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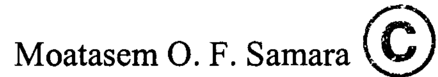
pH Sensitive Liposomes as a Drug Delivery System to Hepatocytes

by


Moatasem O. F. Samara ©

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DEDICATED TO

My loving parents: Omar and Yusra. Your love and faith are a light that guides me through life.

My loving siblings: Mohammad, Maisa, Maysoon, Mahmood and Fayez. I start with you when I count my blessings.

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Abstract

The purpose of this project was to formulate, modify, evaluate and test pH sensitive liposomes targeting the asialoglycoprotein receptor (ASGP-R) found in large numbers exclusively on the surface of hepatocytes. Lactosylceramide was introduced at different concentrations to an oleyl alcohol based formulation. The safety, pH sensitivity, stability and *in vitro* uptake efficiency by murine Hepa 1c1c7 hepatoma cell-line, in which the presence of ASGP-R was confirmed, of the original recipe and modified formulations were evaluated. Liposomes containing 0.5% lactosylceramide were shown to posses efficient targeting properties while retaining desired ones. The *in vivo* biodistribution of original and modified recipes was evaluated and increased liver uptake was confirmed for both recipes. Further tests are required to establish the distribution between the different liver tissues. Negative results were obtained when testing survival of biologically active material after intracellular delivery and further testing is required to confirm or negate these results.

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LIST OF ABBREVIATIONS

%	Percent
λ	Wave Length
μg	micro gram
μl	micro liter
3D	Three dimensional
AF	Asialofetuin
ASGP-R	Asialoglycoprotein Receptor
ATPases	Adenosine Triphosphate Catalyzing Enzyme
BSA	Bovine Serum Albumin
CHEMS	Cholesteryl Hemiscuccinate
cm^2	Square Centimeter
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOPE	Dioleoylphosphatidylethanolamine
EDTA	Ethylenediaminetetraacetic Acid
Egg PC	Phosphatidylcholine (from Chicken, Egg)
ELISA	Enzyme-Linked ImmunoSorbent Assay
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GI	Gastrointestinal

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HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
hr	Hour
HRPO	Horseradish Peroxidase
IgG	Immunoglobulin Class G
IV	Intravenous
Lac-Cer	C8 ß-D-Lactosylceramide
LUVs	Large Unilamellar Vesicles
M.W.	Molecular Weight
MAbs	Monoclonal Antibodies
mg	milligram
MHC-I	Major Histocompatibility Complex Class 1
min	minute
ml	milliliter
MLVs	Multilamellar Vesicles
mM	millimolar
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Normal
nm	nanometer
OAlc	Oleyl Alcohol
°C	Degrees Centigrade
OD	Optical Density

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PBS	Phosphate Buffered Saline
PC membrane	Polycarbonate Membrane
PEG	Polyethylene Glycol
PI	Proeatese Inhibitors
RES	Reticulo-Endothelial System
RNA	Ribonucleic Acid
rpm	Rounds Per Minute
scFv	Single Chain Antibodies
sf	Serum Free
SUVs	Small Unilamellar Vesicles
T-80	Tween 80
^{99m}Tc DTPA	Technetium Diethylenetriamine Pentaacetate
TCPs	Tissue Culture Plates
TMB	Tetramethylbenzidine
TRITC	Tetramethyl Rhodamine Isothiocyanate

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1. Introduction

1.1. Liposomes

1.1.1. General review

Liposomes are microscopic structures consisting of one or more concentric lipid bilayers enclosing an equal number of aqueous compartments (Pagano and Weinstein 1978). They were first described 40 years ago by Bangham (New 1990) and the term liposome was introduced in 1968 (Sessa and Weissmann 1968). They function as a drug delivery system (Allen and Cullis 2004; Crommelin and Storm 2003; Taylor and Newton 1994), a methodology for manipulating drug distribution in the body (Hashida et al. 2001). The benefits of using drug delivery systems, which also apply to liposomes, include: (1) higher availability of the therapeutic agent at the target organ or tissue, (2) specific delivery of a therapeutic agent to specific cellular compartments, (3) protection of the body from the harmful effects of the therapeutic agent at sites other than the target site, (4) protection of the therapeutic agent from the physiological environment on route to the target site of action, and (5) the ability to use higher concentrations of the therapeutic agent as a result of the reduced toxicity effect (Allen and Cullis 2004). Furthermore, the importance of liposomes as drug delivery systems is increased as a result of: (1) being mostly composed of natural materials which are biodegradable, non-immunogenic and non-toxic, (2) they can incorporate large amounts of a wide range of active agents with different properties such as molecular size and solubility characters, (3) they can be used in a wide range of applications such as chemotherapy, vaccines, red blood cells substitute, and diagnostics as well as delivery of traditional therapeutic agents, (4) can be introduced

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intravenously, topically or as an inhalation aerosol, and (5) easy to manufacture both on small scale and large scale and is stable in different storage conditions (Allen 1998; Allen and Cullis 2004; Crommelin and Storm 2003; Goyal et al. 2005; Taylor and Newton 1994; Ulrich 2002).

In general, agents that stand the most to gain from delivery when formulated with liposomes are those which have biological (low therapeutic index, rapid metabolism or unfavorable pharmacokinetics) or physical (low solubility, lack of stability or irritant) properties which can be alleviated through this formulation (Allen 1998).

1.1.2. Liposome Formulation

Molecules can be classified into polar and non-polar molecules based on their solubility and symmetry of their electronic clouds. Polar molecules are soluble in polar solvents such as water and hence are referred to as hydrophilic compounds and non-polar molecules are soluble in organic solvents such as chloroform and are referred to as hydrophobic or lipophilic. Another class of molecules, based on their solubility or electric charge, is amphiphilic molecules which posses a polar and a non-polar group on the same molecule. When amphiphiles are placed in a polar medium such as water, they self assemble or organize so that their polar portion is facing the polar environment while shielding their non-polar portions. As a result a symmetrical structure such as a sphere, as seen in figure 1, is formed with a bi-layer membrane surrounded by the polar solvent and entrapping it at the same time. Examples of amphiphiles are soaps, detergents and polar lipids such as phospholipids, the most common components of liposomes (Lasic 1998; Ulrich 2002).

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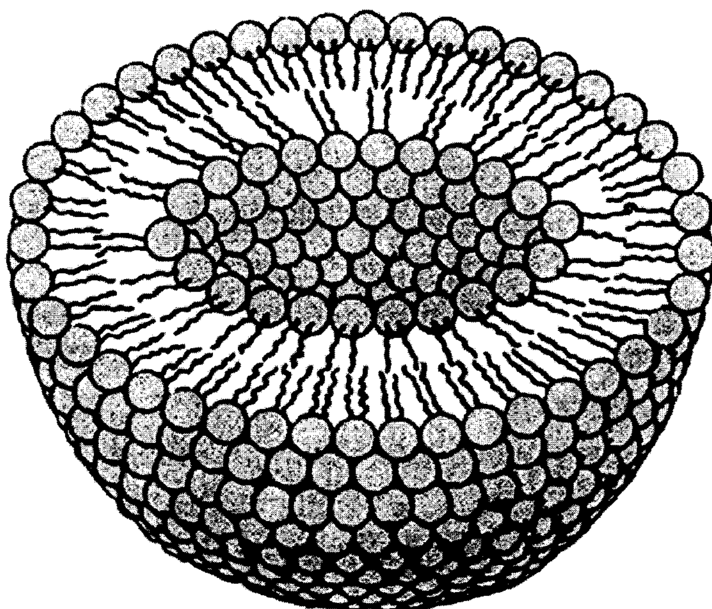

Figure 1: Liposome Structure Formed by Phospholipids
Structure of liposome displaying aggregation of phospholipids forming a sphere shaped bi-layer membrane. (Dhand 2004)

The wide range of molecules which can be loaded into liposomes includes water soluble agents dissolved in the aqueous medium which will be entrapped inside the liposomes while hydrophobic agents can be incorporated into the hydrophobic part of the liposomal bi-layer membrane. Amphiphiles can be incorporated into either part of the liposomes (New 1990; Ulrich 2002).

1.1.3. Production of Liposomes

Several procedures are available for the formulation of liposomes. Hybrids of these procedures can also be used to achieve formulations with desired size, lamellarity (number of alternating layers of phospholipids and aqueous medium) and optimized efficiency of encapsulation. The choice of the formulation procedure is influenced by

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the type of lipids used, the nature of material to be encapsulated or delivered and the availability of equipment as well as practicality and ease of formulation. The most common techniques for formulation of liposomes are (New 1990; Torchilin and Weissig 2003):

- Hand-Shaken Vesicles:

This simple technique is based on drying a thin lipid membrane on the walls of a round bottom flask. The aqueous phase is added in excess resulting in the swelling of the lipid layers. The swelled lipids detach from the solid support and form multilamellar vesicles upon mechanical agitation, resulting from hand shaking and swirling of the round bottom flask. The resulting multilamellar vesicles can be modified through extrusion to become uniform in size and reduce lamellarity. These multilamellar vesicles can also be used in other techniques described below. This technique's main advantage is its simplicity. It is also compatible with a wide range of material to be encapsulated. Hydrophilic material is dissolved in the aqueous hydrating solution while hydrophobic material can be dissolved in the organic solution along with the membrane lipids prior to the drying process to produce the lipid thin film. The major draw back of this technique is the low efficiency of encapsulation for water soluble molecules which is typically in the range of 3 to 10%. The efficiency of entrapment for a lipid soluble molecule is very high and a 100% entrapment can be, theoretically, achieved.

- Sonicated Vesicles:

This technique is applied to reduce the size and lamellarity of multilamellar vesicles (MLVs). It can be used to produce small unilamellar vesicles (SUVs), however it

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should be noted that vigorous and prolonged sonication is needed which can result in degradation of the material to be incorporated into liposomes. It also may result in the de-esterification of the liposomal lipids. A probe sonicator can be directly immersed into the liposomal suspension or alternatively, the liposomal preparation can be put in a vessel and sonicated in a bath sonicator. It should be noted that the sonication process introduces a lot of energy to the liposomal suspension and care should be taken while handling thermolabile material. SUVs prepared through this technique could be unstable at very small sizes and may fuse together forming larger vesicles. Hence it is recommended, if possible, to leave the preparation over night at room temperature to obtain a preparation with stable and defined characteristics.

- Freeze-dried Rehydration Vesicles:

This technique allows for high entrapment efficiency. It is based on the preforming of liposomes through other techniques followed by freeze drying of these liposomes. The lipid bilayers are then rehydrated using an aqueous solution with the agent of interest. This process allows close proximity between lipid bilayers and the agent of interest resulting in greater chances of entrapment. The rehydrated vesicles can then be extruded to obtain uniform size and lamellarity characters.

- Reverse-Phase Evaporation:

This technique yields high encapsulation efficiency as it yields liposomes with large aqueous space to lipid ratio and therefore, high capture efficiency. The technique is based on dispersing the aqueous phase in the form of small droplets within the organic solution of the liposomal membrane phospholipids. This results in the formation of monolayer membranes surrounding the aqueous droplets with the

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hydrophilic portion facing the aqueous phase and the hydrophobic portion facing the organic solvent. This is achieved through the sonication of the aqueous phase in excess of an organic solvent in which the phospholipids intended to form the liposomal membrane are dissolved. The organic solvent is removed by vacuum and a gel like state is formed. At a critical point, the gel will collapse and some of the micelles will disintegrate providing phospholipids to form a second layer for other micelles resulting in bilayer liposomes formed at high encapsulation efficiency. There are several draw backs to this technique which include non-uniformity of size and difficulty in extrusion. It also has the limitation that not all lipids can be used in this formulation. Finally, the aqueous phase material will be exposed to organic solvents, which makes this technique unsuitable for the formulation of material which could be unstable in such conditions, e.g. denaturing of proteins.

- Detergent Depletion:

This technique can yield very uniform liposomes and has very mild conditions which can allow the entrapment of a wide variety of materials which may not survive other techniques such as organic solvents, sonication and extrusion. It is based on the formulation of micelles in the presence of detergents which are then removed through a variety of methods such as dilution, dialysis and gel filtration resulting in liposomes with uniform characters. The size and lamellarity characters of the formed liposomes can be determined through the choice of the used detergent. The size can also be greatly influenced by the rate of depletion of the detergents. The major draw backs of this technique are that it is time consuming and requires a lot of experience.

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1.1.4. Classification of Liposomes

1.1.4.1. Size and Structure

As seen in Figure 2, liposomes are classified into SUVs, LUVs and MLVs based on their diameter size and lamellarity, the number of phospholipid layers alternating with an equal number of aqueous layers entrapped by them:

- **SUVs:** Small Unilamellar Vesicles, these are prepared through sonication and are sometimes referred to as: Sonicated Unilamellar Vesicles. They range in size between 15 and 30 nm, but they will spontaneously fuse unless they are kept in controlled conditions, specifically temperature.
- **LUVs:** Large Unilamellar Vesicles, these are prepared through several techniques which include the fusion of SUVs and the extrusion of MLVs. Other methods include detergent dialysis, reverse evaporation and ethanol injection. LUVs range in size between 100 and 200 nm and could be larger and are generally stable upon storage.
- **MLVs:** Multilamellar Vesicles, these are best described as "onion-like" Liposomes, with layers of the membrane lipids alternating with layers of the aqueous medium.

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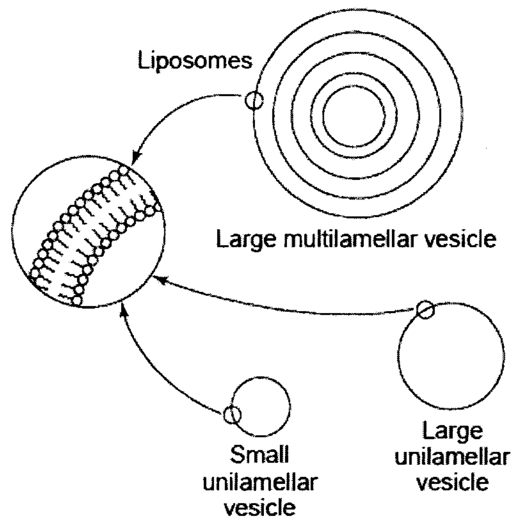

Figure 2: Subtypes of liposomes based on size and lamellarity
Liposomes can be classified based on their structure into large multilamellar vesicles, large unilamellar vesicles and small unilamellar vesicles. (Lasic 1998)

1.1.4.2. Membrane and Functional Properties

- **Stealth Liposomes:**

Liposomes and nanoparticles are cleared rapidly after intravenous injection by the Reticulo-Endothelial System (RES), particularly macrophages and the Kupffer cells of the liver. As a result, therapeutic agents with liposomal formulations may not reach their intended targets. In specific cases, a correlation was established between circulating times and efficacy as it is the case with liposomal doxorubicin and its anti-tumor effect. Stealth liposomes are novel formulations coated with a protective polymer to reduce uptake by the RES. They also have the added benefit of reduced toxicity of the encapsulated agent and allow for a reduced number of injections. The mechanism by which the coating process functions is that it prevents opsinization of the liposomes, a process which facilitates phagocytosis by

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the RES cells. There are reports that suggest a modification to this theory pointing out that there is a limited rapid uptake of stealth liposomes by the RES, suggesting that there is a limited reservoir of proteins capable of penetrating the polymer coating of stealth liposomes. The rate at which stealth liposomes are cleared is most affected by their size and the used polymer, it does not appear to be affected by the dose injected. The most common polymer used in coating stealth liposomes is Polyethylene Glycol (PEG), as a result, Stealth liposomes are sometimes referred to as PEGylated liposomes (Cattel, Ceruti, and Dosio 2004, Gabizon 2001).

- **Thermosensitive Liposomes:**

Thermosensitive liposomes, or temperature sensitive liposomes, are liposomes formulated with lipid constituents which have a relatively sharp phase transition temperatures just above 37 °C. The application of heat at the desired target site, Regional Hyperthermia, results in increased uptake and accumulation of the thermosensitive liposomes, as a result of facilitated extravasation. It also allows the specific release of liposomal content as a result of structural changes and destabilization of the liposomes. This class of liposomes has shown positive results in cancer therapy models with the added advantage of regional hyperthermia. This is already an approved technique for treatment of cancer (Lindner et al. 2004).

- **Magnetic Liposomes:**

Magnetic liposomes are liposomes formulated to incorporate magnetite nanoparticles. The magnetic properties of these particles have allowed the

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use of such formulations in drug targeting, bioseparation and cell sorting in tissue with applications in tissue engineering which allowed the construction of 3D cellular structures. The liposomes containing these nanoparticles can be also targeted through traditional targeting approaches, e.g. immunoliposomes, to specific tissues or tumors allowing them to become contrast agents for Magnetic Resonance Imaging (MRI). They also can be utilized as heating mediators in regional hyperthermia applications (Matteucci and Thrall 2000).

- **Targeted Liposomes:**

The biodistribution of traditional or plain liposomes is generally influenced by characters such as lipid composition and physical characters such as size. In general, the RES is responsible for the rapid uptake of a very large portion of injected liposomes. The options for the route of administration are very limited and the most common route of administration is the intravenous route. Other routes include aerosol and topical application of liposomal preparations. The development of stealth liposomes has greatly reduced the first pass effect and uptake by the RES, allowing more time for distribution to other tissues and organs of the biological system. Introducing molecules in the liposomal membrane is an efficient way of targeting liposomes to a specific organ or tissue. The most commonly used molecules are monoclonal antibodies (MAbs) specific to a cell surface antigen, e.g. a receptor or tumor antigen on the surface of the targeted tissue with limited expression elsewhere in the body. Other common molecules used in

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targeting liposomes are agents with high affinity to cell surface structures, most commonly receptors unique to the targeted tissue and ideally would be involved in endocytosis (Metselaar, Mastrobattista, and Storm 2002; Pagano and Weinstein 1978; Schnyder and Huwyler 2005).

- **pH Sensitive Liposomes:**

pH sensitive liposomes are a special class of liposomes which contain components in their membrane that are sensitive to pH changes in the surrounding environment. These components undergo acid induced destabilization resulting in loss of integrity of the liposomal membrane and the release of liposomal content. pH sensitive liposomes were first formulated to take advantage of the increased acidity at tumor sites. However, the drop in pH around a tumor is limited and not sufficient to cause the release of liposomal content. Furthermore, the drop in pH at tumor sites occurs at the distal parts from the tumor blood supply (Allen and Cullis 2004; Drummond, Zignani, and Leroux 2000). The environment of an endosome or a lysosome does, however, undergo significant acidification to pH levels below 5 through the function of proton-translocating ATPases. Therefore, the destabilizing characters of pH sensitive liposomes membrane components allow improved delivery of liposomal content to the cytoplasm after internalization of liposomes into cells via endocytosis or phagocytosis. As seen in figure 3, the destabilized liposomes could either fuse with the endosomal or lysosomal membrane releasing their content or could become destabilized within the endosome or lysosome releasing the liposomal

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content which could escape into the intracellular environment through pores formed in the endosomal or lysosomal membrane which are caused by the destabilized liposomes (Drummond, Zignani, and Leroux 2000; Venugopalan et al. 2002). In this way, the content of pH sensitive liposomes may escape the fate sometimes faced by content of traditional liposomes which remain entrapped within cellular vesicles or undergo extensive degradation within the lysosome and as a result their activity. This advantage of pH sensitive liposomes made them useful in the delivery of chemotherapeutic agents and membrane impermeable molecules. It is also the reason behind expectations of a possible use for pH sensitive liposomes for the intracellular delivery of biologically active material (Nair et al. 1992).

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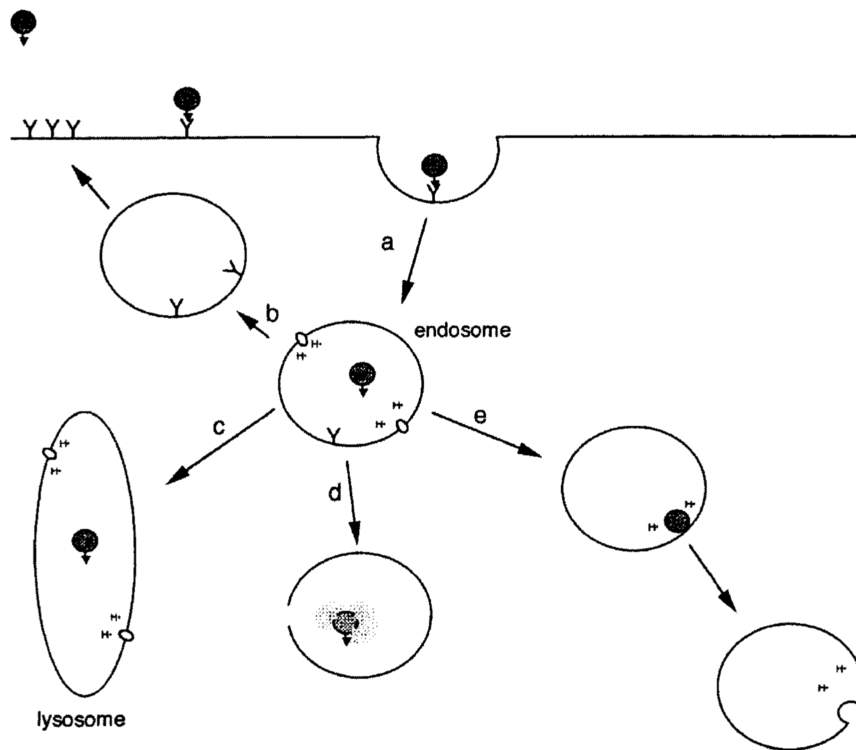

Figure 3: Potential fates of pH-sensitive liposomes following binding to a target cell

Upon binding to cell surface receptors, liposomes can either remain bound at the cell surface, disassociate from the receptor, or accumulate in coated or non-coated invaginations. Following endocytosis (a), liposomes can be delivered to lysosomes (c) where they and their contents may be degraded. Following acidification of the endosomal lumen, pH-sensitive liposomes are designed to either fuse with the endosomal membrane (e), releasing their contents directly into the cytoplasm, or become destabilized and subsequently destabilize the endosomal membrane (d) resulting in leakage of the endosomal contents into the cytosol. Receptors may be recycled back to the cell surface (b) or targeted for degradation in the lysosome (c). (Drummond, Zignani, and Leroux 2000)

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There are four basic classes of pH sensitive liposomes. The first is based on the use of mildly acidic amphiphiles which act as stabilizers at neutral pH. The second class is liposomes containing "caged" lipid derivatives which undergo acid induced hydrolysis of specially engineered chemical bonds resulting in increased destabilization of the membrane and the release of liposomal content. The third class contains pH sensitive peptides or reconstituted fusion proteins which destabilize membranes upon acidification. The fourth and final class is pH titratable polymers which change their confirmation at low pH resulting in membrane destabilization. The first class of liposomes is the most studied class of pH sensitive liposomes but it has the disadvantage of reduced in vivo stability upon contact with serum components. Most reported pH sensitive liposomes preparations are based on dioleoylphosphatidylethanolamine (DOPE) associated with a weakly acidic amphiphile such as cholesteryl hemiscuccinate (CHEMS). DOPE has a cone like structure which results in its propensity to form non-bilayer structures. CHEMS has a stabilizing effect on the bilayer phase at neutral pH, but becomes protonated at acidic pH and thereby losing its negative charge and its electrostatic repulsion based stabilizing ability to the bilayer structure.

Jennifer J. Sudimack et al. reported an oleyl alcohol based pH sensitive liposomal formulation composed of oleyl alcohol, CHEMS, Tween-80 and Egg PC. This preparation has shown increased pH sensitivity as well as

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stability on storage and in serum. The increase of oleyl alcohol concentrations enhanced pH sensitivity while the increase of Tween-80 concentrations enhanced the stability characters at the expense of pH sensitivity. They concluded that a molar concentration of egg PC/CHEMS/T-80/OAlc at 50:50:2:80 gave a balance between pH sensitivity and stability. They also successfully modified this preparation to include folate-PEG-cholesterol to target the folate receptor, found in several cancer types, which resulted in enhanced cytotoxicity of the delivered chemotherapeutic agent (Sudimack et al. 2002).

1.2. Hepatitis C

1.2.1. The disease, its manifestations and infection trends

Hepatitis is the inflammation of the liver. Viral Hepatitis is the inflammation of the liver resulting from a viral infection. Hepatitis C is a type of Viral Hepatitis caused by an RNA virus of the family *Flaviviridae*. The number of world wide infections is estimated to range between 124 million and 170 million people. Estimates based on testing of blood donations provide the number of infections world wide to be as high as 500 million (Clarke 1997). The geographical distribution of this disease is concentrated in Asia and Africa but a high prevalence is also noticed in North America with an estimated 4 million patients in the United States and a quarter of a million patients in Canada (Zou, Tepper, and El Saadany 2000).

Disease transmission occurs through exposure of blood to the virus. The sharing of contaminated syringes between drug abusers is currently the major means by which

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the disease is transmitted. The transfusion of contaminated blood products is still a major cause of infection but it has been decreasing as a result of the emphasis on screening of blood donors and the advances in screening tests and methods. Other, less common, causes of infection include transplantation of organs, occupational exposure and high risk sexual behavior. The disease can progress from acute infection to chronic infection and can cause liver cirrhosis, liver fibrosis and liver cancer eventually leading to death. Cofactors which affect the rate and extent of progression of the disease include age at infection, gender, race, genetics, co-infection with Human Immunodeficiency Virus or Hepatitis B Virus, alcohol and tobacco consumption, obesity and metabolic disease (Butt 2005; Shepard, Finelli, and Alter 2005).

1.2.2. Hepatitis C Virus

The causative agent of Hepatitis C is a single stranded RNA virus. The genome of the virus is composed of 9,500 bases, which carry the code for approximately 3,000 amino acids, figure 4. The proteins produced by the virus once it gains entry to the host cell are classified into structural proteins and non-structural proteins. The structural proteins are the core proteins and envelope proteins which are responsible for the structure of the virus and its ability to gain entry and infect new host cells. The non-structural proteins are proteins which are needed for the virus to replicate and produce new viral particles once it gains entry to host cells (Shepard, Finelli, and Alter 2005).

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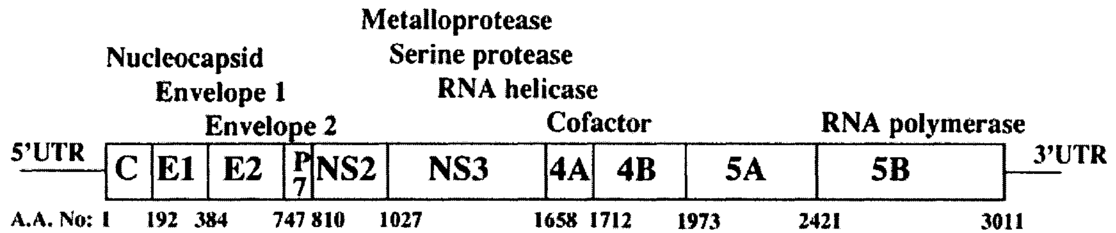

Figure 4: Genomic organization of HCV RNA and corresponding encoded viral proteins

The viral proteins of HCV are divided into structural and non-structural proteins. The non-structural proteins have an important role in the viral replication process; however, the exact function of some of the non-structural proteins is not yet determined. The most targeted structures in the development of therapy approaches are the NS3 protease and the NS5B RNA polymerase (Clarke 1997).

1.2.3. Vaccination

The development of a vaccine which offers protection against infection by the Hepatitis C virus has not yet been achieved. The major problem in the development of such a vaccine is the presence of several sub-types of the Hepatitis C virus with major differences between them. Furthermore, as a result of the mechanism by which the virus replicates, there is a constant change in the structure of the virus resulting in the inability of the immune system to recognize some of the resulting viral particles. As a result of these characters, hepatitis C infections tend to be chronic, which indicates that the immune system is unable to clear them over a prolonged period of time. Furthermore, patients and test animals cleared from the infection are infected again if exposed again to the virus indicating that the immune system does not efficiently recognize and clear the viral particles. However, there are positive signs

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which give hope of the possibility of finding an effective vaccine against this disease. Two of these signs are that 50% of acute viral infections are cleared spontaneously and some individuals, and test animals, show a natural immunity against infection with the virus. These two signs suggest the ability of the immune system, of a part of the population at least, to recognize and effectively defend against the HCV which leads us to believe in the possibility of the development of an effective vaccine (Houghton and Abrignani 2005; Hsu, Abrignani, and Houghton 1999).

1.2.4. Interferon Alfa and Ribavirin antiviral therapy

Interferon Alfa is a natural chemical which has a function in enhancing non-specific immune defense mechanisms against viral infections. Ribavirin is a synthetic compound that interferes with the viral replication and life cycle. Its antiviral activity is not limited to HCV as it exhibits its antiviral effects against a wide range of DNA and RNA viruses. It exhibits its antiviral activity against HCV through one or more of the following mechanisms: the inhibition of an enzyme necessary for viral replication, the depletion of an energy source used in the viral replication and the creation of viral particles which have defects in them, rendering them unable to spread and replicate.

Neither Interferon nor Ribavirin treatments are effective as monotherapy. But high success rates are achieved through a treatment regimen combining the two agents. The efficiency of treatment is influenced by viral and host specific factors. Viral specific factors include the genotype of the infecting virus, the duration of the infection before commencing therapy and the viral levels in the patient. Patient specific factors include age, sex, race, extent of damage to the liver, body weight and

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the presence or absence of other diseases and illnesses. In general, the efficiency of antiviral treatment is 90% in acute cases and 50% in chronic ones (Brok, Gluud, and Gluud 2005; Feld and Hoofnagle 2005; Heathcote and Main 2005; Katzung 2004).
A major setback to this therapy regimen is the high cost associated with it. Currently, the treatment cost of one of the genotypes of the virus is $ 25,000 US for a 48 week course of therapy. It is estimated that the direct medical costs of Hepatitis C therapy in the US over the next decade will exceed $ 10 billion US. Given the high incidence of the disease and high rate of spread of the infections, it is unrealistic to expect this therapy to eradicate this disease (Shepard, Finelli, and Alter 2005). Another setback is the side effects associated with this therapy. The side effects of interferon include thrombocytopenia, nausea, fatigue, headache, alopecia, rash, anorexia, hypotension as well as severe neuropsychiatric side effects. Therefore this therapy cannot be applied to psychotic patients or patients suffering from a wide range of diseases. Furthermore, interferon is abortifacient in addition to causing birth defects and therefore cannot be used in pregnancy. Ribavirin has a wide range of side effects which include anemia, nausea, fatigue, depression, insomnia and pruritis and is not to be administered to patients with anemia, renal failure or heart disease (Katzung 2004).

1.2.5. Liver Transplantation

Hepatitis C is the most common indication for liver transplantation. It is needed when antiviral therapy fails or when the diagnosis of the disease occurs at a late stage of the disease. At this stage significant damage to the liver would have occurred and the function of the liver severely compromised. The demand for organs vastly outstrips

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the supply. Furthermore, the procedure is long, complicated and expensive. The procedure is not a cure for Hepatitis C as recurrent infection is almost certain and occurs in most transplant recipients. A dilemma facing liver transplant recipients is the fact that immunosuppressant therapy cannot be administered or otherwise, Hepatitis C viral re-infection will occur rapidly and result in rapid destruction of the newly transplanted organ. This however results in greater incidence of organ rejection after transplantation. The best approach of solving this problem is controlling the viral infection before and after transplantation to mitigate recurrent disease (Brown 2005).

1.3. The Asialoglycoprotein Receptor

The asialoglycoprotein receptor (ASGP-R) was first isolated and characterized in 1971 by Pricer and Ashwell (Pricer and Ashwell 1971) hence it is sometimes referred to as the Ashwell Receptor. It was later isolated and characterized from the liver of many species including humans. The receptor is itself a glycoprotein. It is found on the surface of hepatocytes almost exclusively, as confirmed by Park *et al.* in a study utilizing fluorescence-activated flow cytometry on various human tissue cell lines using an antibody specific to the H1 subunit of the human ASGP-R. The receptor is in great abundance, approximately providing 500,000 ligand binding sites per hepatocyte (Erion et al. 2005; Kaneta et al. 2004; Kwon, Inoue, and Ha-Kawa 2005; Westerlind et al. 2004).

The ASGP-R serves as an endocytotic receptor (Spiess 1990), responsible for the endocytosis of ligands with galactosylated or lactosylated residues. The endosomes

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are then fused with lysosomes and their contents degraded. The ASGP-R is believed to be recycled and sent back to the surface of the cell membrane (Erion et al. 2005). These characters of abundance, exclusivity and endocytotic function have made the ASGP-R an excellent target for hepatocyte or liver targeted delivery. Asialofetuin, the natural ligand for ASGP-R or synthetic ligands with galactosylated or lactosylated residues have been used to deliver liposomes, recombinant lipoproteins and polymers carrying drugs or gene therapies to the liver. However, there are specific problems with the delivery of liposomes and their contents to the liver such as stability in blood and the degradation of their content following endocytosis (Kim et al. 2005; Westerlind et al. 2004; Wu et al. 1998; Zhang et al. 2005).

1.4. Therapeutic Antibodies

Antibodies are the effector molecules of the humoral part of the adaptive immune system. They are secreted by B cells in different isotypes with varying functions. They perform their natural function through one or more of the following mechanisms: Opsinization for phagcytosis, complement activiation, precipitation and neutralization by binding to antigen thus preventing its interaction with its intended target. The B cells secreting antibodies can be fused with cancerous cells to give rise to immortal cell lines of hybridomas. These cells secrete monoclonal antibodies with the same specificity and binding ability to their target antigen. These unique characters of antibodies have provided polyclonal and monoclonal antibodies with many applications in medicine and research. These applications include *in vitro* and *in vivo* screening and diagnostics, immunological and biological research, specific

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drug delivery, antidotes for drug overdose or toxins as well as many other industrial and commercial applications (Goldsby et al. 2000).

Antibodies in their original role function as effector molecules in the immune system. This role is also applied in passive immunization, the transfer of antibodies or portions of them from an external source to a subject. Passive immunization occurs in nature as in the passive immunization from mother to infant through breast feeding. It is also applied in medicine as the case with antibodies, or fractions of them, to treat digoxin toxicity. Antibodies can also be used to give temporary immunity or as a treatment for the exposure to pathogenic viruses and bacteria or their products (Goldsby et al. 2000).

1.4.1. Intrabodies

1.4.1.1. Introduction and History

An intrabody can be simply defined as an antibody, or fractions of it, generated and functioning inside the cell (Kontermann 2004; Marasco 1997; Stocks 2004). The principle behind this approach and the production of such antibodies is the utilization of functional properties and characteristics of antibodies in an intracellular location. In their natural environment, antibodies generally function in body fluids extracellularly where their specific binding to a molecule can block its action or interaction. In theory, the same functions can be performed by antibodies or fragments of antibodies in the intracellular environment (Kontermann 2004).

The first successful use of antibodies intracellularly was in 1980 when microinjection was used to inject intact antibodies into cells and they were capable of neutralizing

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tumor antigens within the injected cells in that specific experiment. The limitations to this technique include the complexity of this technique and the limited number of cells which could be injected. This defined the limitations where and when it can be used. This has lead to quest to produce the intrabodies within the intended target cells, exploiting recombinant DNA technology. In 1988, intact and functional antibodies were reported to be produced in the cytoplasm of yeast. In 1990 intrabodies were produced within mammalian cells. These antibodies were composed of a heavy chain and light chain which could bind in the cellular cytoplasm and were recognized by anti-idiotypic antibodies. However the resultant antibodies did not demonstrate any functional activity. Over the years to follow, many intrabodies were reported which were produced inside the cells and retained their activities (Stocks 2004).

1.4.1.2. Technical Considerations

The main obstacle in producing intrabodies was the reducing intracellular environment in the cytoplasm and nucleus of the cell. This reducing environment prevented the proper folding and stability of the antibody and the formation of the disulfide bond which gives them their 3D structure and therefore binding function and specificity. The use of smaller Single Chain Antibodies (scFv) as intrabodies has helped in alleviating this problem as there was no need for a disulfide bond between the heavy chains and light chains as in the classical antibodies. Furthermore, either through engineering or through selection from libraries, scFv were selected to be expressed as intrabodies where they could be formed, folded and remain stable in the intracellular environment (Stocks 2004).

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1.4.1.3. Intrabodies and Transbodies

Intrabodies, which are antibodies, or their fragments, produced within the cell through gene therapy approaches, are sometimes referred to as 'transbodies' which are conventionally produced through conventional means of antibody production and subsequently delivered intracellularly. The transbodies approach would have the following advantages:

- They would have proper folding prior to their introduction into cellular cytoplasm. After delivery into cytoplasm their sulfide bonds could be protected within the 3D structure of the antibodies and they would therefore remain stable.
- Their use would not face the ethical and technical limitations of using gene therapy, which is required for intrabodies to be secreted inside cells.
- They have a limited active half-life and therefore their use could be stopped should undesired or harmful effects be observed.

Methods of introducing preformed antibodies into cellular cytoplasm include the fusion of protein transduction domains to the desired antibodies or their fragments. Another possible application is the use of a drug delivery system which is attempted as a part of this thesis (Heng and Cao 2005).

1.4.1.4. Applications of Intrabodies

Due to the fact that antibodies can be generated against virtually any given antigen, a large number of intrabody based approaches have been developed and are showing promise in the treatment of a wide range of diseases and illnesses (Kontermann 2004). The majority of these approaches can be classified into:

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- Cancer Therapy: Through the inhibition of receptor tyrosine kinases, inhibition of intracellular signal transduction pathways, interference with the apoptotic pathway and through addressing nuclear targets and extracellular proteinases.
- Treatment of Infectious diseases: Intrabodies targeting structural or non-structural viral proteins can interrupt their replication and infection cycles (Marasco 2001). The most promising results in this track are intrabodies targeting viral proteins of the HIV-1 (Aires da Silva et al. 2004; Bai et al. 2003)and HCV viral proteins (Zemel et al. 2004) with positive results in disrupting the viral life cycle and replication.
- Intracellular Immune Suppression: Two approaches are attempted, the first in reducing attack by host T cells through intrabodies attacking the MHC-I molecules in the donor tissue or organ. The second approach is *via* using intrabodies against the enzyme α1,3-galactosyltransferase thus reducing the α1,3-galactosylation of surface antigens in donor cells which are recognized by host xenoreactive natural antibodies.
- Treatment of Diseases caused by altered proteins production: an example is the approach to treat Huntington's disease with intrabodies specific to the polyproline stretch in the N-terminus in the huntingtin protein thus preventing protein aggregation and resultant death of nerve cells.

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2. Hypothesis and Objectives

Hypothesis

Oleyl Alcohol based pH sensitive liposomes can be formulated to incorporate lactosylceramide (Lac-Cer) at concentrations which both induce uptake by the liver while retaining desired properties of pH sensitivity and stability.

This liposomal preparation is safe and stable and can enhance delivery of therapeutic molecules into hepatocytes and could be used to deliver biologically active material intracellularly without loss of their activity.

Objectives

The main objectives of this project are:

- To formulate pH sensitive liposomes with lactosylceramide while retaining their desired properties.
- To evaluate the formulated liposomes for pH sensitivity, stability and toxicity.
- To evaluate the formulated liposomes for their efficiency in targeting the liver to deliver liposomal content *in vitro* and *in vivo.*
- To evaluate survival of biologically active material after their intracellular delivery by pH sensitive liposomes.

The long term objectives of this approach are to obtain a drug delivery system which can specifically, efficiently and safely deliver its content to hepatocytes in the liver. The use of this drug delivery system could improve biodistribution profiles of therapeutic molecules resulting in improved therapeutic efficiency and toxicity profiles. It could also alter the intracellular delivery conditions and

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efficiency of these molecules allowing new uses for chemical and biological molecules as well as enhancing and improving the efficacy of others.

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3. Experimental

3.1. Materials

Asialofetuin, Calcein, CHEMS (Cholesteryl Hemiscuccinate), FITC (Fluorescein Isothiocyanate), Goat anti-Rat IgG, HRPO (Horseradish Peroxidase), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Protease Inhibitors Cocktail, Protein G, standard mouse IgG, Sulforhodamine B and TRITC (tetramethyl rhodamine isothiocyanate) were purchased from Sigma Aldrich, Canada. Balb/c mice were purchased from the Health Sciences Animal Services at the University of Alberta. Bio-Rad Protein Assay Kit was purchased from Bio-Rad, USA. DMEM, FBS and Trypsin EDTA were purchased from Gibco, Canada. Egg PC (Phosphatidylcholine), C8 ß-D-Lactosylceramide (Lac-Cer), Oleyl Alcohol and polycarbonate membranes were purchased from Avanti Polar Lipids, USA. Sephadex G-25, Sephadex G-50, Spephadex G-75, Sephadex G-100 and Sephadex G-200 were purchased Amersham Pharmacia Biotech, Sweden. ^{99m}Tc-DTPA was purchased from the Cross Cancer Institute in Edmonton, Alberta. TMB substrate was purchased from Kirk-Guard Perry Laboratories, USA.

Common lab chemicals including acetate, boric acid, BSA (Bovine Serum Albumin), Chloroform, DMSO (Dimethyl Sulfoxide), Glycine, Isopropyl Alcohol, KCl, KH_2PO_4, Na_2HPO_4, NaOH, Sodium Azide, Triton X-100 and Tween-80 were purchased from major chemical suppliers such as Fisher Scientific, Sigma and Fluka.

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3.2. Overview of Experimental Strategy, Design and Rationale

The purpose of this project was to design a drug delivery system specific to hepatocytes as they are the target of HCV infections and many other liver diseases. We elected to focus our research on liposomes as a drug delivery system due to the many advantages they offer as mentioned earlier in section 1.1.1. Furthermore, pH sensitive liposomes were selected as they significantly enhance the delivery of their content once it arrives at its intended intracellular destination. pH sensitive liposomes are also expected to allow the delivery of biologically active material intracellularly which could open the door to many applications in medicine and provide us with new options of treatment for many different medical conditions. One of these options could be the delivery of antibodies intracellularly as discussed in section 1.4.1. addressing intrabodies.

A formulation of oleyl alcohol based liposomes was selected as the basis for our formulation since it exhibits increased pH sensitivity and stability in serum in comparison with older pH sensitive liposomal formulations (Sudimack et al. 2002). Lac-Cer was selected as a targeting molecule because of literature evidence of improved uptake by hepatocytes as a result of its incorporation (Spanjer and Scherphof 1983). Furthermore, it is commercially available and its incorporation into a liposomal formulation is simple and does not require lengthy or complicated processes.

The hand shaken method, which is the simplest method of preparing liposomes, was used in the literature describing the formulation of OAlc liposomes. This method has lower requirements in terms of equipment (New 1990). It also can be formulated

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under mild conditions of preparation which makes it suitable to incorporate proteins and other materials which could otherwise be damaged or denatured (Torchilin and Weissig 2003).

The first series of experiments were designed to optimize the formulation of the pH sensitive liposomes and incorporating Lac-Cer into the formula while maintaining the desired pH sensitivity and stability characters of the formulation.

The use of calcien in NaOH as a hydrating agent, allowed the testing and monitoring of the pH sensitivity, measured as the specific percentage of liposomal content released in a fixed period of time as a function of pH change. It also allowed evaluation of stability, measured as the specific percentage of liposomal content released as a function of time during storage or under physiologic conditions. Calcein has a self-quenching character when present at high concentrations. Liposomes hydrated with a Calcein at a high concentration should show very limited fluorescence as long as their content is entrapped. If liposomal content leaks or was released into the solution in which the liposomes are suspended, calcein becomes diluted and resulting in detectable fluorescence. The total calcein content of liposomes can be determined by releasing their content into the suspending solution. This can be achieved through disrupting liposomal membranes by adding a detergent (Ishida et al. 2005).

The size distribution and uniformity of liposomal preparations were evaluated in early studies.

The second series of experiments were *in vitro* testing of uptake of liposomes loaded with a fluorescent probe by a hepatoma cell line. The experiments were designed to

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determine the influence, if any, of the different concentrations of Lac-Cer incorporated into the liposomal formulation, by a hepatoma cell line. The cell line used, Hepa 1c1c7, was chosen as it was available to our group and had desirable properties of rapid growth and attachment to the walls of culture plates. The presence of the ASGP-R receptor in this cell line was tested for by testing its uptake of asialofetuin (AF), a known molecule with high affinity for binding and endocytosis by the ASGP-R (Wu et al. 1998). *In vitro* toxicity tests were also incorporated into our experiments to ensure the safety of using Lac-Cer in our formulation as there are reports in the literature of ceramide induced toxicity (Siskind and Colombini 2000; Stover and Kester 2003). One of the reports specifically uses the very same cell line used in my *in vitro* studies (Reiners and Clift 1999).

In vivo biodistribution assay of the formulated liposomes, with and without Lac-Cer, was conducted with liposomes loaded with radioactive ^{99m}Tc-DTPA. This probe was selected because of its short half life making it safer to use. In addition, the biodistribution patterns of the free molecule are available in literature (Liu et al. 2002).

Finally, a series of tests were devised to test for the survival of biologically active material and the retention of their activity after intracellular delivery by liposomes. The initial experiments tested the survival of YP4 anti-HRPO MAbs as a model for intracellular antibodies and HRPO as an example of a biologically active substance, an enzyme. YP4 MAbs were selected as they are available at our lab and they are rat anti-HRPO IgG antibodies, therefore, the detection of their intracellular survival and retention of their biological activity could be done within the same test. HRPO was

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selected since the assay of its activity is readily available and its use is widespread. Furthermore, the availability of YP4 in our lab allowed the testing for the retention of HRPO's structure if it retained its activity after intracellular delibery. In later experiments we also included a protease inhibitor cocktail (PI) and FITC as part of the liposomal formulation. The PI was intended to facilitate the survival of liposomal proteins by preventing its degradation by lysosomal proteases, while FITC was used to confirm the uptake of the liposomes and their content. Due to the possible toxic effect of PI in the formulations, the safety and toxicity of the liposomes containing them was evaluated using the MTT test (Coligan 1991).

3.3. Liposomes Preparation and Evaluation

3.3.1. Development of Liposomes

Procedure 1

The individual components of the liposomal membrane were dissolved in chloroform at 1 ml each and they were combined in a completely dry 100 ml round bottom flask. The chloroform was removed, leaving a thin uniform lipid film on the walls of the round bottom flask. This was done using a rotoevaporator set to rotate at medium speed, the round bottom flask was heated using a water bath set at 40 °C and the vacuum was controlled to prevent bubbling. The round bottom flask was kept under vacuum for a minimum of 1 hr up to 3 hr. The dry film was hydrated with 4 ml of the appropriate hydrating medium using the hand shaken method. The resulting liposomes were sonicated using a probe sonicator set at power level 3 for a total of 1 minute divided into 6 equal sessions. Six cycles of freezing and thawing were

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performed using a dry ice bath and a room temperature water bath. Finally, the liposomes were extruded 4 or 5 times through an extruder (Lipex Biomembranes Extruder, Vancouver, BC, Canada), using a water heating jacket unit to 50 °C, fitted with a polycarbonate membrane of a desired pore size. The pressure needed for extrusion was applied through regulated pressurized nitrogen. The flow rate of the extrusion process was monitored and if needed, the polycarbonate membrane was replaced. The suspension from this process was run through an appropriate size exclusion column using PBS as a running buffer and the fractions were collected and the amount of the agent of interest in the different fractions was measured. The fractions corresponding to the liposomes were pooled together and used in further tests and experiments.

The preparations made with this procedure used 80 mM Calcein in 1 N NaOH as the hydrating solution and the mixture was passed through a 100 nm polycarbonate membrane.

The components of the liposome lipid membrane were the following:

Component	**Mole ratio**	**M.W.**	**Weight (mg)**
Egg PC	50	762.1	19
CHEMS	50	486.7	12
Tween-80	2	1310	1.3
Oleyl Alcohol	80	268.5	11

Several preparations of liposomes were prepared with or without Lac-Cer at one of the concentrations as enumerated in the following table:

Component	**Mole ratio**	**M.W.**	**Weight (mg)**

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Lac-Cer 5%	10	749.98	3.75
Lac-Cer 1%	2	749.98	0.75

Procedure 2

The components of the liposomal membrane were dissolved in chloroform at 1 ml each and they were combined in a completely dry 100 ml round bottom flask. The chloroform was removed, leaving a thin uniform lipid film on the walls of the round bottom flask, as described earlier. The dry film was hydrated with 2 ml of the appropriate hydrating medium using the hand shaken method. The resulting liposomes were sonicated using a bath sonicator for 4 min. Six cycles of freezing and thawing were performed using a dry ice bath and a room temperature water bath. Finally, the liposomes were extruded an several number of times (15 or 17 extrusions) using an Avanti Polar Lipids, USA, Mini Extruder. The extruder was heated using a solid heating block unit to 50 to 60 °C, fitted with a polycarbonate membrane of a desired pore size. The pressure needed for extrusion was applied manually. The polycarbonate membrane integrity after this process was confirmed under the microscope and if damaged, the extrusion step was repeated. The suspension from this process was run through an appropriate size exclusion column using PBS as a running buffer and the fractions were collected and the amount of the agent of interest in the different fractions was measured. The fractions corresponding to the liposomes were pooled together and used in further tests and experiments.

The preparations made with this procedure used the following hydrating solutions:

- 80 mM Calcein in 1 N NaOH
- 200 µg/ ml FITC in PBS

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- PBS

Polycarbonate membranes used in this procedure were: 50 nm, 80 nm, 100 nm and 200 nm in pore size.

The components of the liposomes lipid membrane were the following:

Component	Mole ratio	M.W.	Weight (mg)
Egg PC	50	762.1	19
CHEMS	50	486.7	12
Tween-80	2	1310	1.3
Oleyl Alcohol	80	268.5	11

Several preparations of liposomes were prepared with or without Lac-Cer at one of the concentrations as enumerated in the following table:

Component	Mole ratio	M.W.	Weight (mg)
Lac-Cer 1%	2	749.98	0.75
Lac-Cer 0.5%	1	749.98	0.375
Lac-Cer 0.2%	0.4	749.98	0.15

Procedure 3

The components of the liposomal membrane were dissolved in chloroform (1 ml each) and they were combined in a completely dry 100 ml round bottom flask. The chloroform was removed, leaving a thin uniform lipid film on the walls of the round bottom flask, as described earlier. The dry film was hydrated with 2 ml of the appropriate hydrating medium using the hand shaken method. The resulting liposomes were sonicated using a bath sonicator for 4 min. Six cycles of freezing and thawing were performed using a dry ice bath and a room temperature water bath. Finally, the liposomes were extruded 15 or 17 times using an Avanti Polar Lipids, USA, Mini Extruder. The extruder was heated using a solid heating block unit to 50 °C, fitted with a polycarbonate membrane of a desired pore size. The pressure needed

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for extrusion was applied manually. The polycarbonate membrane integrity after this process was confirmed under the microscope and if damaged, the extrusion step was repeated. The suspension from this process was run through an appropriate size exclusion column using PBS as a running buffer and the fractions were collected and the amount of the agent of interest in the different fractions was measured. The fractions corresponding to the liposomes were pooled together and used in further tests and experiments.

Polycarbonate membranes used in this procedure were: 100 nm and 200 nm in pore size.

The preparations made with this procedure used the following hydrating solutions:

- 200 µg/ ml FITC in PBS
- 200 µg/ ml HRPO in PBS
- 500 µg/ ml HRPO in PBS
- 500 µg/ ml HRPO in PBS and 1 X Protease Inhibitors Cocktail
- 500 µg/ ml HRPO in PBS and 2 X Protease Inhibitors Cocktail
- 1 mg/ ml YP4 MAbs in PBS spiked with FITC
- 2 mg/ ml FITC labeled YP4 MAbs in PBS
- Tc^{99} DTPA

The components of the liposome lipid membrane were the following:

Component	Mole ratio	M.W.	Weight (mg)
Egg PC	50	762.1	9.5
CHEMS	50	486.7	6
Tween-80	2	1310	0.65
Oleyl Alcohol	80	268.5	5.5

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Several preparations of liposomes were prepared with or without Lac-Cer at concentration of 0.5%

3.3.2. Size Analysis

100 µl of the pooled liposomes was diluted into 4 ml of filtered PBS in a disposable cuvette. The size of the liposomes was measured by dynamic light scattering technique with Zetasizer 3000 (Malvern, UK). More liposomes were added if needed as indicated by the analysis device. The device was set to determine size based on intensity.

3.3.3. Optimization of Extrusion Step

A thin lipid film was prepared and hydrated as mentioned above using PBS. During the extrusion step, a 100 µl sample was taken after extrusions 11, 13, 15 and 17 and diluted with PBS into a total volume of 4 ml. Size analysis was performed to determine the optimal number of extrusions for every polycarbonate membrane pore size.

3.3.4. Determination of pH Sensitivity

A 100 µl aliquot of the liposomal preparation, with the lipid mixture of interest, prepared using 80 mM Calcein in 1 N NaOH for hydration, was serially diluted to $1{:}10^5$ with a final volume of 4 ml in a disposable cuvette, in one of the following buffers: PBS buffer pH 7.3 (136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4) or sodium acetate buffer (100 mM NaCl, 10 mM acetate) pH 5.0, 5.5,

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6.0, and 6.5. The pH was adjusted using NaOH or HCl. After a 10 minute incubation at room temperature, the fluorescence was measured at excitation λ = 490 nm, and emission λ = 520 nm using a fluorometer (FluoroMax, Spex, USA). The liposomes were then disrupted by adding 200 μl 2% Triton X-100 in PBS to make a final concentration of 0.1% and the fluorescence measurement was taken. PBS was used as a blank. The ratio of the fluorescence before and after disrupting the liposomes corresponds to the amount of free dye that leaked from the liposomes.

3.3.5. Evaluation of Stability at 4 °C in PBS

A liposomal preparation, with the lipid mixture of interest, was prepared using 80 mM Calcein in 1 N NaOH for hydration and the liposomes were stored at 4 °C in a sealed glass container. At Days 0, 1, 7, 14 and 28 (day 0, is after elution through the size exclusion column) a 100 μl sample was taken and serially diluted to $1:10^5$ in PBS with a final volume of 4 ml in a disposable cuvette. The fluorescence was measured at excitation λ = 490 nm, and emission λ = 520 nm using Fluorometer (FluoroMax, Spex, USA). Then the liposomes were disrupted by adding 200 μl 2% Triton X-100 in PBS to make a final concentration of 0.1% and the fluorescence measurement was taken. PBS was used as a blank. The ratio of the fluorescence before and after disrupting the liposomes corresponds to the amount of free dye that leaked from the liposomes.

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3.3.6. Evaluation of Stability at 37 °C in FBS

A liposomal preparation, with the lipids mixture of interest, was prepared using 80 mM Calcein in 1 N NaOH for hydration. A 100 µl sample of the preparation was diluted with FBS into a total of 1 ml in a 2 ml ependorf tube and incubated in a water bath set at 37 °C for 1 hr. At the end of the incubation, a 100 µl sample was serially diluted with PBS into a final dilution of $1:10^5$ ($1:10^4$ dilution of the liposomes in FBS suspension where the liposome stock was diluted into a 1:10 dilution) with a final volume of 4 ml in a disposable cuvette. The fluorescence was measured at excitation λ = 490 nm, and emission λ = 520 nm using fluorometer (FluoroMax, Spex, USA). Then the liposomes were disrupted by adding 200 µl 2% Triton X-100 in PBS to make a final concentration of 0.1% and the fluorescence measurement was taken. PBS was used as a blank. The ratio of the fluorescence before and after disrupting the liposomes corresponds to the amount of free dye that leaked from the liposomes.

3.4. *In vitro* Uptake Testing

3.4.1. Tissue Culture of Hepa 1c1c7 Cell Line

Growth in 75 cm^2 Tissue Culture Flasks

A cell stock was removed from liquid nitrogen and quickly thawed in a water bath. The DMSO in the cell stock was removed by diluting the cell stock with 5 ml of serum free DMEM in a 15 ml centrifuge tube. The cell suspension was centrifuged at 1200 rpm for 3 min and the supernatant was aspirated. The cells were resuspended in 12 ml of 10% FBS DMEM and transferred into a 75 cm^2 tissue culture flask and incubated at 37 °C and 5% CO_2. The media was changed after 24 hr. Approximately

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48 h later the cell confluence was determined under the light microscope and if needed, the medium was aspirated and 12 ml of fresh 10% FBS DMEM was added. When the cells reached full confluence, they were washed with sterile PBS and treated with 1X 5 ml Trypsin EDTA and incubated at 37 °C and 5% CO_2 for 5 min. The cell suspension in Trypsin EDTA was diluted with 7 ml of 10% FBS DMEM and mixed well using a sterile pipette. The flask content was transferred into 3 new 75 cm^2 tissue culture flasks and the volume in each flask was adjusted into a total of 12 ml with 10% FBS DMEM. The flasks were incubated at 37 °C and 5% CO_2 for 72 hr with one change of media to reach full confluence.

Growth in 12, 24 and 96 Well Tissue Culture Plates

The cells in a fully confluent 75 cm^2 tissue culture flask were washed with sterile PBS and treated with 5 ml Trypsin EDTA and incubated at 37 °C and 5% CO_2 for 5 min. The cell suspension in Trypsin EDTA was diluted with 7 ml of 10% FBS DMEM and mixed well using a sterile pipette. A 4 ml aliquot of the flask content was diluted into 25 ml with 10% FBS DMEM and mixed well. For 12 well TCPs, 2 ml of the cell suspension was transferred into each well of a 12 well tissue culture plate. The cells were incubated at 37 °C and 5% CO_2 for 24 hr. They were then viewed under the light microscope and the media changed and allowed a further 24 hr incubation to reach full confluence. For 24 well TCPs, 1 ml of the cell suspension was transferred into each well of 24 well TCP. The cells were incubated at 37 °C and 5% CO_2 for 24 to 36 hr to reach full confluence. The cells are viewed under the light microscope and the media changed after 24 hr of incubation if needed and a further 12 hr of incubation allowed to reach confluence. For 96 well TCPs, 200 µl of the cell

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suspension were transferred into each well of a 96 well tissue culture plate well using a multi-channel pipette. The cells were incubated at 37 °C and 5% CO_2 for 24 hr to reach full confluence.

Preparation of Cell Stocks (Working Cell Banks)

The cells in a fully confluent 75 cm^2 tissue culture flask were washed with sterile PBS and treated with 5 ml Trypsin EDTA and incubated at 37 °C and 5% CO_2 for 5 min. The cell suspension in Trypsin EDTA was diluted with 7 ml of 10% FBS DMEM and mixed well using a sterile pipette. The cell suspension was transferred to a 15 ml centrifuge tube and centrifuged at 1200 rpm for 3 min. The cells were resuspended with 3 ml 10% DMSO and 10% FBS DMEM and split into 3 cryogenic vials. The vials were kept at – 20 °C for 2 hr and then transferred into liquid nitrogen for storage.

3.4.2. Labeling of Proteins

The proteins to be fluorescently labeled were dissolved in FITC labeling buffer (0.05 M Boric Acid, 0.2 M NaCl, pH 9.2) at a concentration of 2 mg/ ml. A 20 μl aliquot of 5 mg/ ml of FITC in DMSO was added to the protein in the labeling buffer and allowed to react at room temperature for 2 hr in the dark. Subsequently, the solution was either:

- transferred to a dialysis bag and dialyzed against PBS pH 7.4 over 48 hr with 3 changes.

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- or run through a Sephadex G-25 size exclusion column, bed volume 15 ml, 12 cm height and 1 cm diameter. The fractions were collected and their fluorescence measured at absorption = 495 nm and emission = 520 nm.

The same procedure was used to label proteins with TRITC.

3.4.3. Protein Estimation

Concentrations of standard mouse IgG protein were prepared at 1, 0.8, 0.6, 0.4, 0.2, 0.1 mg/ml. The Bio-Rad protein assay reagent was diluted to a 1 X concentration with H_2O. 190 μl of the reagent was added to each well of a 96 well tissue culture plate. A 10 μl aliquot of the different concentrations of standard protein were added to the plate at 3 wells/ concentration of standard or test samples. The plate was kept for 5min at room temperature and the absorbance was read at 595 nm using a microplate reader (VMax Kinetic Microplate Reader, Molecular Devices, California, USA).

3.4.4. Confirmation of Asialoglycoprotein Receptor

The Hepa 1c1c7 hepatoma cell culture fully confluent in 24 well TCP was washed with sterile PBS twice. Solutions of TRITC labeled BSA or AF at 2, 10 and 50 μg/ ml in serum free DMEM were prepared. The 1 ml of the test solutions /well was added and a total of 4 wells/ concentration of each protein were used. The negative control included wells incubated with serum free DMEM. After 2 hr of incubation at 37 °C and 5% CO_2, a 100 μl from the supernatant of each well was diluted into 4 ml 0.2% Triton X-100 in PBS and transferred into a disposable fluorescence cuvette for measurement. The plates were washed twice with PBS and then 1 ml/ well of 0.2%

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Triton X-100 in PBS was added to the plates. After gentle shaking for 2 hr, the samples were diluted into 4 ml of 0.2% Triton X-100 in PBS and transferred into disposable fluorescence cuvettes for measurement. The samples were read using a fluorometer (FluoroMax, Spex, USA) at absorption = 495 nm and emission = 520 nm.

3.4.5. Toxicity Testing

The 96 well plates containing fully confluent Hepa 1c1c7 cells were prepared. Serum free DMEM was used to prepare solutions containing 1 mg, 100 μg, 10 μg and 1 μg /ml of total lipids for each liposomal preparation. The tissue culture plate was washed with 200 μl/ well of PBS and 100 μl/ well of the test solutions was added, with a total of 6 wells/ test. The cells were incubated for 2 (or 4) hr at 37 °C and 5% CO_2. The cells were washed with PBS 2 times with 200 μl/ well. 100 μl/ well of 0.5mg /ml MTT in serum free DMEM was added to each well and the plates were incubated for 2 hr at 37 °C and 5% CO_2. The solution content of the wells was later removed and 100 μl/ well of isopropyl alcohol were added. The plate was put on a plate shaker and left to shake gently for 1 hr. The optical density of the solution in each well was measured at 550 nm using a microplate reader (VMax Kinetic Microplate Reader, Molecular Devices, California, USA).

3.4.6. Testing of Liposome Uptake by Hepatoma Cells

Fully confluent 24 well tissue culture plates with the hepatoma cells were prepared. Solutions containing 50 μg/ ml of total lipids of liposomes encapsulating 200 μg/ ml

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of FTIC in PBS were prepared using serum free DMEM. The cell plates were washed with sterile PBS and then 1 ml of the test solutions /well were added with a total number of 6 wells/ test. The negative control used was neat serum free DMEM. After 2 hr of incubation at 37 °C and 5% CO_2, 100 μl from the supernatant was diluted into 4 ml 0.2% Triton X-100 in PBS and transferred into a disposable fluorescence cuvette for measurement. The plates were washed twice with PBS and then 1 ml/ well of 0.2% Triton X-100 in PBS was added to the plates. After gentle shaking for 2 hr, the samples were diluted into 4 ml of 0.2% Triton X-100 in PBS and transferred into a disposable fluorescence cuvette for measurement. The samples were read using a fluorometer (FluoroMax, Spex, USA).

3.5. Biodistribution Determination

Liposomes encapsulating Tc^{99} DTPA were prepared as described in previous sections. Insulin syringes were filled with approximately 100 μl/ syringe, the exact amount was determined/ syringe, with a total of 10 syringes/ preparation for every liposomal preparation. Five syringes were used/ test and 3 were used for reference and 2 were kept in case they were needed. A total of 5 female balb/c mice, ages between 8 and 10 weeks and weighing 15 to 22 gm/ mouse, were kept at the site of the experiment overnight. Each mouse received an intravenous tail vein injection and was euthanized after 10 min (or 1 hr) by cervical dislocation. The mice were dissected and the following organs or tissues were collected in scintillation vials: blood, heart, lungs, thyroid, liver, spleen, stomach, GI tract and kidneys. The urine was also collected and the carcass was split into upper and lower parts which were

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also collected in scintillation vials. The tissue content of each scintillation vial was weighed and the radioactivity was measured using a gamma counter (1480 Wizard 3 Gamma Counter, Wallac, USA)

3.6. Survival of Biologically Active Material

3.6.1. Production and Purification of YP4 MAbs

3.6.1.1. YP4 anti-HRPO hybridoma culture

A stock of YP4 hybridoma was removed from liquid nitrogen storage and thawed in a water bath. The content was suspended in 10 ml of serum free DMEM in a 15 ml centrifuge tube. The hybridoma cells were precipitated by centrifugation at 1200 rpm for 3 min. The supernatant was aspirated and the cell pellet was resuspended using 15 ml of 10% FBS DMEM. The cell suspension was transferred into a 75 cm^2 tissue culture plate and incubated at 37 °C and 5% CO_2 with daily monitoring under the microscope. When sufficient growth in the cell population was obtained, the cells were used to seed 175 cm^2 tissue culture plate with 120 ml of 5% FBS DMEM/ plate. The cells were allowed to grow until they died or until the medium color changed to yellow.

3.6.1.2. Affinity chromatography purification using protein G

A 2 ml bed volume protein G column was equilibrated with PBS. The supernatant was filtered with Whatman filter paper. The pH of the supernatant was checked and if it was too low, the supernatant was mixed with 2 X PBS pH 7.2 – 7.4. The supernatant was run through the column allowing the antibodies to bind to protein G. The column was then washed with PBS to remove unbound proteins. Fractions of the washing were collected and the OD was measured at 280 nm. The process was continued until the absorption was at baseline. The bound IgG was eluted with 0.1M glycine.HCl pH 2.8 and 1 ml fractions were collected in test tubes already containing 1 ml of 1.5M Tris.Cl pH 8.9 and the OD of each fraction was measured at 280 nm. The fractions with an OD of higher than 0.1 were pooled together and dialyzed

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against PBS. The final product was concentrated using Centricon tubes. The protein G column was equilibrated with 0.03% sodium azide in PBS and stored at 4 °C.

3.6.1.3. ELISA Testing for YP4 MAbs:

An ELISA plate was coated with 100 μl of 4 μg/ ml Goat anti-Rat IgG/ well over night at 4 °C. The wells were washed three times with PBS using the wash bottle method. The wells were then blocked with 200 μl of 3% BSA in PBS for 2 hr at room temperature. The wells were washed with PBS using the wash bottle method three times. A 100 μl aliquot of the test solution was added/ well and incubated for 1 hr at room temperature. The wells were washed with PBS using the wash bottle method three times. Subsequently, 100 μl of 1 μg/ ml HRPO/ ml in PBS / well were added and incubated for 1 hr at room temperature. The wells were washed with PBS using the wash bottle method three times. Lastly, 100 μl of TMB substrate/ well was added and allowed to react for 20 min. The plates were read using a microplate reader (VMax Kinetic Microplate Reader, Molecular Devices, California, USA)

3.6.2. Survival of YP4 MAbs

Fully confluent hepatoma cells in 24 well tissue culture plates were prepared. Solutions containing 50 or 100 μg/ ml of total lipids of liposomes encapsulating YP4 MAbs at 500 μg, 1 mg or 3 mg/ ml in PBS were prepared using serum free DMEM. The cell plates were washed with sterile PBS and then 1 ml of the test solutions /well were added with a total number of 4 wells/ test (n = 4). The cell plates were incubated at 37 °C and 5% CO_2 for the test period (1, 2, 4 or 8 hr) and then 100 μl of the supernatant of each well was collected and diluted with Triton X- 100 in PBS into a final concentration of 0.2% Triton X- 100 in PBS. The wells were washed twice with

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PBS and 1 ml of 0.2% Triton X- 100 in PBS was added/ well and the plates were put on a plate shaker for 1 hr. The supernatant and the cell extracts were tested by ELISA for functional YP4 MAbs.

3.6.3. HRPO and HRPO with Protease Inhibitors Cocktails

Fully confluent 24 well tissue culture plates of hepatoma cells were prepared. Solutions containing 50 or 100 μg/ ml of total lipids of liposomes encapsulating 200 μg or 500 μg/ ml of HRPO (or HRPO with 1 X or 2 X of Protease Inhibitors Cocktail) in PBS were prepared using serum free DMEM. The cell culture plates were washed with sterile PBS and then 1 ml of the test solutions /well were added with a total number of 4 wells/ test (n = 4). The cell plates were incubated at 37 °C and 5% CO_2 for the test period (1, 2, 4 or 8 hr) and then 100 μl of the supernatant of each well was collected and diluted with Triton X- 100 in PBS into a final concentration of 0.2% Triton X- 100 in PBS. The wells were washed twice with PBS and 1 ml of 0.2% Triton X- 100 in PBS was added/ well and the plates were put on a plate shaker for 1 hr. A 100 μl of the supernatant or cell extract were transferred/ well into a 96 tissue culture plate and 100 μl of TMB substrate were added/ well and were allowed to react for 20 min. The plates were read using a microplate reader (VMax Kinetic Microplate Reader, Molecular Devices, California, USA)

Additional liposomal preparations with biologically active material were spiked with a small amount of FITC. In the case of these liposomes, an extra plate of cells was used to estimate the uptake of liposomes by the cells as described in the earlier section.

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4. Results and Discussion

4.1. Liposomes Preparation and Evaluation

4.1.1. Efficiency of Encapsulation

The efficiency of encapsulation ranged between 4 and 11% for most of the various preparations, which corresponds to the average efficiency reported in literature for the hand shaken method (New 1990). The efficiency does not seem to be influenced by the presence or absence of Lac-Cer or its concentration, except for the 5% Lac-Cer containing liposomes which were very difficult to prepare and yielded a very low encapsulation. It also did not seem to be influenced by the total lipids at the levels we used, the extruders we used, or the type of hydrating agent used. It does however, seem to be influenced by the uniformity of the lipid film produced through the drying process which varied with different durations.

The extrusion through the lypex extruder was easier and fewer extrusions were required. The water jacketed heating system allowed constant control of the temperature during the extrusion process. The pressure provided through compressed nitrogen gas required less physical strength and labor for the extrusion process. The negatives of this extruder were that it required relatively large volume preparations, required more assembly and cleaning and there was a significant loss from the amount initially extruded to generate the final product due to splashing and frothing. The Avanti mini-extruder had the advantages of simple assembly and maintenance, the ability to use smaller volumes which was more economical, and most of the extruded material was retrieved at the end of the process. This allowed the preparation of formulations with expensive biochemicals. Its disadvantages were that

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it required physical strength and labor for extrusion, the heating system was a heating block where the temperature was not completely controlled and it was not very safe for the operator. It also required a lot of experience for the operator to balance the applied pressure between extruding the products and not puncturing the polycarbonate membrane. The filters needed to be checked for punctures or tears under the microscope after each extrusion process and if any defects were observed, the extrusion process was repeated.

4.1.2. Efficiency of Liposome Separation

The separation process required the use of different sizes of exclusion columns packed with different materials depending on the hydrating material used. The separation efficiency of the different columns differed based on the material used in the hydrating solutions, figures 5,6,7,8. It was not dependant on the presence, absence or the concentration of Lac-Cer in the formulation (data not shown) and was not affected by the size of the prepared liposomes. The separation profiles confirmed the successful formation of liposomes, which appear as a separate peak in the earlier fractions as a result of their large size. The removal of free agents which were not encapsulated into liposomes form a clear separate peak. Furthermore, the separation profiles can be used in the case of most agents to determine the efficiency of encapsulation. In the case of liposomes encapsulating HRPO, it was not possible to determine the efficiency of encapsulation as OD is not directly proportional to the amount of enzyme in each fraction and some of the readings exceeded the sensitivity of the plate reader. Attempts to fluorescently label HRPO were not successful.

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Adding a free dye to the hydration medium could not assist in the estimation of the efficiency of encapsulation for HRPO as the free dye arrives in later fractions compared to HRPO as a result of their relative small size. Better separation of HRPO liposomes or efficient separation of liposomes encapsulating YP4 antibodies was not achieved despite the use of size exclusion columns with the highest Sephadex grade available at our lab. Different separation columns were used including Sephadex G-200 35 X 1.5 cm column, which is the highest Sephadex number available to us, but did not yield an efficient separation. The early fractions contained a peak linked closely to the second peak. These fractions are expected to contain liposomes encapsulating YP4 antibodies as well as free antibodies. The fractions containing liposomes were selected based on visual observation of the milky appearance associated with suspended liposomes in a buffer.

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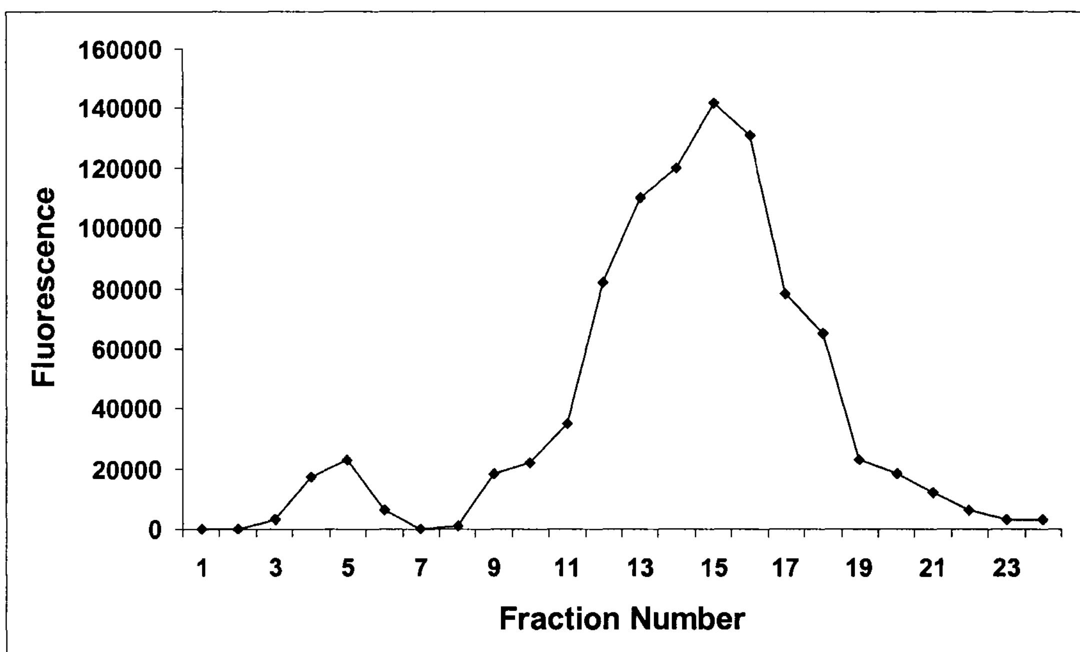

Figure 5: Purification profile of liposomes containing FITC with a Sephadex G-50 Column

Separation profile of plain liposomes encapsulating 200 μg/ ml FITC in PBS, extruded through a 100 nm PC membrane, from free dye using a Sephadex G-50 14 X 1 cm column. Two peaks were observed with fractions separating them with minimal fluorescence (efficient separation).

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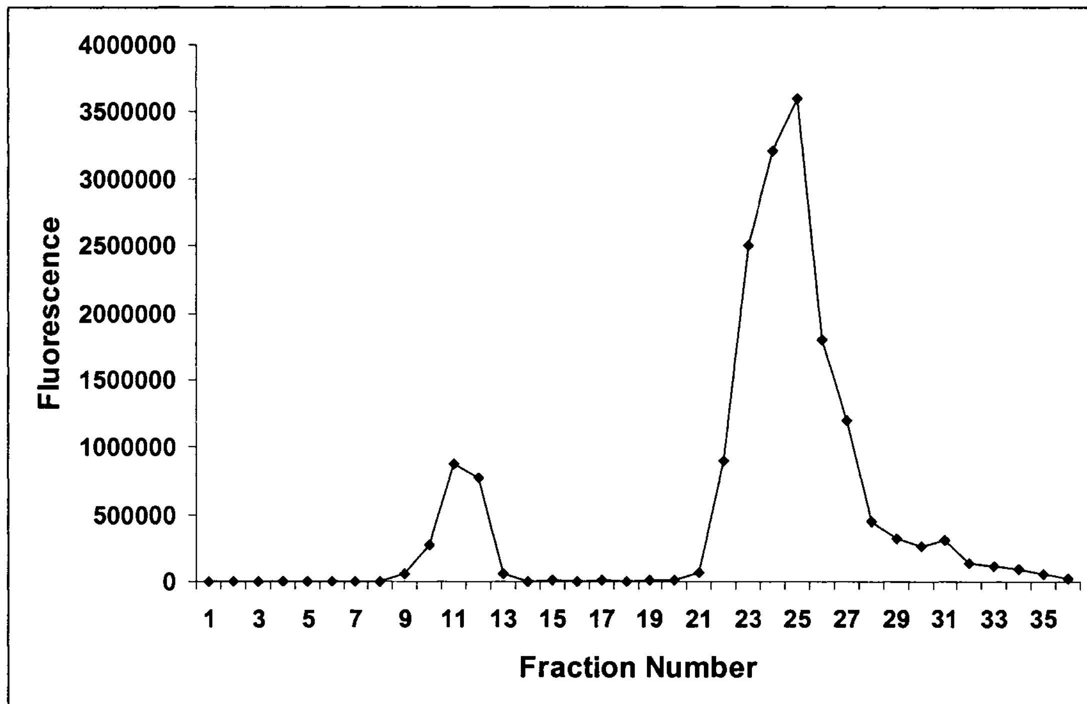

Figure 6: Purification profile of liposomes containing Calcein with a Sephadex G-75 Column

Separation profile of plain liposomes encapsulating 80 mM/ ml Calcein in 1 N NaOH, extruded through a 200 nm PC membrane, from free dye using a Sephadex G-75 35 X 1.5 cm column. Two peaks were observed with fractions separating them with minimal fluorescence (efficient separation).

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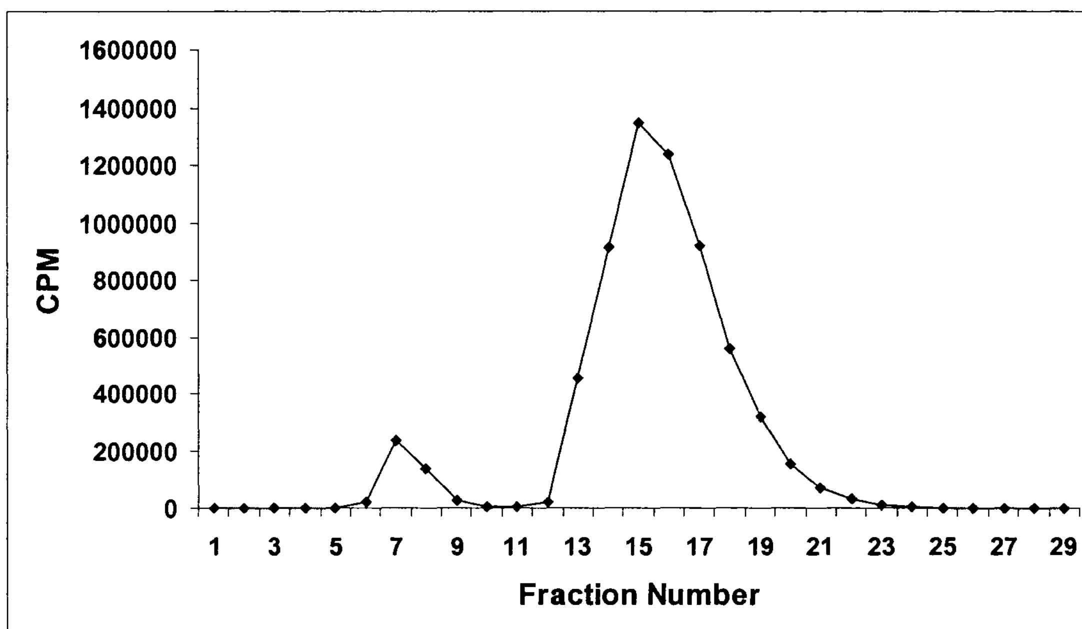

Figure 7: Purification profile of liposomes containing ^{99m}Tc DTPA with a Sephadex G-50 Column

Separation profile of plain liposomes encapsulating Tc^{99} DTPA at pH 7.3, extruded through a 100 nm PC membrane, from free isotope using a Sephadex G-50 PD-10 8 X 1 cm column. Two peaks were observed with fractions separating them with minimal fluorescence (efficient separation).

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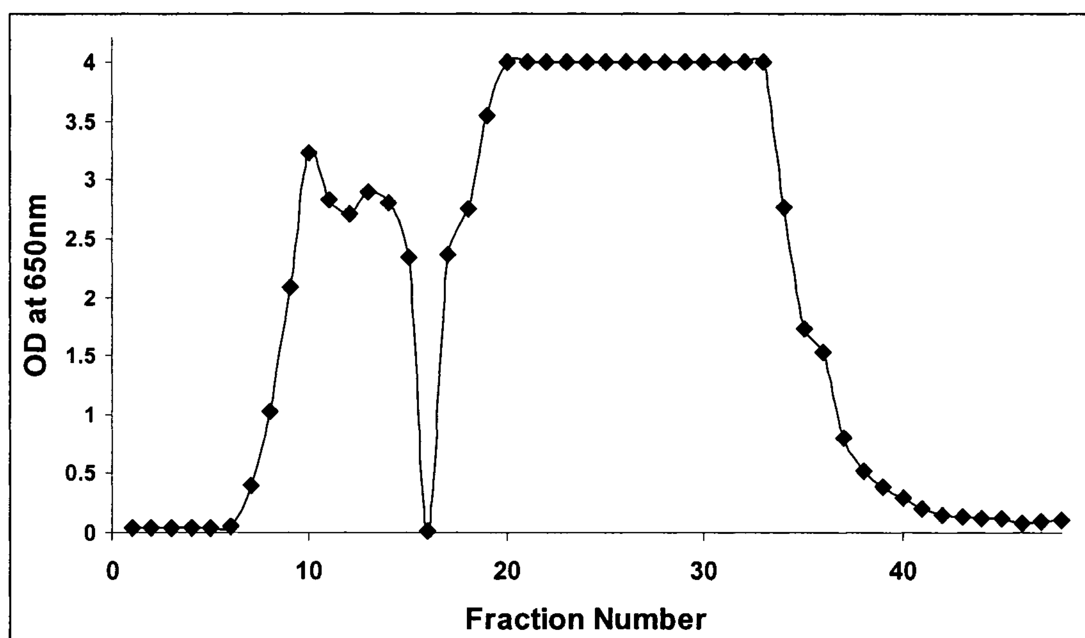

Figure 8: Purification profile of liposomes containingHRPO with a Sephadex G-200 Column

Separation profile of plain liposomes encapsulating 500 μg/ ml HRPO in PBS, extruded through a 100 nm PC membrane, from free protein using a Sephadex G-200 35 X 1.5 cm column. Two peaks were observed but there were no fractions separating them (acceptable separation).

4.1.3. pH Sensitivity

The first set of liposomes prepared containing 0, 1 and 5% of lactocylceramide, figure 9, confirmed the reproducibility of the data published in the literature for the original formulation. The data also indicated that the addition of lactosylceramide to the liposome formulation reduced the pH sensitivity of the preparation in a concentration dependent manner. Furthermore, it is apparent from the initial amount of Calcein released at pH 7.3 that the presence of lactosylceramide has reduced the stability of the preparation as evident from the high fluorescence in the 5% lactosylceramide containing liposomes.

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It became apparent that the concentration of lactosylceramide needs to be titrated, within the recipe for the liposomes, to identify concentrations at which acceptable pH sensitivity remains. The later batches of liposomes contained 0, 0.2, 0.5 and 1% of lactosylceramide as a mole ratio within the formulation. Two sets of liposomes were prepared. The first was extruded through a 100 nm polycarbonate membrane and the second was extruded through a 200 nm polycarbonate membrane. Both were tested for pH sensitivity and it was observed that at the molar concentrations of 0.2 and 0.5% of lactosylceramide used in the formulation, acceptable pH sensitivity was maintained, figures 10 and 11. There was a small reduction of pH sensitivity compared to that of liposomes prepared according to the original formula without the addition of lactosylceramide (Sudimack et al. 2002). The liposomal formulations containing 0.2 and 0.5% lactosylceramide were selected based on these pH sensitivity characteristics for further evaluation as described in later sections.

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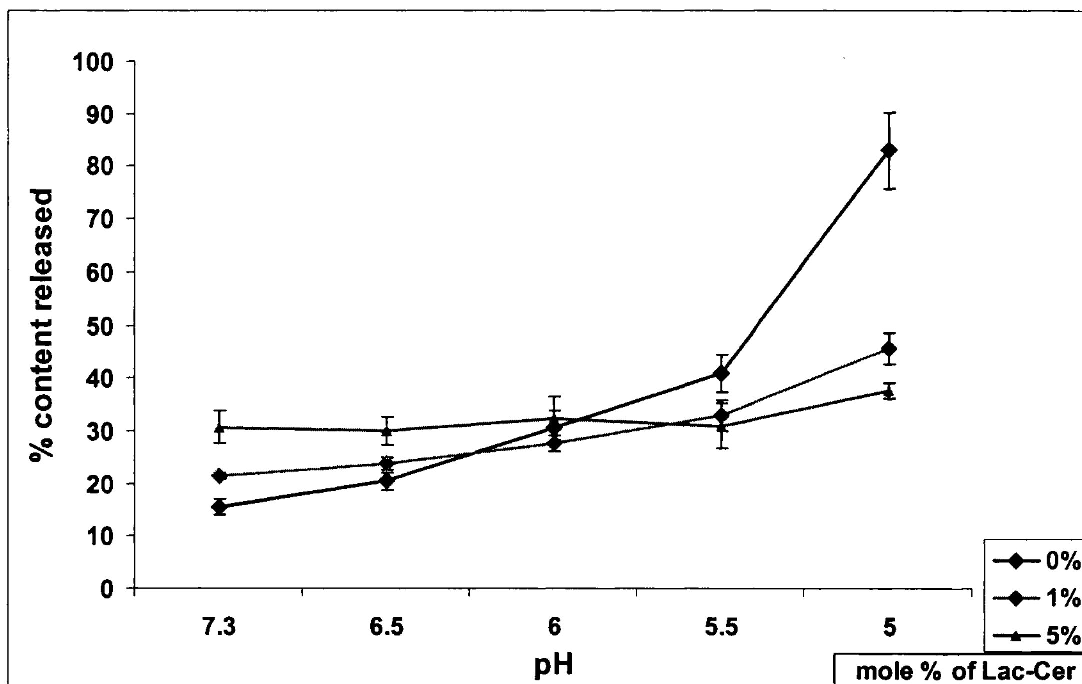

Figure 9: pH dependant release of liposomal content from 100 nm liposomes

Percentage of Calcein released from OAlc based liposomes containing 0, 1 and 5% mole Lac-Cer of their total lipids. The liposomes were passed through a 100 nm polycarbonate membrane fitted on a lypex extruder. The liposomes were incubated in PBS pH 7.3 or Citrate buffer pH 6.5, 6, 5.5 or 5 for 10 min, after serial dilutions, at room temperature. The fluorescence of released Calcein was measured and the liposomes were lysed using Triton X-100 at 0.1% for measurement of total (n = 3).

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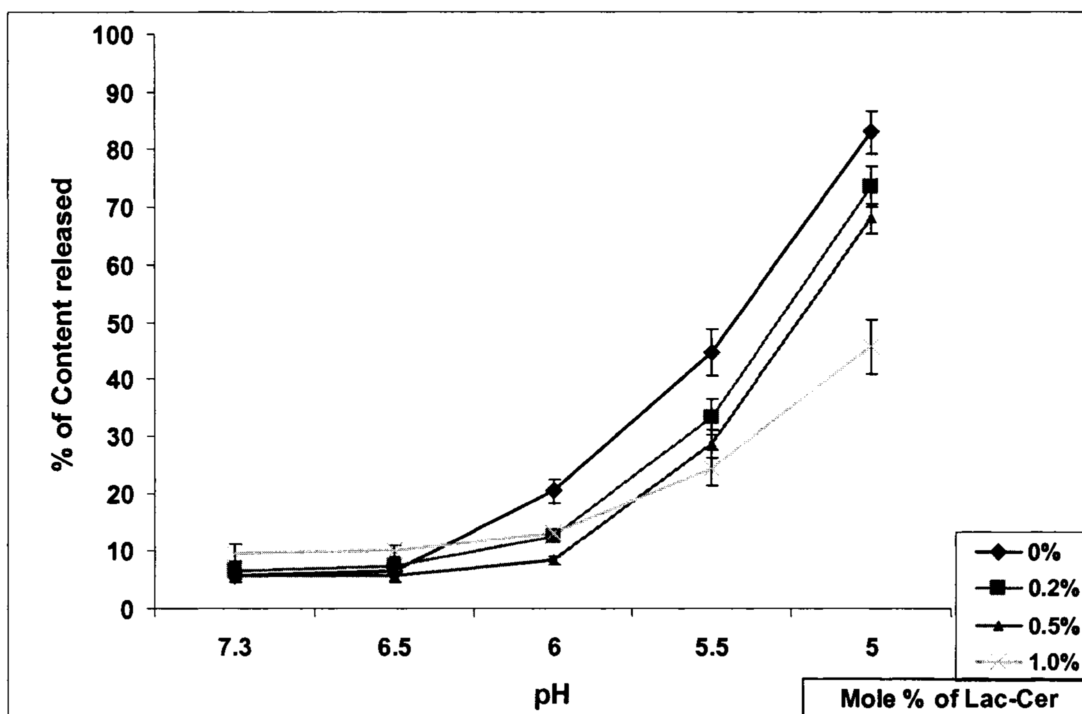

Figure 10: pH dependant release of liposomal content from 100 nm liposomes

Percentage of Calcein released from OAlc based liposomes containing 0, 0.2, 0.5 and 1% mole Lac-Cer of their total lipids. The liposomes were passed through a 100 nm polycarbonate membrane fitted on an Avanti mini-extruder. The liposomes were incubated in PBS pH 7.3 or Citrate buffer pH 6.5, 6, 5.5 or 5 for 10 min, after serial dilutions, at room temperature. The fluorescence of released Calcein was measured and the liposomes were lysed using Triton X-100 at 0.1% for measurement of total fluorescence (n = 3).

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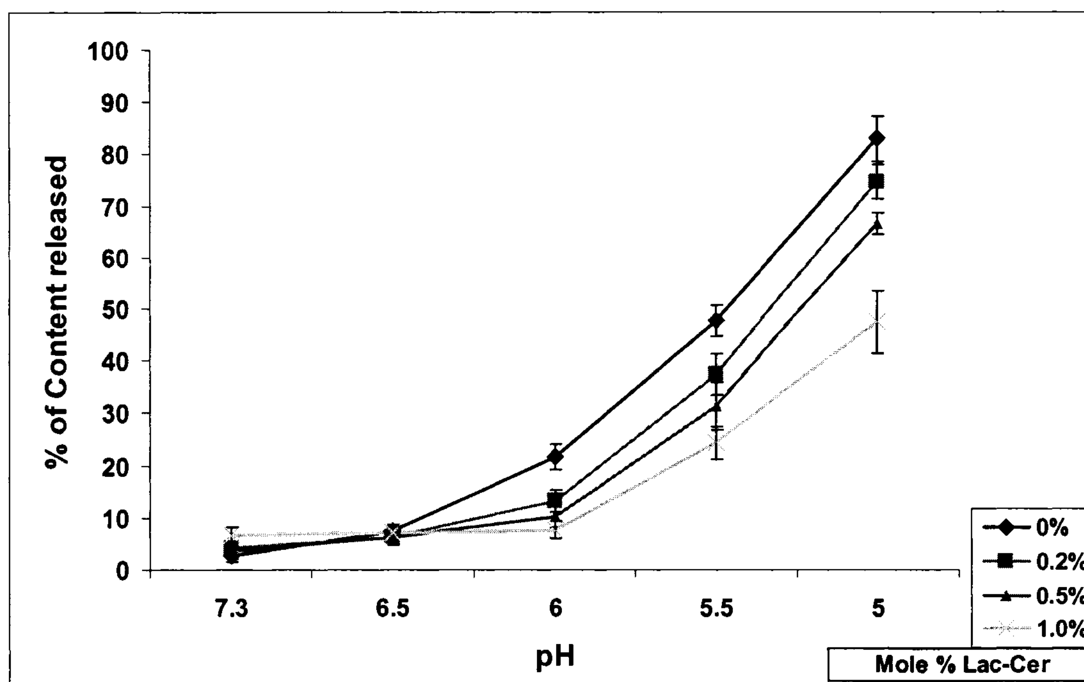

Figure 11: pH dependant release of liposomal content from 200 nm liposomes

Percentage of Calcein released from OAlc based liposomes containing 0, 0.2, 0.5 and 1% mole Lac-Cer of their total lipids. The liposomes were passed through a 200 nm polycarbonate membrane fitted on an Avanti mini-extruder. The liposomes were incubated in PBS pH 7.3 or Citrate buffer pH 6.5, 6, 5.5 and 5 for 10 min, after serial dilutions, at room temperature. The fluorescence of released Calcein was measured and the liposomes were lysed using Triton X-100 at 0.1% and the total fluorescence was measured (n = 3).

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4.1.4. Size Uniformity

The Avanti Polar Lipids Mini-Exruder was used for the preparation of the various liposomal preparations of liposomes, the size analysis profiles of which are shown in figure 12. The number of extrusions recommended by the manufacturer to achieve desired and uniform liposomal sizes was 11 extrusions. When the extrusion process was performed according to this protocol, two peaks were observed upon size analysis according to intensity, figure 12.1, indicating insufficient extrusion to achieve the desired size and uniformity characters. Further extrusions, with varying numbers of repetitions, showed that acceptable size uniformity was achieved through a higher number of extrusions of 17 and 15 extrusions for the sizes of 100 and 200 nm respectively, figures 12.2, 12.3, 12.4, and the procedure was modified accordingly.

Despite the modifications to the process, differences were still observed between the desired liposomal diameter and the actual average size of the prepared liposomes. Liposomes extruded through a 100 nm membrane had an average diameter of about 120 nm and liposomes extruded through a 200 nm membrane had an average diameter of about 170 nm. Furthermore, repeated attempts to produce liposomes at 50 and 80 nm sizes were not successful despite increasing the number of extrusions up to 31 extrusions, figures 12.5 and 12.6. As a result, it was not possible to test the effect of different sizes of liposomes on uptake as originally planned. An Added advantage of the size analysis process is that it provides confirmation of the formation of liposomes utilizing the modified procedure.

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Figure 12: Size distribution profiles of liposomes extruded through PC membranes with different pore sizes

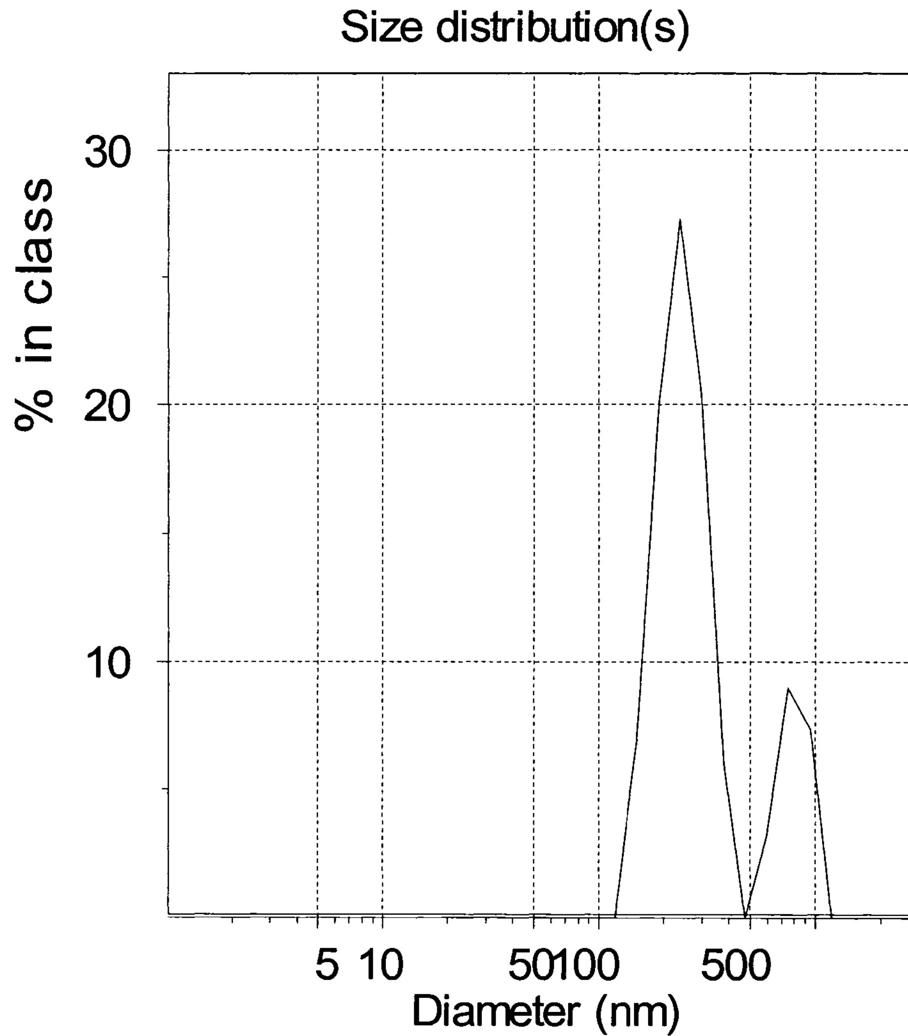

Figure 12.1 Size analysis of 0% Lactosylceramide liposomes extruded through a 200 nm PC membrane fitted on an Avanti mini extruder 11 times

Peak	Area	Mean	Width
1	80.5	243.9	165.3
2	19.5	799.5	407.9

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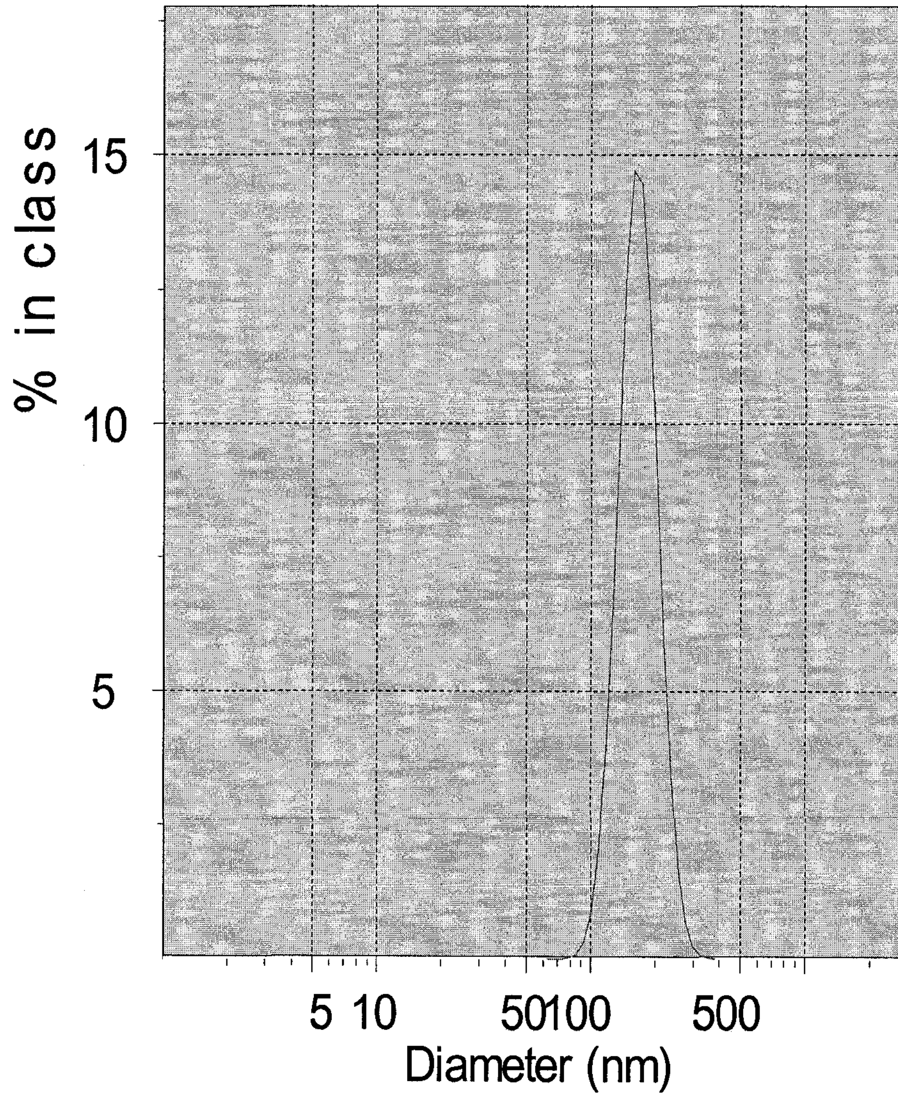

Figure 12.2 Size analysis of 0.2% Lactosylceramide liposomes extruded through a 200 nm PC membrane fitted on an Avanti mini extruder 15 times

Peak	Area	Mean	Width
1	100	168.2	83.2

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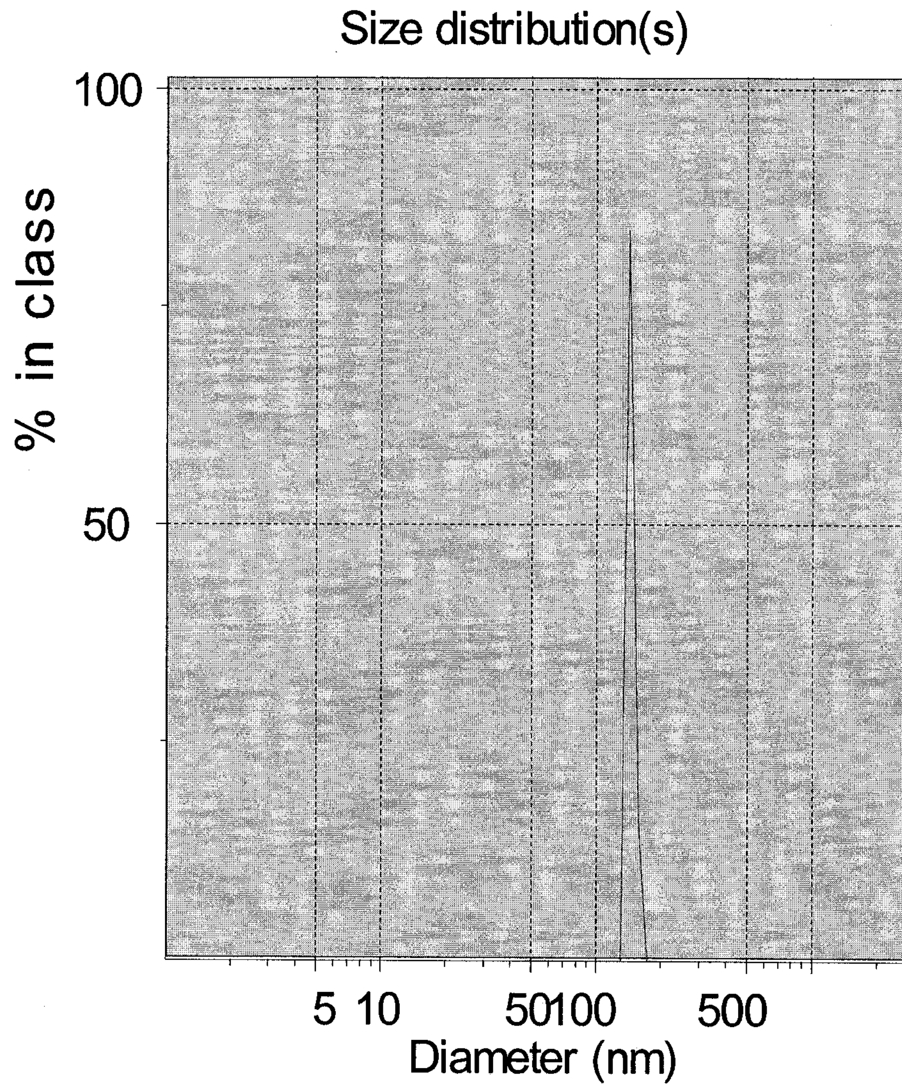

Figure 12.3 Size analysis of 0.2% Lactosylceramide liposomes extruded through a 100 nm PC membrane fitted on an Avanti mini extruder 15 times

Peak	Area	Mean	Width
1	100	145	16.1

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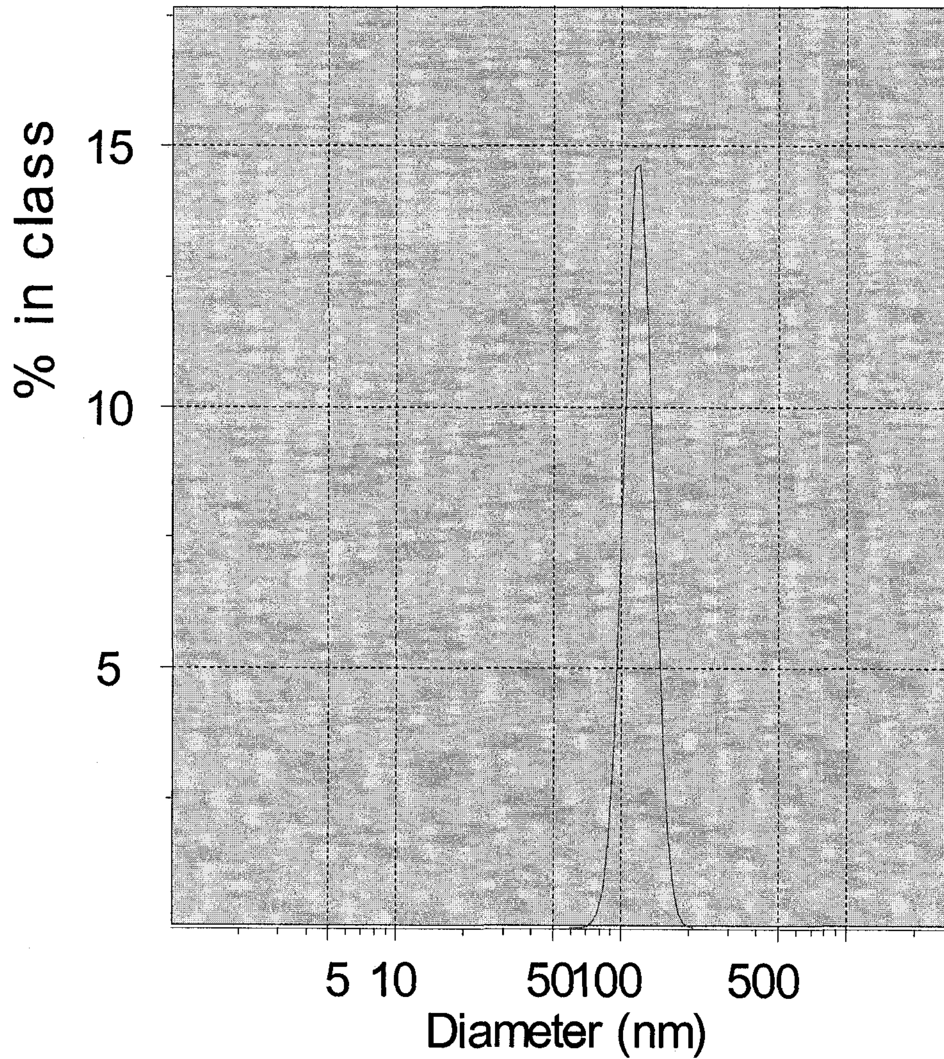

Figure 12.4 Size analysis of 1% Lactosylceramide liposomes extruded through a 100 nm PC membrane fitted on an Avanti mini extruder 17 times.

Peak	Area	Mean	Width
1	100	119.9	42.2

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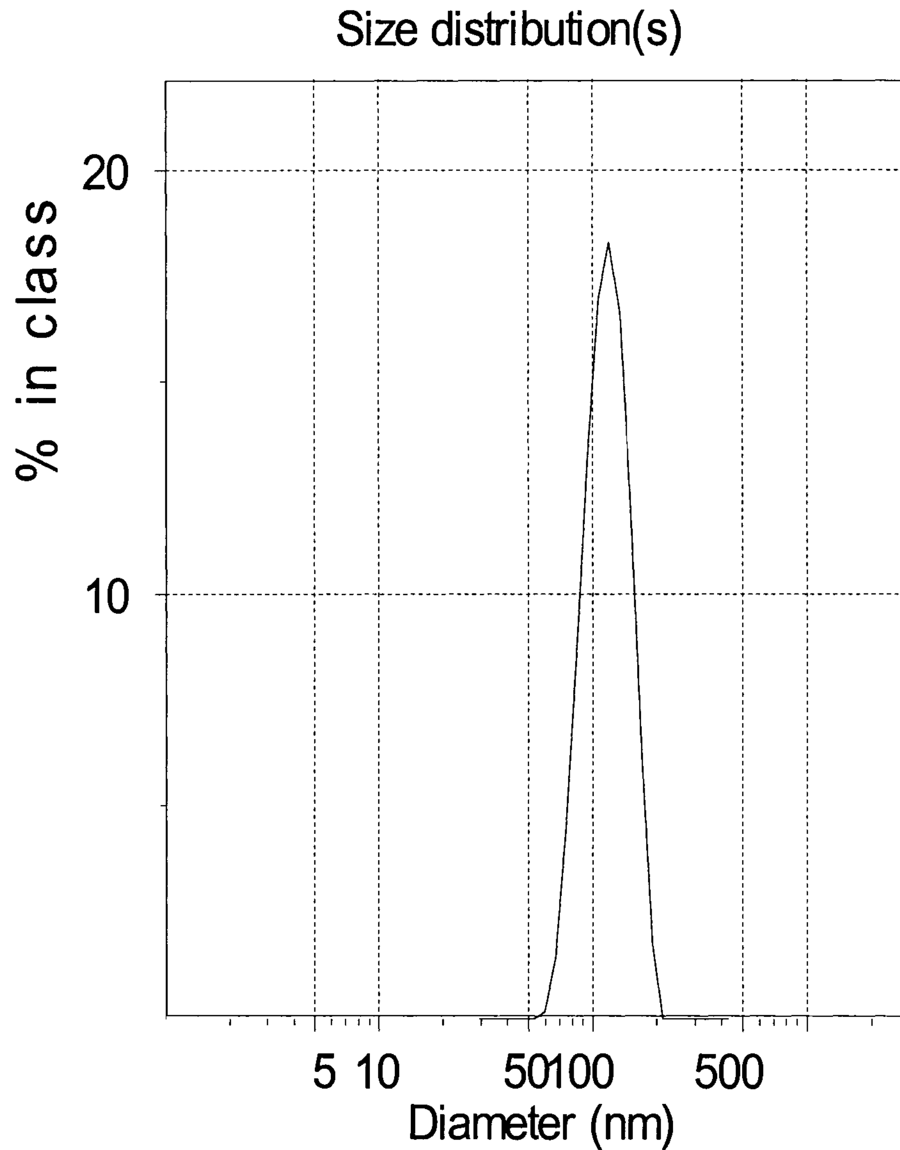

Figure 12.5 Size analysis of 0% Lactosylceramide liposomes extruded through an 80 nm PC membrane fitted on an Avanti mini extruder 17 times.

Peak	Area	Mean	Width
1	100	118.5	74.8

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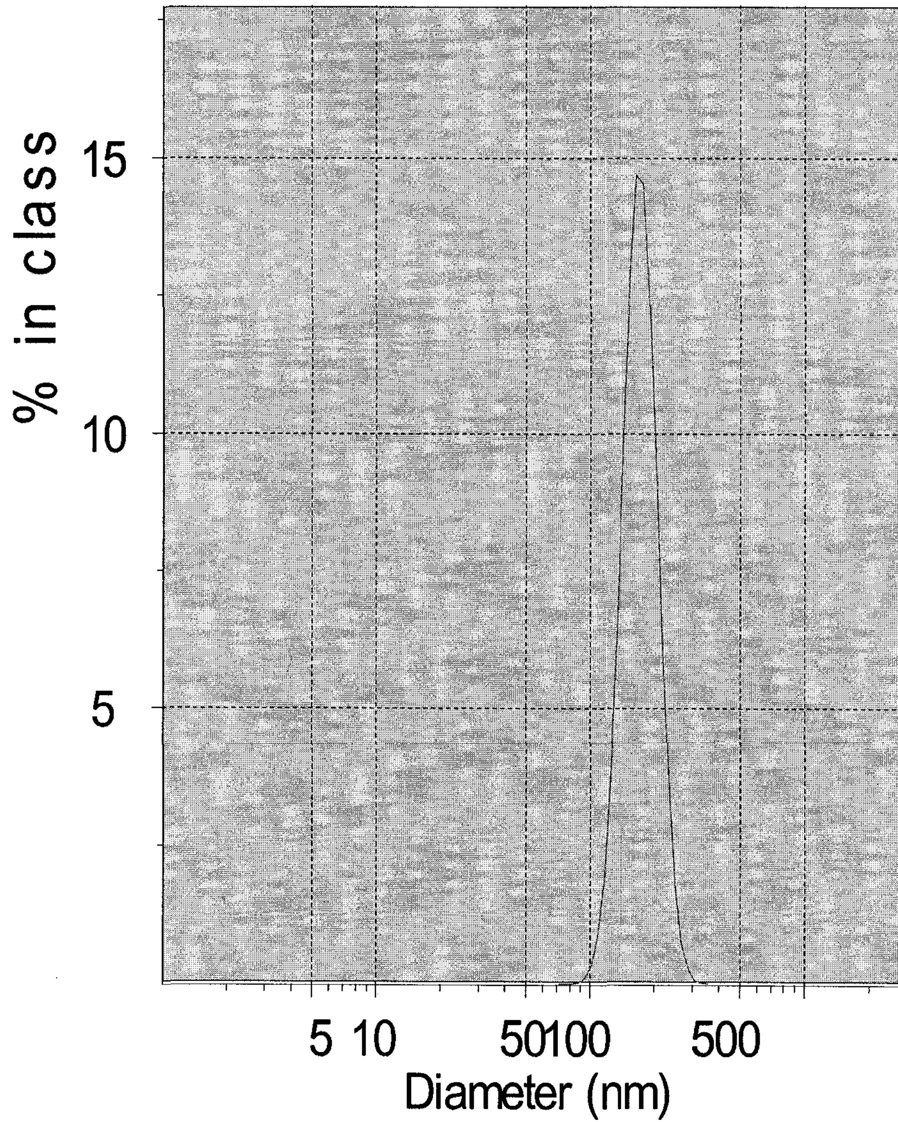

Figure 12.6 Size analysis of 0% Lactosylceramide liposomes extruded through a 50 nm PC membrane fitted on an Avanti mini extruder 17 times.

Peak	Area	Mean	Width
1	100	172.6	76.6

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4.1.5. Stability in Serum

The stability of the liposomal preparations in simulated biological conditions was evaluated by incubation in FBS at 37 °C for 1 hr. The high release of content during incubation indicated a decrease in stability. The results indicated a decrease in stability of liposomes that is dependant on the concentration of lactosylceramide in the formulation, figures 13 and 14. From the observed results, it was judged that the loss of stability in 1.0% Lac-Cer liposomes was too high to allow their use *in vivo* while 0.2 and 0.5% Lac-Cer liposomes still maintained acceptable stability. These results were similar with the testing of liposomes prepared in different sizes.

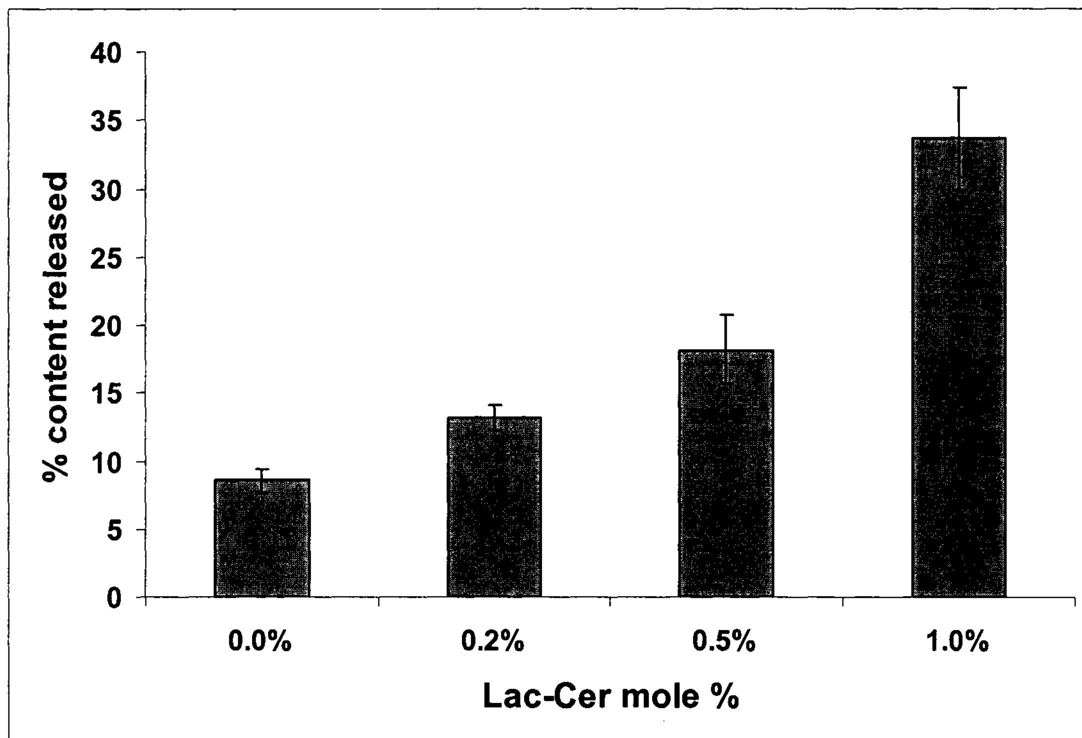

Figure 13: Stability of 100 nm liposomes at physiologic conditions

The percentage of calcein released from liposomes, containing 0, 0.2, 0.5 and 1% Lac-Cer encapsulating 80 mM of calcein in NaOH and extruded through a 100 nm PC membrane fitted on an Avanti mini extruder, after incubation in FBS for 1 hr at 37 °C (n = 3).

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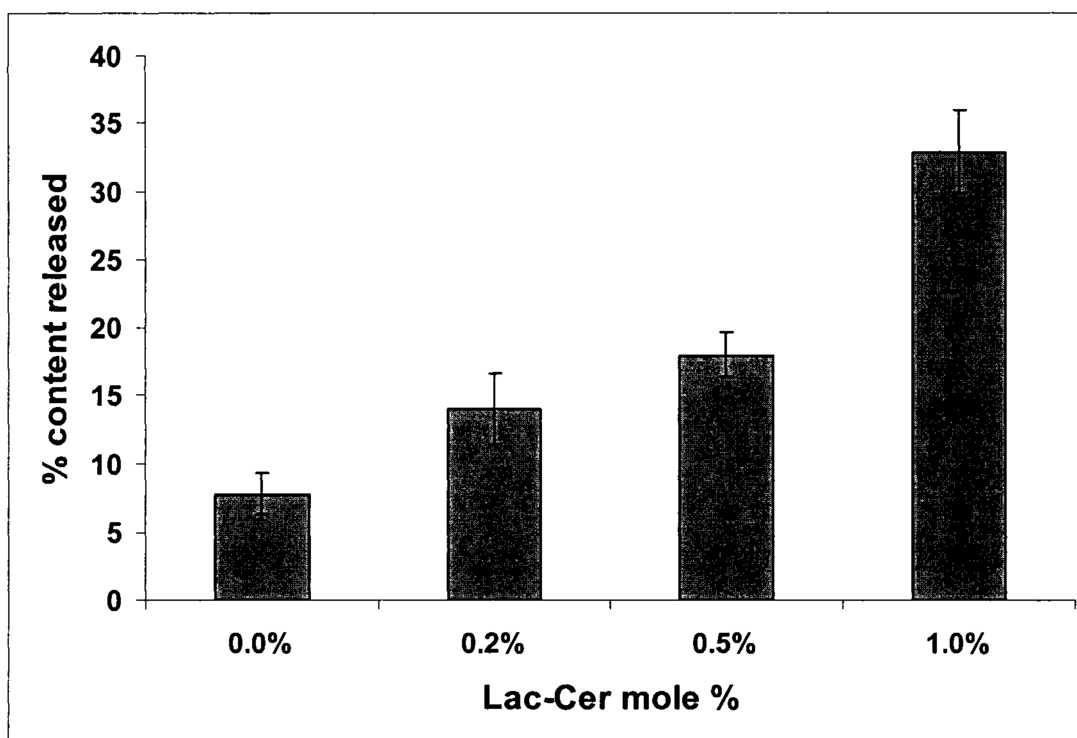

Figure 14: Stability of 200 nm liposomes at physiologic conditions

The percentage of calcein released from liposomes, containing 0, 0.2, 0.5 and 1% Lac-Cer encapsulating 80 mM of calcein in NaOH and extruded through a 200 nm PC membrane fitted on an Avanti mini extruder, after incubation in FBS for 1 hr at 37 °C (n = 3).

4.1.6. Stability upon Storage

The results of liposomal stability upon storage suggest gradual but constant release of liposomes content upon storage in PBS in the fridge at 0 to 4 °C, figures 15 and 16. The amount of released content was directly affected by the molar concentration of lactosylceramide in the prepared liposomes. Based on the observed rate of release, we concluded it is best to perform the desired tests on the prepared liposomes within the first two weeks of their preparation and preferably within the first week. For further testing after this duration of time, new batches should be prepared or the existing

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batches should be run through a size exclusion column once again. However, this will result in further dilution of the liposomes which should be taken into account when carrying out further tests.

Furthermore, we concluded that for a series of experiments that would involve different sizes of liposomes with different concentrations of Lac-Cer, which would require several days to prepare them, it is a better practice to start initially by preparing the liposomes which do not contain lactosylceramide and then proceed to the preparations which would contain it, starting with the lowest molar concentrations. This ensures the lowest possible amount of free, un-encapsulated, material in the liposomal suspensions when used in the designed experiments.

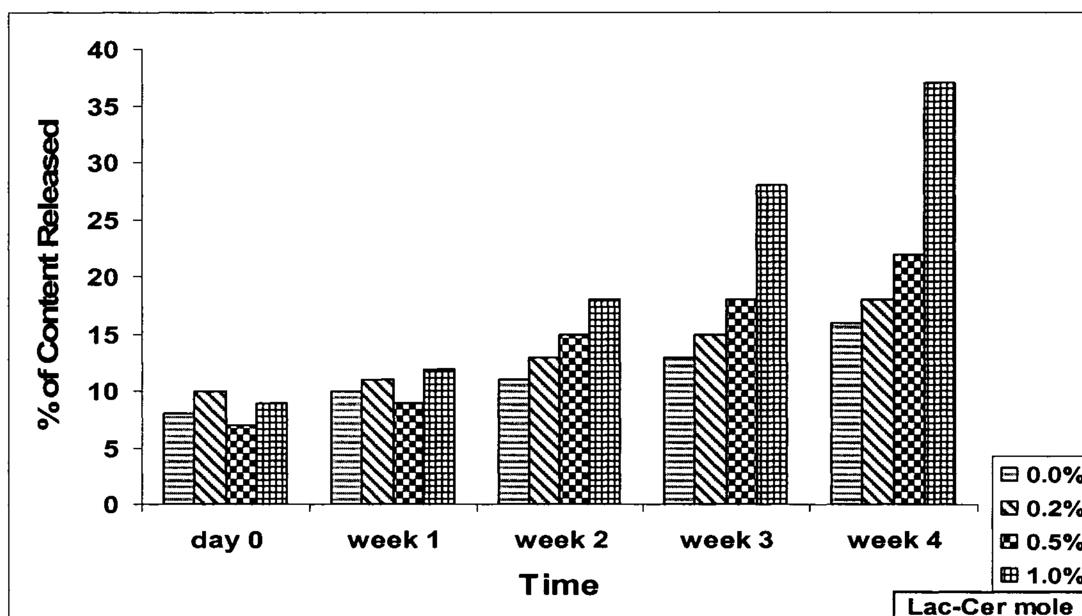

Figure 15: Stability of 100 nm liposomes at storage conditions

The percentage of calcein released from liposomes, containing 0, 0.2, 0.5 and 1% Lac-Cer encapsulating 80 mM of calcein in NaOH and extruded through a 200 nm PC membrane fitted on an Avanti mini extruder, after incubation in PBS for different time periods at 4 °C.

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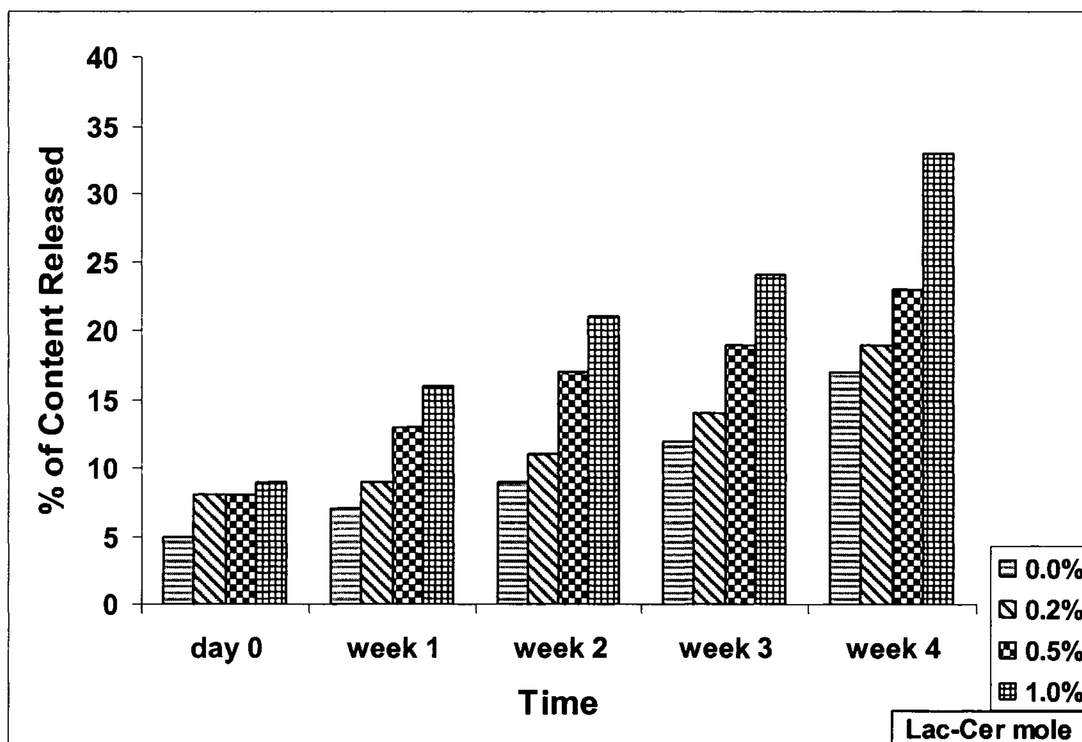

Figure 16: Stability of 200 nm liposomes at storage conditions

The percentage of calcein released from liposomes, containing 0, 0.2, 0.5 and 1% Lac-Cer encapsulating 80 mM of calcein in NaOH and extruded through a 200 nm PC membrane fitted on an Avanti mini extruder, after incubation in PBS for different time periods at 4 °C.

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4.2. Liposomes *in vitro* Uptake

4.2.1. Toxicity Testing

Evaluation of the *in vitro* toxicity of the liposomes allowed us to establish the concentration, at which liposomes testing with the hepatoma cell line is safe to perform. From the results, figure 17, we decided to use the liposomes at a 50 μg/ml concentration.

We also concluded that the addition of lactosylceramide at the concentrations we used does not add to the toxicity of the liposomes, alleviating fears of ceramide induced toxicity as reported in the literature (Reiners and Clift 1999), which was at higher concentrations of ceramides.

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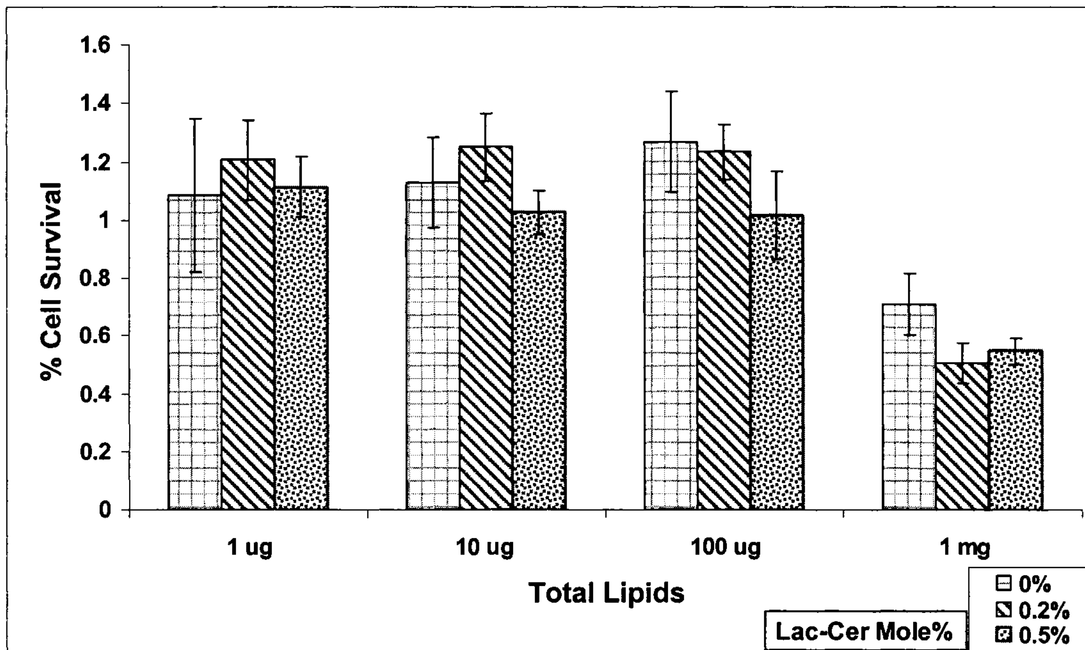

Figure 17: Toxicity of Lac-Cer liposomes

MTT test results after treatment of Hepa 1c1c7 cells in a TCP with liposomal preparations encapsulating PBS. The liposomes were formulated using different concentrations of Lac-Cer. Different concentrations of total lipids of these preparations were suspended in serum free DEMEM media. The cells were incubated with the treatments for 2 hr at 37 °C and 5% CO_2 before applying the MTT test to determine cell survival (n = 6).

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4.2.2. Demonstration of the Presesnce of ASGP-R on Hepatoma Cells

Asialofetuin (AF) is a well established ligand that binds to the ASGP-R and is often described as a natural ligand for it. Hence this probe was labeled with FITC and incubated with the hepatoma cell cultures. As a control, FITC-labeled BSA was used/ BSA was particularly chosen since albumin is one of the few proteins in serum which is a non-galactosylated protein. The results observed show that the uptake of AF, which has a high affinity to the ASGP-R, is higher than the uptake of BSA, which is used as a control, at each of the different concentrations used, figure 18. Furthermore, the percentage of AF taken up by the hepatoma cell line decreases as the concentration increases suggesting that the higher uptake is mediated by a receptor mechanism that is becoming saturated as the concentration increases. The percentage of BSA taken up by the cells remains constant at these concentrations suggesting that the uptake process is a result of cellular uptake or association of the culture medium and its content as a part of the feeding process for the cultured cells, figure 18. These observations along with the observed, and expected, increase in liposomal delivery into cells when lactosylceramide is added to the formulation, figures 19 and 20, confirm the presence of the ASGP-R receptor in the Hepa 1c1c7 cell line used in our experiments. This conclusion is further supported by an extensive literature search which gave no indication that the ASGP-R is absent from this commonly used cell line.

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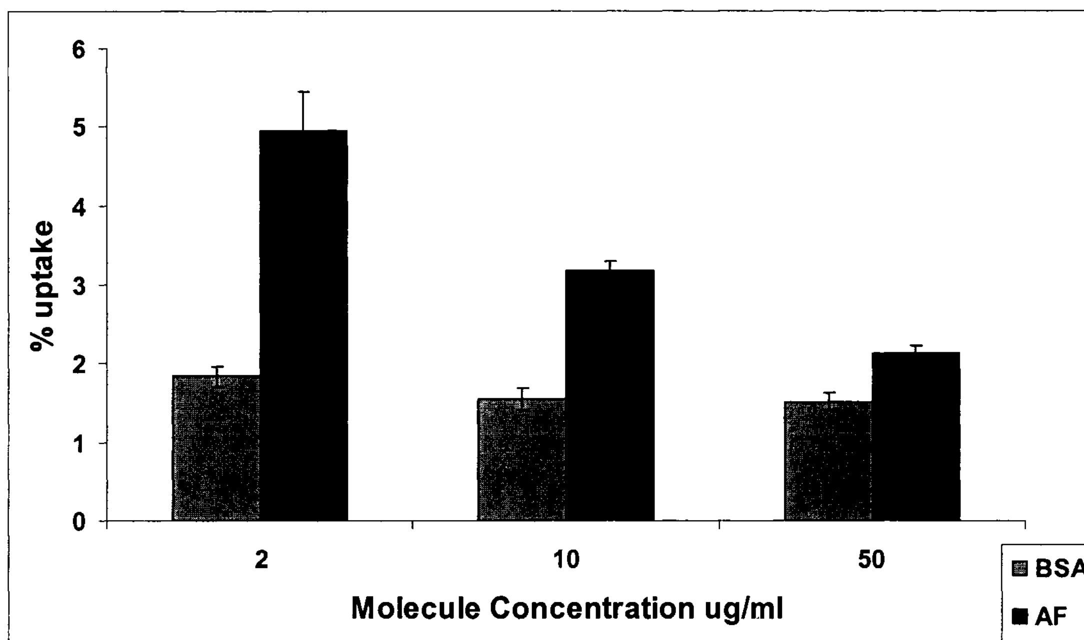

Figure 18: Uptake of labeled molecules by Hepa 1c1c7 cells

Uptake of FITC labeled AF and BSA at different concentrations in serum free DMEM by Hepa 1c1c7 cells in a TCP after a 2 hr incubation at 37 °C and 5% CO_2 (n = 3).

4.2.3. Uptake of Liposomes by a Hepatoma Cell Line

A significant increase in uptake was observed in liposomes containing lactosylceramide at a molar concentration of 0.5% and this increase was not observed at the 0.2% concentration when compared to the original formulation of liposomes. figures 19 and 20 and table. There was no significant difference in uptake between the two different sizes of liposomes used, the 100 and the 200 nm liposomes when comparing them at the same Lac-Cer concentrations. These findings support the hypothesis that the incorporation of lactosylceramide into the pH sensitive liposomes can promote the increase of uptake by hepatocytes while maintaining desired pH sensitivity and stability characters. The data also suggests that at the lower

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concentration of 0.2%, pH sensitivity and stability were maintained but selective uptake by hepatocytes was not achieved compared to control liposomes which lacked the Lac-Cer as part of its composition.

For subsequent experiments, the molar concentration of lactosylceramide at 0.5% was used. The formulation with the 0.2% concentration was determined to be less efficient with regards to hepatocyte binding.

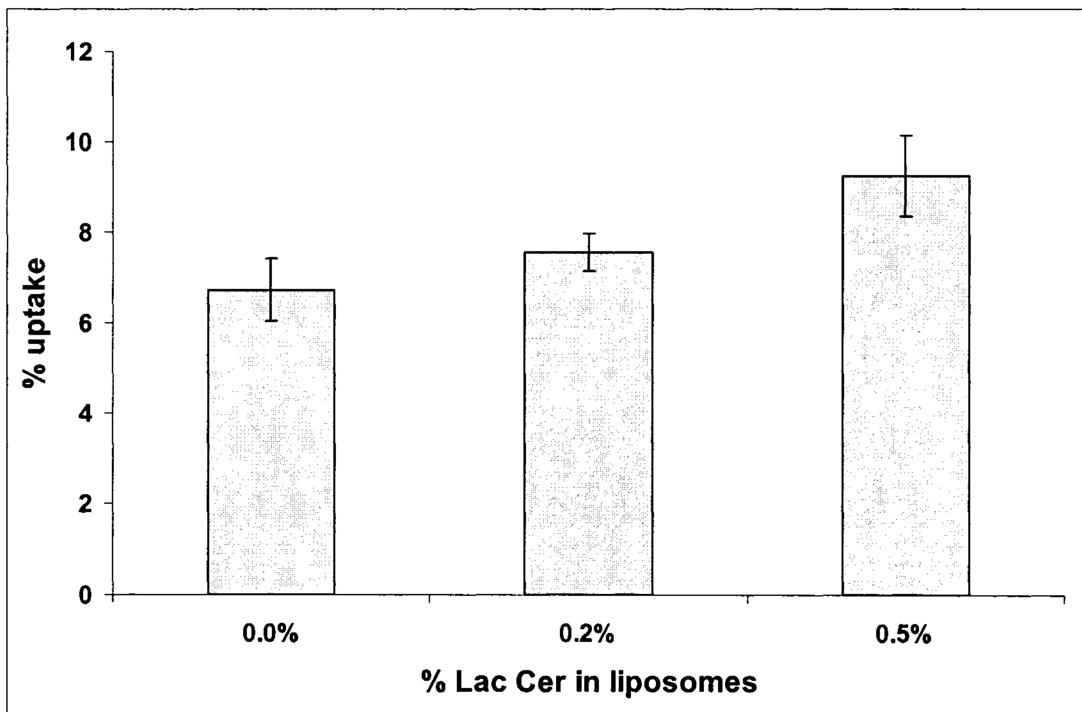

Figure 19: Uptake of 100 nm liposomes by a hepatoma cell line

Uptake of liposomes, encapsulating 200 μg/ ml FITC in PBS and extruded through a 100 nm PC membrane. The liposomes were diluted in serum free DMEM at 50 μg/ ml of total lipids and incubated with Hepa 1c1c7 cells in a TCP for 2 hr at 37 °C and 5% CO_2 (n = 6).

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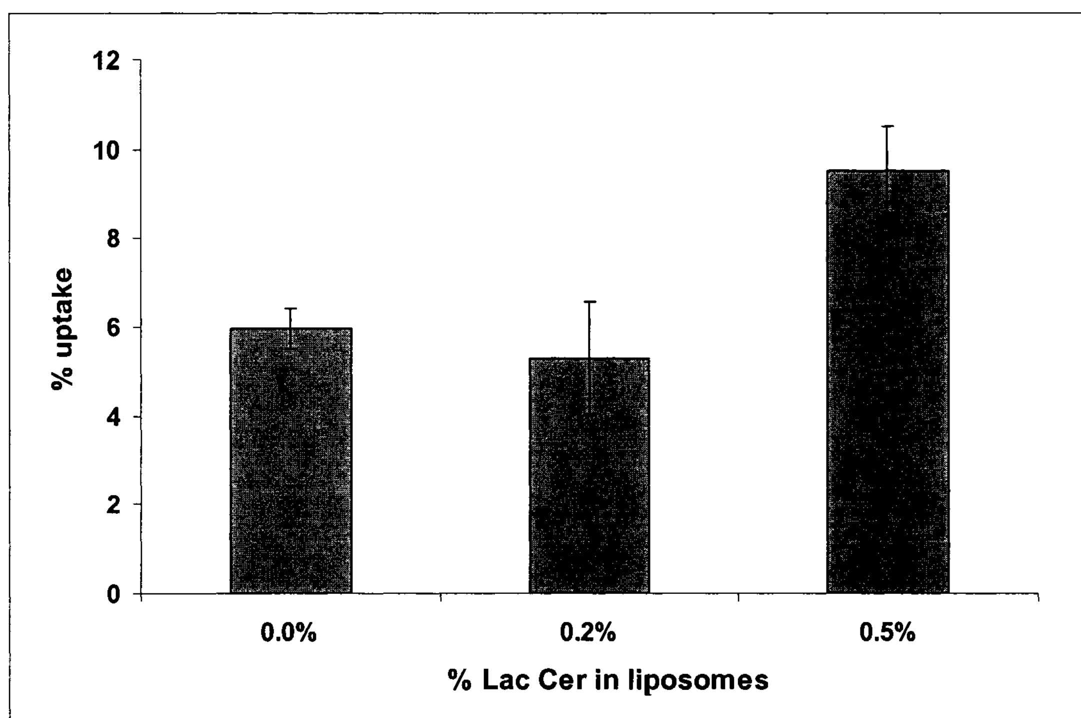

Figure 20: Uptake of 200 nm liposomes by a hepatoma cell line

Uptake of liposomes, encapsulating 200 µg/ ml FITC in PBS and extruded through a 200 nm PC membrane. The liposomes were diluted in serum free DMEM at 50 µg/ ml of total lipids and incubated with Hepa 1c1c7 cells in a TCP for 2 hr at 37 °C and 5% CO_2 (n = 6).

Table: Statistical analysis of uptake of different liposomal formulations by Hepa 1c1c7 hepatoma cells

Group 1		Group 2		P Value	Significance
Diameter nm	Lac-Cer content%	Diameter nm	Lac-Cer content%		
100	0.0	100	0.5	0.005	YES
100	0.0	100	0.2	0.033	YES
100	0.2	100	0.5	0.011	YES
200	0.0	200	0.5	0.0002	YES
200	0.0	200	0.2	0.261	NO
200	0.2	200	0.5	0.001	YES
100	0.0	200	0.0	0.155	NO
100	0.5	200	0.5	0.668	NO

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4.3. Liposomes *in vivo* Biodistribution

In figures 21, 22, 23, and 24, there was an observed increase in the uptake of the liposomes containing Tc^{99} DTPA by the liver compared to that of the free agent in the literature (Liu et al. 2002, 399-404). However, there was no significant difference observed in the uptake of liposomes containing lactosylceramide and the control liposomes, used as a control, by the liver. There was a difference between the uptake of the preparations of liposomes at the 15 min test by the kidneys and spleen as the uptake by the spleen decreased while the uptake by kidneys decreased with the addition of lactosylceramide as compared to that of the control formula. However this difference did not last after 1 hr of biodistribution.

The biodistribution and clearance of liposomes and their content were very rapid, suggesting a first pass effect. There was little difference within the biodistribution profiles and amount of the agent remaining within the test animals when comparing the profiles of 15 min to 1 hr. The majority of radioactivity was cleared out of the body into the urine. It is not clear if the large portion of Tc^{99} DTPA in urine was a result of direct release of the agents from the liposomes in the blood or if it was release of the free agent into the blood stream after the uptake of liposomes by the different organs and tissues. Tc^{99} DTPA has a very high rate of renal clearance from plasma and is used to evaluate renal function.

The experiment was not designed and does not answer the question of was there a significant change in the tissue distribution within the liver among the various cell types. The literature suggests a rapid uptake of liposomes by Kupffer cells within the

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liver and it is not clear if the use of lactosylceramide has shifted the uptake within the liver into hepatocytes. These cells are expected to increase their uptake in the case of the liposomes containing lactosylceramide which targets the ASGP-R found in large numbers at the cell surface of hepatocytes.

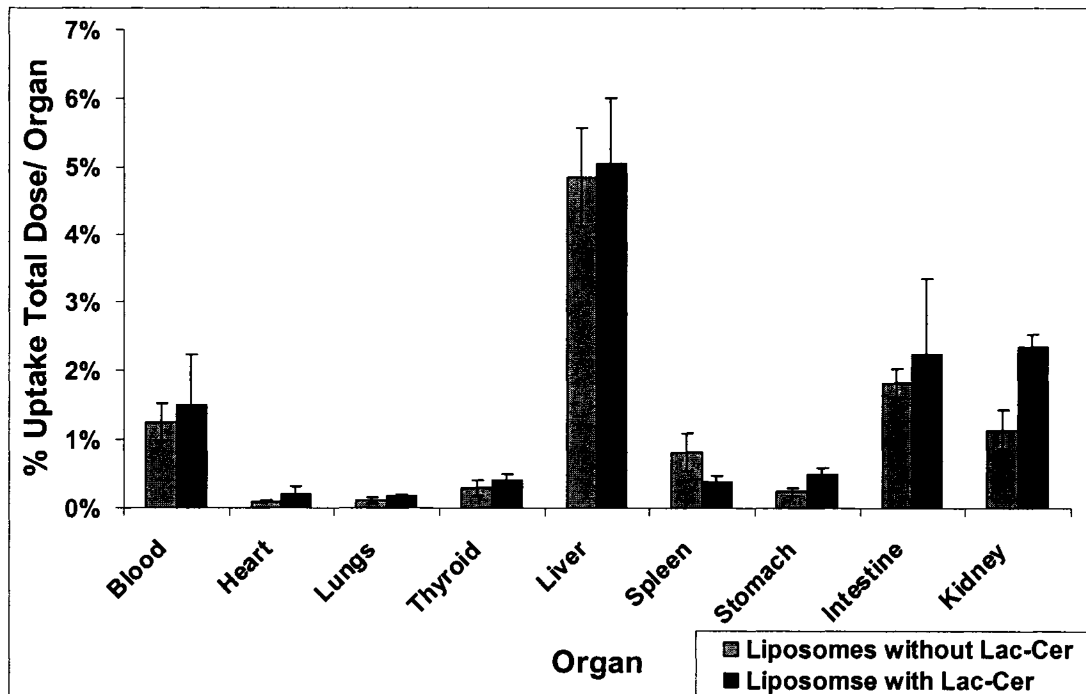

Figure 21: Uptake of liposomes by organs after 15 min from administration
Biodistribution, as portion of total dose /organ, of liposomes containing 0 and 0.5% Lac-Cer encapsulating Tc^{99} DTPA within female balb/C mice after 15 min from administration through IV route (n = 5).

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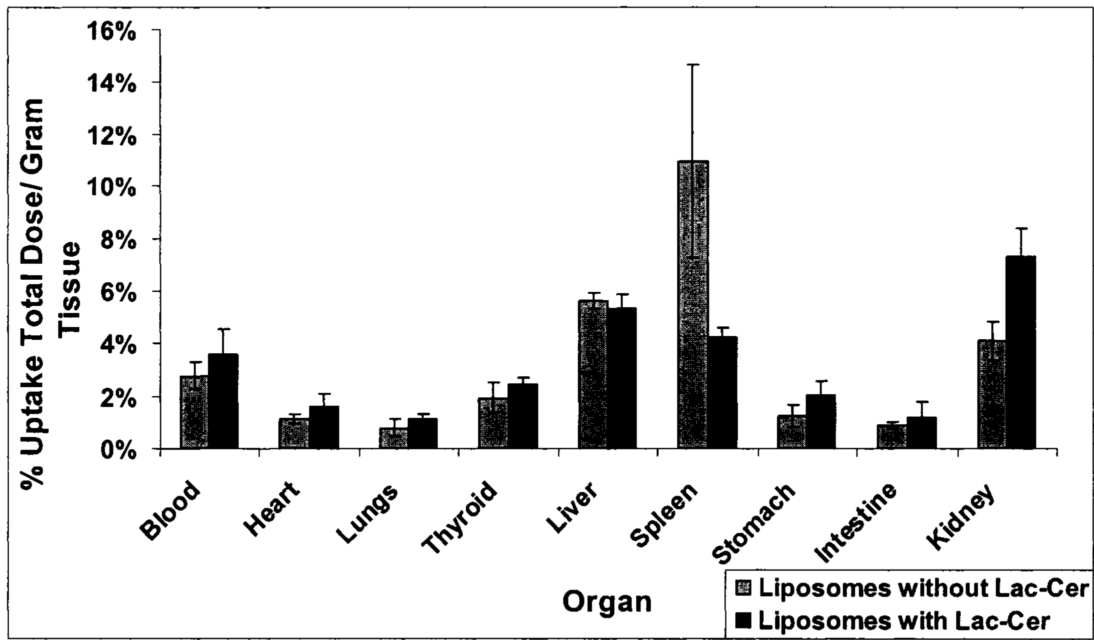

Figure 22: Uptake of liposomes by organs (as a function of tissue weight) after 15 min from administration

Biodistribution, as portion of total dose / gram of tissue, of liposomes containing 0 and 0.5% Lac-Cer encapsulating Tc^{99} DTPA within female balb/C mice after 15 min from administration through IV route (n = 5).

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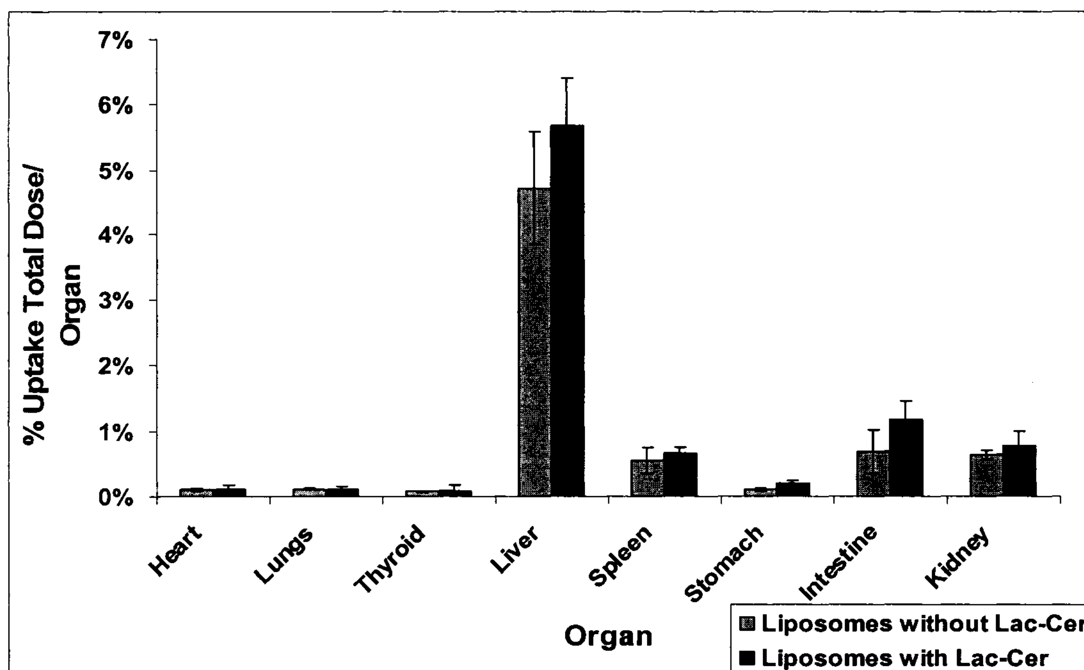

Figure 23: Uptake of liposomes by organs after 1 hour from administration

Biodistribution, as portion of total dose /organ, of liposomes containing 0 and 0.5% Lac-Cer encapsulating Tc^{99} DTPA within female balb/C mice after 1 hr from administration through IV route (n = 5).

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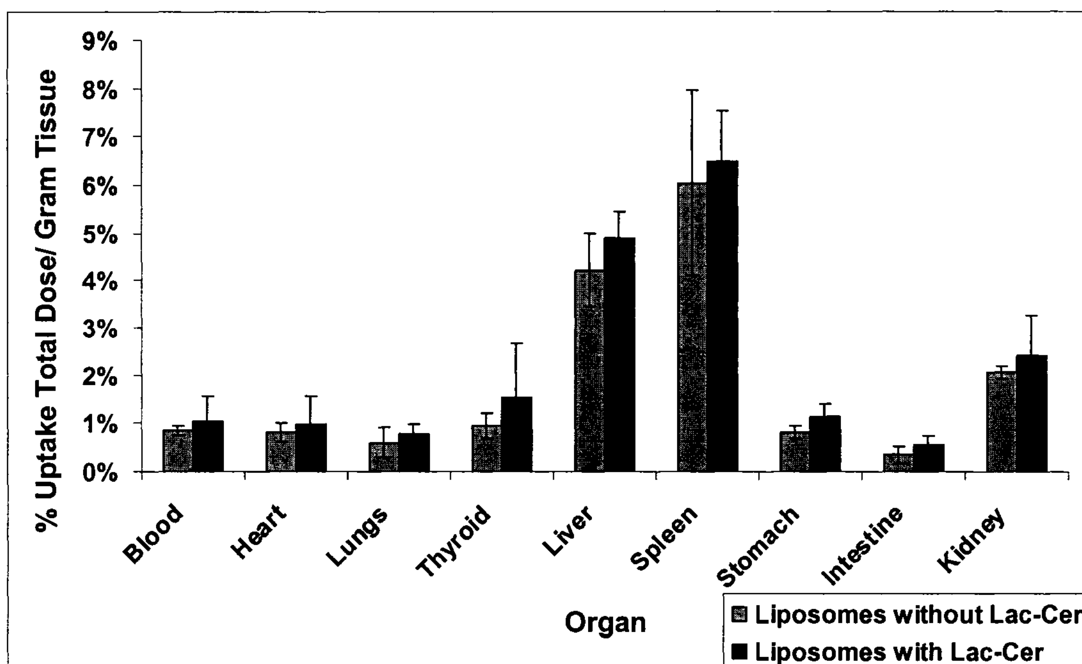

Figure 24: Uptake of liposomes by organs (as a function of tissue weight) after 1 hr from administration

Biodistribution, as portion of total dose / gram of tissue, of liposomes containing 0 and 0.5% Lac-Cer encapsulating Tc^{99} DTPA within female balb/C mice after 1 hr from administration through IV route (n = 5).

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4.4. Survival of Biologically Active Material After Intracellular Delivery

4.4.1. YP4 MAbs

4.4.1.1. Production and Purification of YP4 MAbs

YP4 antibodies were purified from supernatants of hybridoma cultures using a protein G column. In total, 12 mg of YP4 antibodies, which are rat anti HRPO MAbs, were prepared from several batches, figure 25 is the purification profile for one of these batches, and were concentrated into 1 or 2 mg/ ml in PBS for use as a hydrating solution in the preparation of liposomes.

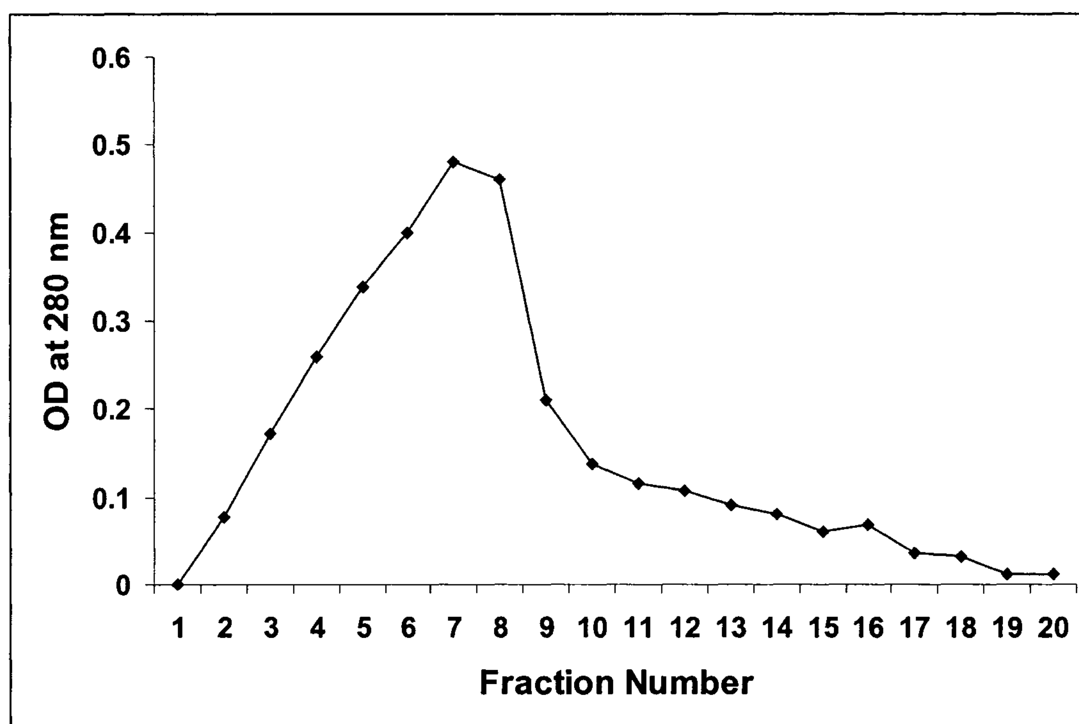

Figure 25: YP4 Antibody Protein G Separation

Separation profile of YP4 MAbs from a 2 ml Protein G column, the source of the MAbs was 400 ml of supernatant from growing YP4 hybridoma in 5% FBS in DMEM.

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4.4.1.2. YP4 ELISA

From the ELISA test, figure 26, it was concluded that the optimum concentration of Goat anti Rat antibodies for coating the ELISA plate wells is 4 µg/ ml. The sensitivity of the ELISA test was confirmed through ELISA testing which suggests it is possible to detect YP4 activity at the low concentration of 0.005 µg/ ml. The results from the later tests, data not shown, to optimize the ELISA conditions indicate no difference in the results between the different alterations in the coating with the primary antibodies, blocking, washing and incubation with the test molecule used by the different researchers within our group. Therefore, the procedure we used was selected based on convenience and practicality.

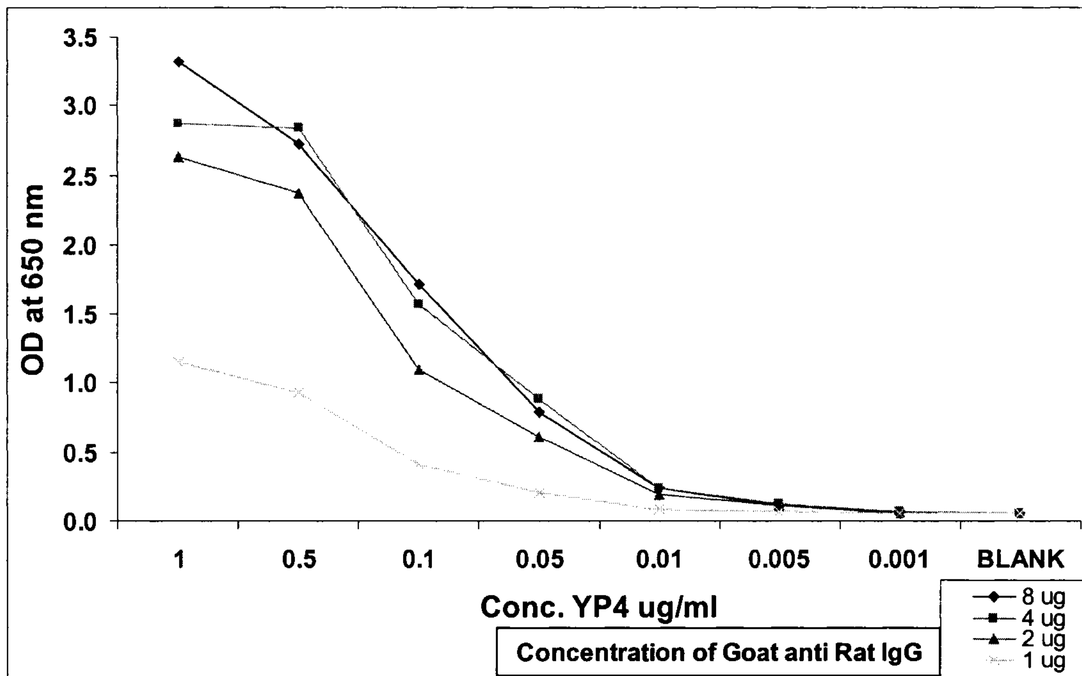

Figure 26: ELISA Optimization for YP4 Detection

ELISA test for YP4 MAb against an ELISA plate coated with different concentrations of Goat anti Rat antibodies at concentrations of 1, 2, 4 and 8 µg/ ml.

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4.4.1.3. YP4 Survival After Intracellular Delivery

No survival of YP4 MAbs activity was observed despite using various times of incubation and very high concentrations of MAbs in the hydrating solution, figure 27. These results along with the results of other survival tests suggest that the tested biologically active material with the used pH sensitive liposomes formulations do not allow for the survival of biologically active material from lysosomal degradation after intracellular delivery.

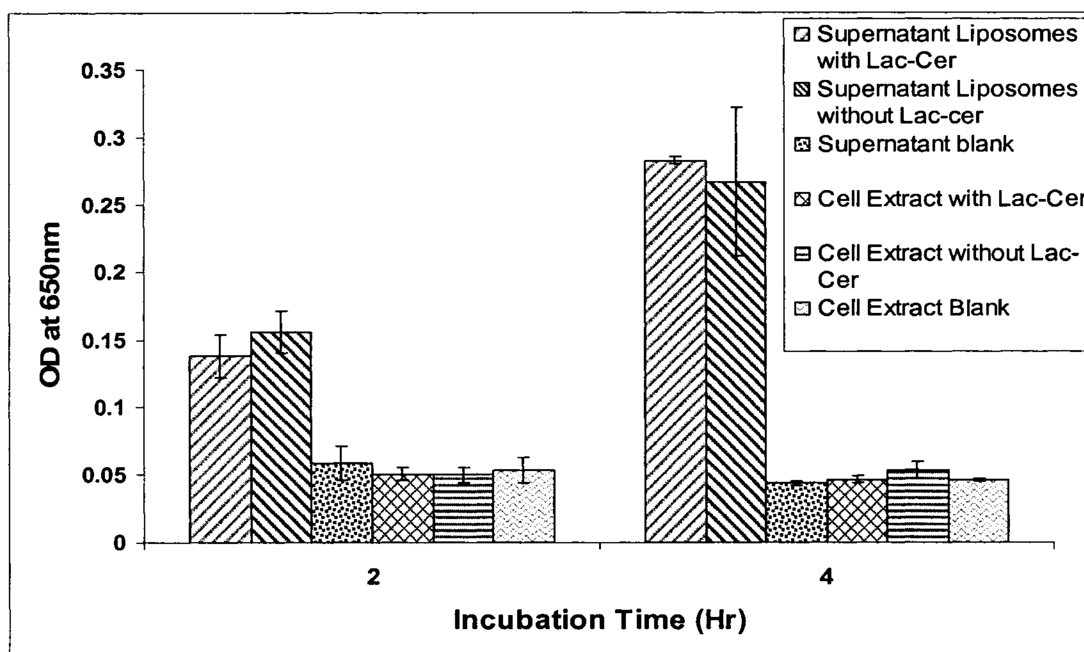

Figure 27: YP4 Survival After Intracellular Delivery

The ELISA values after incubation for 20 min at room temperature with different test solutions from the YP4 survival test. pH sensitive liposomes containing Lac-Cer at 0 and 0.5% encapsulating YP4 at 1 mg/ ml were formulated. The liposomes were suspended at 50 μg/ ml for the 2 hr test and 100 μg/ ml for the 4 hr test of total lipids in serum free DMEM and were incubated with Hepa 1c1c7 hepatoma cells over 2 and 4 hr at 37 °C and 5% CO_2. After the incubation, the supernatants were removed and the cells were washed and then their content was extracted lysing the cells with 0.2% Triton X-100 in PBS (n = 3).

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4.4.2. HRPO

4.4.2.1. Survival after Intracellular Delivery

No activity of HRPO was detected in the extract of the hepatoma cells incubated with the different liposomal preparations, figure 27. Different approaches were tested to allow the detection of biological activity of HRPO, if any, after intracellular delivery into the test hepatoma cell line. These included the use of different concentrations of HRPO in the hydrating solution, the use of different concentrations of total liposomal lipids suspended in serum free DMEM to allow for uptake by the cells and extended incubation times to allow increased uptake of liposomes and their contents by cells. The presence of HRPO activity in the cells was tested, either immediately after the removal of treatment solutions or after periods of incubation of the cells in serum free DMEM. No HRPO activity was observed in any of the cell extracts, indicating that either no uptake of liposomes or their content occurred or very rapid degradation of liposomal HRPO after intracellular delivery.

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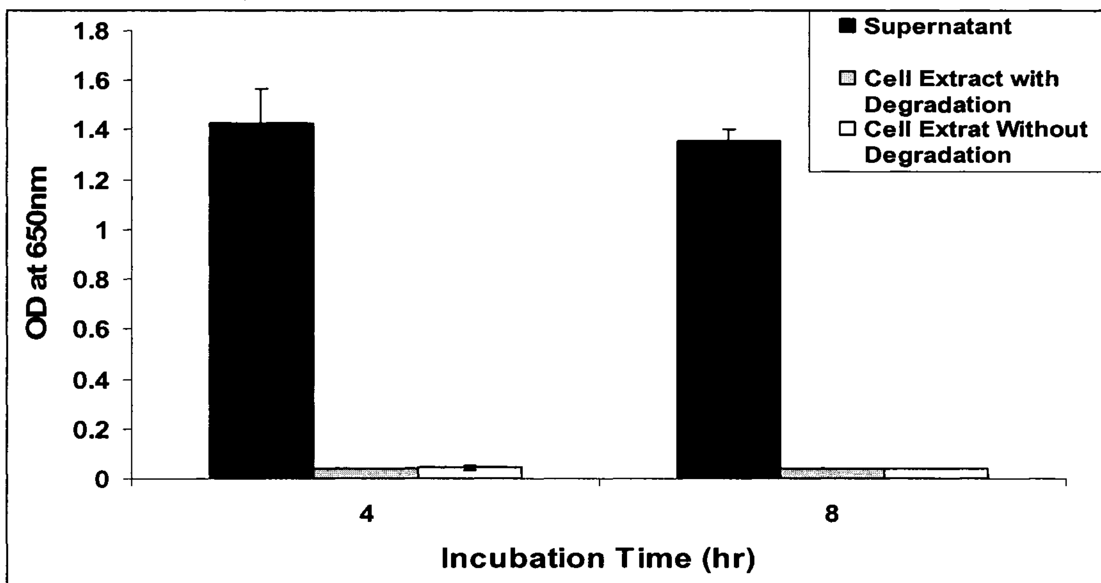

Figure 28: Survival of HRPO After Intracellular Delivery

The OD of TMB after incubation for 20 min at room temperature with different test solutions from the HRPO survival test. pH sensitive liposomes were formulated encapsulating HRPO. The liposomes were suspended in serum free DMEM and incubated with Hepa 1c1c7 hepatoma cells over 4 or 8 hr at 37 °C and 5% CO_2. The different samples tested were: The supernatant of liposomes in serum free DMEM diluted with 0.2% Triton X-100 in PBS and the extract of hepatoma cells lysed with 0.2% Triton X-100 in PBS. The cell content were extracted immediately after the removal and washing of treatments or after 30 min, while incubating in serum free DMEM, to allow for the degradation, if any, of HRPO delivered by liposomes into the hepatoma cells (n = 3).

4.4.3. HRPO with Protease Inhibitors

4.4.3.1. Toxicity Testing

The MTT toxicity test, figure 29, confirmed the safety of using the liposomal preparation at the test concentrations even after the addition of PI to the hydrating solution used in the formulation of liposomes. It also suggested that the liposomes can be used at higher levels of total lipids if needed without causing cytotoxicity.

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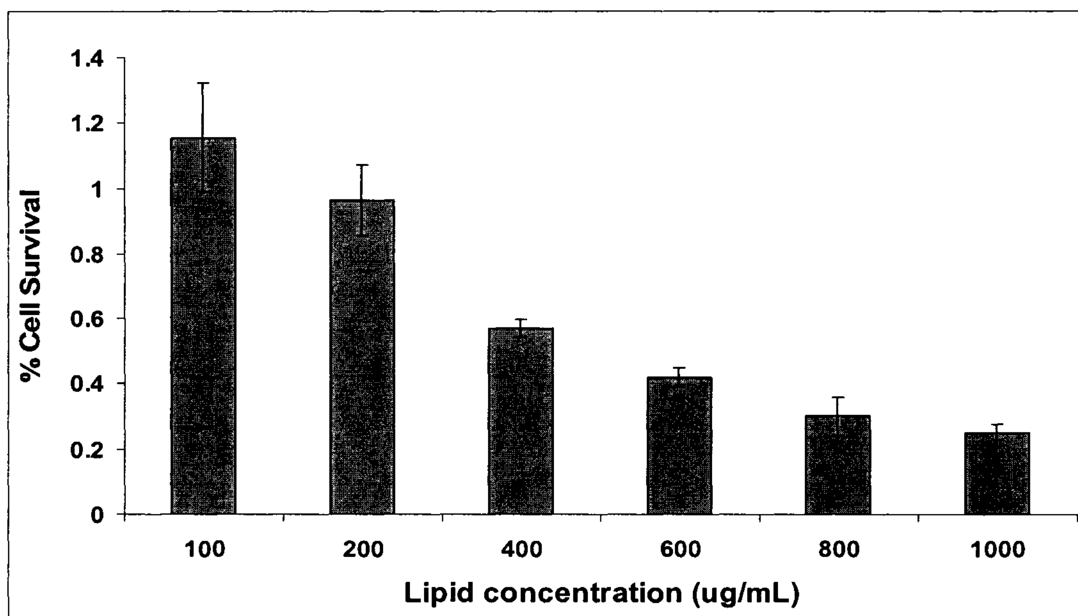

Figure 29: Toxicity Test for PI 0.5% Lac-Cer Liposomes

MTT test for Hepa 1c1c7 cells treated with liposomes containing 0.5% Lac-Cer and encapsulating HRPO at 500 μg/ ml, 1 X Protease Inhibitors cocktail and a trace amount of FITC suspended in serum free DMEM and incubated for 2 hr at 37 °C and 5% CO_2 (n = 6).

4.4.3.2. Survival after Intracellular Delivery

The liposomal preparations were modified by adding a Protease Inhibitors Cocktail to the hydrating solution, in the hope this would prevent lysosomal degradation of liposomal content after delivery. FITC was also added to the liposomal preparation to confirm uptake of liposomes and their content, the labeling of HRPO itself with FITC was not possible. No survival of activity was detected despite the addition of PI at 1 X , figure 30, concentration in the hydrating solution and using liposome suspensions at concentrations as high as 200 μg/ ml of total lipids in serum free DMEM. Uptake was confirmed, figure 31, and was consistent with observed uptake in earlier uptake tests. We suspect that the failure of PIs to retain biological activity could be a result

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of the amount being delivered through liposomes is not sufficient to inhibit lysosomal proteases. The 1 X concentrations of PIs inside the liposomes is diluted within the much larger lysosomes and therefore the PI no longer exhibit their function. The use of PIs at 1 X concentration in the treatment media is not practical as it cannot be applied to *in vivo* tests. The use of PIs at a much higher concentration in the hydration solution for the preparation of the liposomes is not practical and could become toxic to cells upon internalization.

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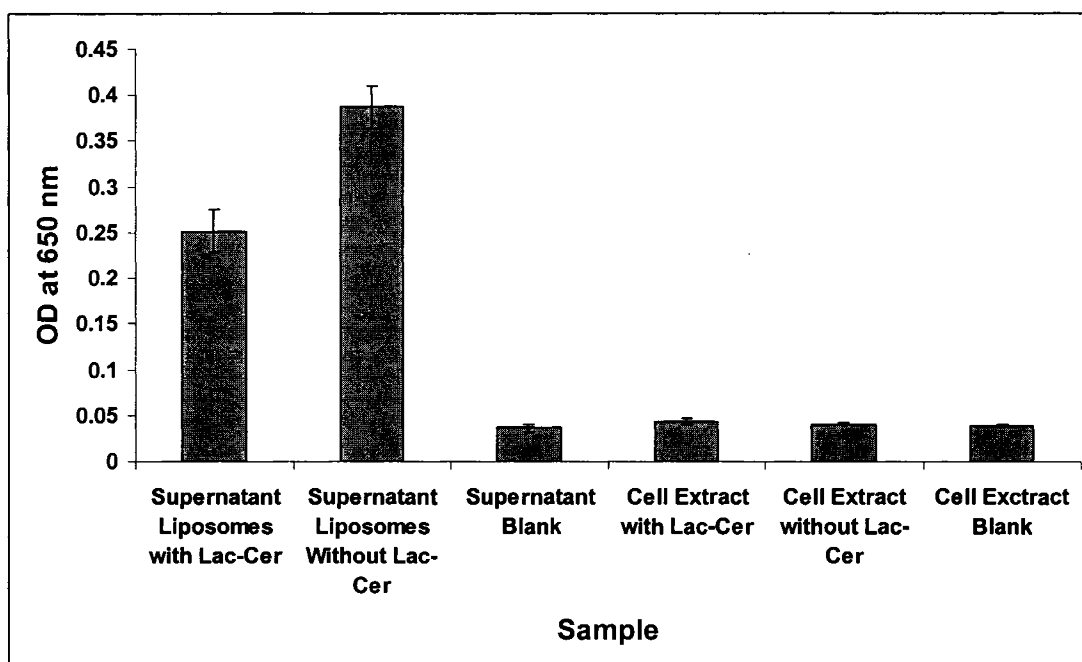

Figure 30: Survival of HRPO after Intracellular Delivery in Liposomes with PI and FITC

The OD of TMB after incubation for 20 min at room temperature with different test solutions. pH sensitive liposomes without Lac-Cer or with Lac-Cer at 0.5% encapsulating HRPO, Protease Inhibitors and FITC were formulated. The liposomes were suspended at 100 µg/ ml of total lipids in serum free DMEM. The test solutions were incubated with Hepa 1c1c7 hepatoma cells over 2 hr at 37 °C and 5% CO_2. The different samples tested were: the extract of hepatoma cells which were washed after the removal of treatments, then incubated for 30 min in serum free DMEM and finally lysed with 0.2% Triton X-100 in PBS, and a blank and their supernatants diluted with 0.2% Triton X-100 in PBS (n = 3).

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4.4.3.3. Uptake of Liposomes Containing HRPO and Protease Inhibitors

The confirmation of liposomal uptake, figure 31, does confirm that the lack of observed biological activity is a result of degradation of liposomal content after intracellular delivery rather than a failure in the delivery of the liposomes and their content.

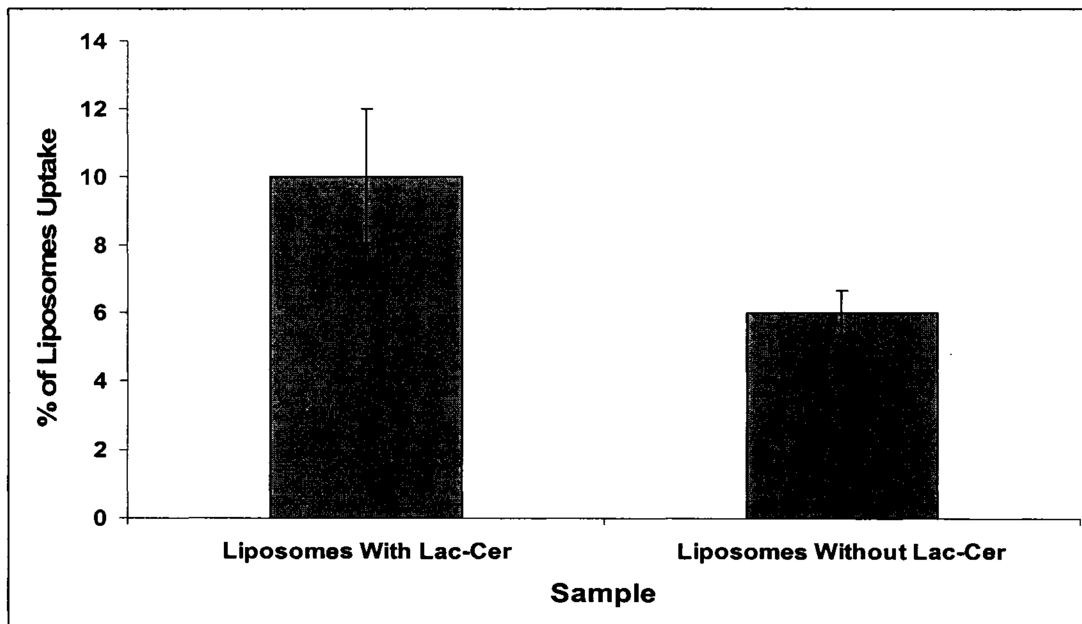

Figure 31: Uptake of Liposomes encapsulating HRPO, FITC and PI

Uptake of liposomes, encapsulating HRPO at 500 µg/ ml with 1 X protease inhibitors cocktail and FITC in PBS The liposomes were diluted in serum free DMEM at 50 µg/ ml of total lipids. The samples were incubated with Hepa 1c1c7 cells in a TCP, for 2 hr at 37 °C and 5% CO_2.

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5. Summary and Conclusions

Several successful formulations of liposomes with and without lactosylceramide, at different concentrations were prepared. The different preparations were successfully used to encapsulate FITC, Calcein and Tc^{99} DTPA as well as proteins including HRPO, YP4 and PI and were used *in vitro* and *in vivo* experiments. The data reported in the literature was reproduced and the liposomal formula was modified to successfully incorporate lactosylceramide at varying concentrations. The presence of lactosylceramide in liposomes pH influenced sensitivity and stability in a concentration dependant manner. The lactosylceramide molar concentrations of 0.2 and 0.5% provided formulations with desired pH sensitivity and stability in serum and storage characteristics. The formulation also allowed production of liposomes with 100 and 200 nm average diameters, but smaller liposomes were not stable and could not be formulated.

The safety of the liposomal formulations was evaluated and showed that there was no added toxicity as a result of adding lactosylceramide to the formulations at the concentrations employed. We also concluded it was safe to incubate our test hepatoma cell line with up 100 μg/ ml of total lipids of liposomes. Uptake of liposomal preparations at 100 and 200 nm diameters incorporating lactosylceramide at 0.2 and 0.5% was tested by a hepatoma cell line *in vitro*. The presence of lactosylceramide at the 0.5% molar concentration significantly increased the uptake of liposomes by the hepatoma cells when compared to the original formulation suggesting an enhanced binding and internalization via the ASGP-R. The size variation had no influence on hepatoma cell uptake *in vitro*. *In vivo* testing of the

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biodistribution of liposomes encapsulating a radioactive probe, showed a change in the biodistribution pattern from the free isotope favoring increased uptake by the liver. There was no observed difference in the quantitative uptake of liposomes by the liver in the presence or absence of lactosylceramide. However, based on our *in vitro* uptake tests, it is likely that the liposomes with lactosylceramide may preferentially accumulate within the haptocytes within the liver. In the absence of lactosylceramide, the Kupfer cells could also be a major sink for the administered liposomes.

Biologically active materials were successfully encapsulated within liposomes and could be delivered intracellularly. However, we could not observe any survival of these biologically active materials or their activity after delivery, even after the incorporation of protease inhibitors into the liposomal formulations.

6. Future Directions

- Further testing for the survival of biologically active material after their intracellular delivery by pH sensitive liposomes using ultrasensitive substrates.
- The use of alternative pH sensitive liposomes formulations to deliver biologically active material intracellularly.
- Histological studies to determine the distribution patterns of liposomal content between different cell types within the liver.
- Testing the efficiency and benefits of delivering therapeutic agents, such as chemotherapeutic agents, antiviral agents and gene therapy, delivered in our liposomal preparation to the liver.

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- Testing for the stability of antibodies in reducing conditions simulating the intracellular environment and the design of antibodies that would be stable in such an environment should normal antibodies be unstable in such conditions.

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