>0.4925 ± 0.009</td>
<td>41.686 ± 19.2436</td>
</tr>
<tr>
<td>Mannosylated Liposomes</td>
<td>0.4975 ± 0.009</td>
<td>1.177 ± 0.0962</td>
</tr>
<tr>
<td>Plain Liposomes</td>
<td>0.5035 ± 0.006</td>
<td>6.138 ± 0.310</td>
</tr>
</tbody>
</table>
Figure 12: Fluorescent and bright field images of cryosections of rat liver. The above images are the cryosections of rat liver observed under 10X. Shown in the first panel are fluorescent images in the order cationic liposomes, mannosylated liposomes, plain liposomes and the second panel shows the corresponding bright field images of the cryosections in the same order.

The method of calculation of the fluorescent intensities of the images of cryosections of rat liver was the same as that of rat heart. The measurements are as shown in Table 11.

Table 11: Fluorescent intensities of cryosections of rat liver treated with Rhodamine labeled liposomes

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Mean area (pixel units)</th>
<th>Mean fluorescent intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic Liposomes</td>
<td>0.499 ± 0.009</td>
<td>55.98 ± 13.98</td>
</tr>
<tr>
<td>Mannosylated liposomes</td>
<td>0.4935 ± 0.0049</td>
<td>41.67 ± 16.53</td>
</tr>
<tr>
<td>Plain Liposomes</td>
<td>0.4795 ± 0.0057</td>
<td>42.604 ± 3.07</td>
</tr>
</tbody>
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5. Discussion

The results in section 4.1 show that liposomes prepared by ‘sonication’ and ‘sonication with extrusion’ showed tremendous decrease (by almost 90%) in intraliposomal DFO concentration after extensive dialysis. The particle size of liposomes prepared by ‘sonication’ and ‘sonication with extrusion’ increased by almost 5 times in diameter after dialysis which implies that these liposomes might have become leaky during dialysis which led to loss of encapsulated DFO and thus show a decreased intraliposomal concentration. On the other hand, liposomes prepared by ‘extrusion’ retained almost 30% of the encapsulated DFO after dialysis. Figure 4 also shows that liposomes prepared by ‘extrusion’ remained intact in size even after extensive dialysis.

On the basis of these results ‘extrusion’ was selected as the appropriate method for the preparation of liposomes after reverse phase evaporation. These studies fulfill Specific Aim 1 which was to optimize the method of preparation of small unilamellar vesicles.

The mean intraliposomal concentration of DFO of all types of liposomes was 8.1 ± 1.9mg/ml before dialysis and 2.06 ± 0.28 mg/ml after dialysis and the mean encapsulation efficiency was found to be 6.88 ± 0.5%.

The polydispersity index of the liposomes was in the range 0.035 – 0.16 which suggests that liposomes had a very tight size distribution peak. This shows that the liposomes were in the range 90 – 160 nm and had a homogenous distribution. The liposomes maintained the same size even after dialysis attributing to the rigidity of the liposome bilayer.

The positive zeta potential value of the cationic liposomes confirmed the presence of DOTAP on their surface. The negative zeta potential of pegylated liposomes confirmed the presence of PEG on their surface and was close to the doxil zeta potential. The plain liposomes had a neutral zeta potential. As seen in Table 8 the zeta potential measurements for cationic liposomes were less positive after dialysis and more negative for the remaining liposomes after dialysis which implies that non-encapsulated DFO had some effect on the surface charge of liposomes.

These results satisfy Specific Aim 2 which was to characterize the six liposomal preparations.

In vitro study was conducted on J774A.1 murine macrophage cell line to demonstrate increased binding of mannosylated liposomes to macrophages compared to plain and cationic liposomes and on BT 20 human mammary adenocarcinoma cell line to demonstrate decreased binding of mannosylated liposomes to non-macrophage cell line compared to plain and cationic liposomes. As shown in Figure panel (A) J774A.1 cells treated with plain (mean intensity 32.51) and cationic (mean intensity 64.13) liposomes had low fluorescent intensity when compared to mannosylated
liposomes (mean intensity 173.39). As shown in panel (B) BT 20 cells treated with mannosylated liposomes (mean intensity 14.80) had low fluorescent intensity compared to plain (mean intensity 42.68) and cationic (mean intensity 31.84) liposomes.

These results indicate that mannosylated liposomes show increased binding to J774A.1 cells which express mannose receptors on their surface and show decreased binding to BT 20 cells which do not express mannosylated receptors which implies that these liposomes are mannose receptor specific.

Fluorescence microscopy of J774A.1 also showed that mannosylated liposomes showed increased binding to macrophage cell line compared to cationic and plain liposomes as seen in Figures 10 (A) & (B).

These results complete Specific Aim 3 which is to evaluate the binding of mannosylated liposomes to macrophage and non macrophage cells.

In the in vivo studies conducted, Sprague-dawley rats were treated with cationic, mannosylated and plain liposomes. Fluorescent microscopy of cryosections of heart demonstrated that rats treated with cationic liposomes had high distribution in heart with highest fluorescent intensity (41.686 ± 19.2436). Plain liposomes on the other had displayed very low (6.138 ± 0.310) distribution in the heart whereas mannosylated liposomes had a nearly negligible (1.177 ± 0.0962) distribution in heart.

Cationic liposomes also displayed the highest fluorescent intensity (55.98 ± 13.98) and distribution in the liver as seen in the fluorescent images in Figure 11. This suggests that cationic liposomes show nonspecific targeting to the liver. Plain liposomes also exhibit nonspecific targeting to the liver but with much less intensity (42.604 ± 3.07) compared to the cationic liposomes. Though plain liposomes have liver as their natural target, cationic liposomes can be more useful in nonspecific targeting to the liver as they accumulate in greater amounts in the liver. Mannosylated liposomes on the other hand show a more localized distribution in the liver as seen in Figure 11. This implies that mannosylated liposomes target specific cells of the liver. Since the fluorescent image display cells which are somewhat spindle shaped which resemble the kupffer cells in the liver it can be said that mannosylated liposomes target kupffer cells in the liver.

The results of the in vivo experiment accomplish Specific Aim 4 which was to demonstrate targeting of mannosylated and cationic liposomes to rat liver and heart respectively.
6. Summary and Conclusion

The liposomes were characterized for their size, zeta potential and incorporation capacity to be as close as possible to doxil, which is a commercially marketed liposomal formulation of doxorubicin. The fully characterized liposomes were then evaluated for their targeting efficiency by in vitro and in vivo experiments. These experiments indicated that mannosylated liposomes can be used to target macrophages which are major stores of iron in iron intoxicated patients. Experiments also displayed that cationic liposomes can be used for non specific targeting to the liver, mainly hepatocytes which can be used as a potential treatment for hepatotoxicity due to iron overload in thalassemia major patients. Cationic liposomes can also be used to target the heart as seen in the in vivo experiments and thus can be used to treat iron induced cardiomyopathy which is the main cause of mortality in thalassemia major patients.

It can be concluded that surface modification of liposomes can help in targeting the liver specifically and nonspecifically. The experiments conducted in this study helped in proving the hypothesis that novel surface modified liposomes can be used to target specific organs in the body for potential use in iron chelation therapy. The novel DFO loaded liposomes can help improve the current DFO infusion therapy for iron chelation which is tedious and expensive for patients.
7. Bibliography


12. www.emdchemicals.com


17. Vicente J. Caride and Barry L. Zaret, Liposome accumulation in regions of experimental myocardial infarction. 


19. Fakhrul Ahsana, Isabel P. Rivashb, Mansoor A. Khana, Ana I. Torres Suárezb. Targeting to macrophages: role of 
   physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by 

   (1993).


   (2005).


24. Praneet Opanasopit, et al., Inhibition of liver metastasis by targeting of immunomodulators using mannosylated 

25. Ghosh P. and Bacchawat, B. K., Grafting of different glycosides on the surface of liposomes and its effect on 
   the tissue distribution of $^{125}$I-labeled $\gamma$-globulin encapsulated in liposomes. *Bichimica et Biophysica Acta.* 632: 
   562-572 (1980).

26. Francis Szoka, Jr., and Demetrios Papahadjopoulos, Procedure for preparation of liposomes with large internal 
   aqueous space and high capture by reverse-phase evaporation. *Proceedings of the National Academy of 
   Sciences, USA* 75 (9):4194-4198 (1978).

27. Instructions and protocol for PD-10 desalting columns. 
   2130800BB.pdf

28. Martha Sartor, Dynamic light scattering to determine the radius of small beads in Brownian motion in a 
   solution. University of California, San Diego. 2003


Appendix 1: Radiation Safety Training
Radiation Safety Training & Exam for Initial Workers

Northeastern University Office of Environmental Health & Safety

On April 30, 2009, Parita Ghia successfully completed the "Radiation Safety Training & Exam for Initial Workers (For people that will be working with radioactive materials)" program on the web, answering 90% of the 10 questions correctly (At least 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: Record Locator: tw0000000006537 (Session).

Refresher Radiation Training for SEALED sources (e.g. Co-57, Ru-106, PT-197)

Northeastern University Office of Environmental Health & Safety

On March 17, 2010, Parita Ghia successfully completed the "Refresher Radiation Training for SEALED sources (e.g. Co-57, Ru-106, PT-197)" program on the web, answering 100% of the 10 questions correctly (At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: Record Locator: tw0000000009754 (Session).
Appendix 2: Occupational Health & Safety for Working with Animals in Research and Teaching.

In June 2009, Parita Ghia completed the following requirements for working with Animals in Research and Teaching:

- Participated in the training program
- Is added to the research protocol
- Received Animal Facility Orientation from DLAM Staff

For any questions, contact Sean Sullivan, Director of NU-IACUC & DLAM at (617)-373-3958 or s.sullivan@neu.edu
Appendix 3: Laboratory Safety Training

Chemical Hygiene Training Part 1 - Right to Know

Northeastern University Office of Environmental Health & Safety

On April 16, 2010, Parita Ghia successfully completed 01: "Chemical Hygiene Training Part 1 - Right To Know" program on the web, answering 93% of the 15 questions correctly (At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: To complete this training you must also take Part 2

Record Locator: tw0000000009911 (Session).

Chemical Hygiene Training Part 2 - Lab Safety and Hazardous Waste Management

Northeastern University Office of Environmental Health & Safety

On April 16, 2010, Parita Ghia successfully completed 02: "Chemical Hygiene Training Part 2 - Lab Safety and Hazardous Waste Management" program on the web, answering 100% of the 10 questions correctly (At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: Record Locator: tw0000000009916 (Session).