LIPOSOME FORMATION BY ADIPOSE TISSUE DERIVED LIPIDS AND ITS APPLICATIONS

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CERTIFICATE

This is to certify that the thesis entitled " **LIPOSOME FORMATION BY FROADIPOSE DERIVED LIPIDS AND ITS APPLICATIONS**" submitted by Ms. ANKITA SARANGI in partial fulfilment of the requirements for the degree of Bachelor of Technology in BIOMEDICAL embodies the bonafide work done by her in the final semester of her degree under the supervision of the undersigned. The thesis or any part of it has not been submitted earlier to any other University / Institute for the award of any Degree or Diploma.

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CONTENT

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ABSTRACT

A liposome is an artificially-prepared vesicle composed of a lipid bilayer. This project aims to produce liposomes using human adipose tissue derived lipids (ATDL). Furthermore, the study attempts to find the alternative use of discarded human adipose tissue after the liposuction operation. Conventionally prepared liposomes are usually made up of phosphatidylcholine and cholesterol, two major constituents of cell membrane. The presence of other phospholipids like phosphatidylserine, phosphatidylinositol, phosphatidylethonolamine and sphingophospholipids of the cells and organelles membranes may mimic the natural cell membrane and stabilise the liposomes further and increase its efficiency as intracellular drug delivery vehicle. These other useful phospholipids could be extracted from the discarded adipose tissues.

The lipids were separated from the human adipose tissue by Folch Method (Folch et al., 1957) and characterised using FTIR. Thin film hydration method was adapted to produce liposomes with 2 different sources, lecithin- cholesterol and ATDL. The liposomes were analysed under light microscope and characterised using DLS. Then these liposomes were loaded with trehalose, a known disaccharide and cryoprotectant. Moreover, the drug encapsulation efficiency and effect of trehalose loaded liposomes on the cryopreservation of red blood cells were compared between conventional and the proposed method. The liposomes made up of ATDL are smaller in size and have less encapsulation efficiency as compared to conventional liposomes. The conventionally produced liposomes loaded with trehalose have very high cryoprotective properties (Viability-85% RBCs) but at the same time the cryoprotective properties of ATDL liposomes loaded with trehalose is also quite significant (viability-70% RBCs).The lower cryoprotective ability of the ATDL liposomes may be due to the lower encapsulation efficiency of it for the water soluble drugs such as trehalose.

CHAPTER 1: INTRODUCTION

1.1- Liposome production , application and advantages

Liposomes are microscopic vesicles that consist of an aqueous centre with a phospholipid bilayer. Liposomes were first described in 1961 as a model of cellular membranes. They were discovered in 1961 by Alec D. Bangham who was at that point studying phospholipids and blood clotting, and since then liposomes became very versatile tools in biology, biochemistry and medicinal biology. Bangham discovered that phospholipid combined with water immediately forms a bi-layered sphere because one end of each molecule is water soluble or hydrophilic, while the opposite end is water insoluble or hydrophobic.

1.1.1 History:

The history of liposomes is usually divided into three periods: genesis, middle age and modern era.

a) Genesis (1968-75)

Liposome's physiochemical characterisation was done in this period and thin lipid film hydration method was also developed which could prepare multilamellar vesicles (MLVs).The resemblance of liposomes to biological membrane has made it a subject for study of the nature of biological membranes.

b) Middle age (1975-85)

Basic research increased the understanding of liposome stability and interaction mechanism within the system thus increasing their utility. This period also saw the rise of various other liposome preparation techniques. Also, the phsio-chemical characteristics of the liposomes were vastly known in this period thus improving their performance as drug carrier systems.

c) Modern era (1985 onwards)

Today, liposomes are used successfully in various disciplines such as biology, chemical science even mathematics and physics. The first liposomal pharmaceutical product received FDA approval in the mid-1990 was Ambisome, a parenteral amphotericin- B based liposomal product. Currently there are at least 8 liposomal pharmaceutical products currently marketed with many more in various stages of clinical trials. The widespread liposome research is promising many more products in the near future.

1.1.2 Types of Liposomes:

Vesicle type	Abbreviation	Diameter size	No. of lipid bilayer
Unilamellar vesicle	UV	All size range	One
Small Unilamellar vesicle	SUV	20-100nm	One
Medium Unilamellar vesicle	MUV	More than 100nm	One
Large Unilamellar vesicle	LUV	More than 100nm	One
Giant Unilamellar vesicle	GUV	More than 1 micro meter	One
Oligolamellar vesicle	OLV	0.1-1 micro meter	Approx 5
Multilamellar vesicle	MLV	More than 0.5	5-25
Multivesicular vesicle	MV	More than 1 micro meter	Multi compartmental structure

Table 1: Type of liposomes based on size (Abdus Samad et. al., 2007)

1.1.3 Liposome Preparation methods:

Liposomes are prepared by various methods:

1. Passive loading- In this method the encapsulated drug or other substances is loaded before or during the formation of the vesicle

- Mechanical dispersion method
    - Lipid film hydration by hand shaking non hand shaking or freeze drying
    - Micro emulsification

    - Sonication
    - French pressure cell
    - Membrane extrusion
    - Dried constituted vesicles
    - Freeze thawed liposomes
- Solvent evaporation method
    - Ethanol injection
    - Ether injection
    - Double emulsion vesicles
    - Reverse phase evaporation vesicles
    - Stable plurilamellar vesicles
- Detergent removal methods
    - Detergent (cholate, alkylglycoside, trito n X-100) removal from mixed micelles
    - Dialysis
    - Column chromatography
    - Dilution
    - Reconstituted sandal virus enveloped vesicles

2. Active loading- in this method encapsulated drugs or other substances are loaded after the vesicle is formed.

1.1.4 Properties of Liposomes:

The size, the lamellarity (Unilamellar or Multilamellar) and lipid composition of the bilayers influence many of their important properties like the fluidity, permeability, stability and structure; these can be controlled and customized to serve specific needs. These properties are also influenced by external parameters like temperature, ionic strength and the presence of certain molecules nearby. The liposome surface can also be modified to achieve various goals:

- Attachment of hydrophobic polymers can result in long circulating liposomes.
- Attachment of label allows the monitoring of the fate of liposomes in a body or cell.
- Entrapment of positively charged ions allows DNA binding and efficient cell transfection

- Attachment of either antigen or antibody leads to the formation of liposome-based immunoassay system

The properties that make them useful for different applications are:

- Surfactant property
- Structural stability on dilution of the liposomes
- Varying permeability of the bilayer when exposed to different molecules.
- Ability to entrap both water soluble (hydrophilic) and insoluble (hydrophobic) substances and deliver them into desired targets.

1.1.5 Applications of Liposomes:

Application of liposomes are in various fields such as drug delivery, topical drug encapsulation, treatment of human immunodeficiency virus infections, enhanced antimicrobial efficacy, lipotransfection ,liposome vaccines. Liposome can administered orally or there are liposome aerosols present these days, topical application as well lymphatic targeting with liposomes is also possible. Liposomes are also being used in nucleic acid therapy, in eye disorders, as vaccine adjuvant, for brain targeting and many other applications.

Applications of liposomes can be divided into two categories: therapeutic and diagnostic depending upon the constituents of liposomes drugs or various markers. The benefits and limitations of liposome loaded with drug critically depend on the interaction of liposomes with cells in vivo after their administration.

In vitro and in vivo studies of interactions of liposomes with cells have shown that the interaction is either simple adsorption or endocytosis. Fusion of liposomes with cell membranes is very rare. The fourth possible interaction is exchange of bilayer constituents with components of cell membranes.

Liposomes provide several advantages in delivering genes to cells. (a) Liposomes can combine with negatively and positively charged molecules equally. (b) Liposomes offer protection to the encapsulated DNA from harmful processes and environment. (c) Liposomes can carry large pieces of DNA, pieces as large as a chromosome. (d) Liposomes can be

targeted to specific cells or tissues. The ability to chemically synthesize a wide variety of liposomes has resulted in a highly adaptable and flexible system capable of gene delivery both *in vitro* and *in vivo*.

Cationic liposomes are positively charged liposomes, which interact (bind) with the negatively charged DNA to form a stable complex. Cationic liposomes have a positively charged lipid and a co-lipid. Co-lipids, which are the helper lipids, are required for stabilization of liposome complex. A variety of cationic liposomes are commercially available and many others are under development. One of the most frequently used positive charged lipids is lipofectin. PH-sensitive, or negatively-charged liposomes, enclose DNA rather than complexing with it. Since both the DNA and the lipid are negatively charged, repulsion rather than complex formation occurs. Yet, some DNA does manage to get entrapped within the aqueous interior of these negatively charged liposomes. To date, cationic liposomes have been much more efficient at gene delivery both than pH-sensitive liposomes.PH-sensitive liposomes have the potential to be much more efficient at *in vivo* DNA delivery than their cationic counterparts.

1.1.6 Marketed Liposomal Products:

Various marketed formulations of liposomes are:

Product	Drug	Company	Use
1.Myocet	Liposomal doxorubicin	Cephalon (UK) Limited and Triveni interchem private limited	Combinational therapy for treatment of recurrent breast cancer
2.Doxil, Caelyx	Liposomal doxorubicin	Sequus Pharmaceuticals,Inc.,C.A.	treatment of refractory Kaposi's sarcoma, recurrent breast cancer and ovarian cancer

3.LipoDox	Liposomal doxorubicin	Sun Pharma Global and Oddway international	treatment of refractory Kaposi's sarcoma, recurrent breast cancer and ovarian cancer
4.Thermodox	Liposomal doxorubicin	Celsion corporation	treatment of liver cancer and also recurrent chest wall breast cancer
5.DaunoXome	Liposomal Daunorubicin	NeXstar Pharmaceuticals,Inc.,CO	treatment of Kaposi's sarcoma
6.Ambisome	Liposomal Amphoteracin B	NeXstar Pharmaceuticals,Inc.,CO	treatment of fungal infection
7.Marqibo	Liposomal vincristine	Talon therapeutics	treatment of metastatic malignant uveal melanoma
8.Visudyne	Liposomal verteporfin	Novartis Pharmaceuticals UK Ltd and ciba-vision-ophthalmics	treatment of age-related macular degeneration, pathologic myopia and ocular histoplasmosis.
9.DepoCyt	Liposomal cytarabine	Enzon Pharmaceuticals, Inc, MundiPharma, including its independent but associated companies, in Europe and most other countries, two Pacira cGMP manufacturing facilities	Treatment of neoplastic meningitis and lymphomatous meningitis.

10. DepoDur	Liposomal morphine sulphate	EKR Therapeutics, Flynn Pharma(UK), Pacira cGMP manufacturing facilities	treatment of postoperative pain
11. Arikace	Liposomal amikacin	Insmed Incorporated	treatment of lung infections due to pathogens
12. Lipoplatin	Liposomal cisplatin	Regulon Inc	Treatment of lung, head and neck, ovarian, bladder and testicular cancers.
13. LEP-ETU	Liposomal Paclitaxel	NeoPharm, Inc	It is used for treatment of ovarian, breast and lung cancer.
14. Epaxal	Hepatitis A vaccine	Crucell UK Ltd, Crucell Italy S.r.l.,Masta ltd	It is hepatitis A vaccine
15. Inflexal V	Influenza vaccine	Crucell UK Ltd, Crucell Italy S.r.l.,	It is influenza vaccine
16. Abelcet	Liposomal Amphotericin B	The liposome company,NJ	It is used to treat infections caused by fungi and yeasts.
17.Amphocil	Liposomal Amphotericin B	Sequus pharmaceuticals,Inc,C.A	It is used to treat serious fungal infections, which can occur throughout the body
18. MiKasome	Liposomal amikacin	NeXstar Pharmaceuticals,Inc.,CO	An agent in clinical trials for treating complicated UTIs, acute infections in cystic fibrosis,

			nosocomial pneumonia
19. DC99	Liposomal doxorubicin	Liposome Co.,NJ,USA	Used to treat cancer
20. ELA-Max	Liposomal lidocaine	Biozone labs,CA,USA	Lidocaine is a local anesthetic .Lidocaine topical is used to reduce pain or discomfort caused by skin irritation

Table 2: Liposomal formulations already present in market and their applications

1.2 Adipose Tissue

Adipose tissue is a type of connective tissue that is a major storage site for fat in mammals in the form of triglycerides. Adipose tissue in mammals is of two different types: white adipose tissue and brown adipose tissue. The amount and distribution of each type of adipose tissue vary from species to species. In adult mammals, adipose tissue contains mostly lipid-filled cells called adipocytes. In addition to adipocytes, adipose tissue also contains stromal-vascular cells including fibroblastic connective tissue cells, leukocytes and pre-adipocytes. Excess of adipose tissue is the main reason for obesity in mammals; obesity does not depend on total body weight it actually depends on total body fat which constitutes mainly of adipocytes.

The lipid droplets in adipose tissue can unioculor or multiocular. Unioculor cells have a large single lipid droplet, and the mitochondria are pushed outwards, towards the cell membrane. The unioculor cells are mostly 25-200 microns in size. Multiocular cells have many lipid droplets, the droplet may be 25 microns and the cell size is about 60 microns.

About 60-85% the weight of adipose tissue constitute of lipids most of them being (90-99%) trigycerides, a trace amounts of phosolipids, cholesterol, digyleceride is also present. This lipid mixture contains six fatty acids, namely myristic, palmitoleic, oleic, plamitic, stearic,

oand linoleic. The rest of the weight of white adipose tissue is composed of water (5 to 30%) and protein (2 to 3%).

1.2.1 Cell membrane:

Cell membrane is a biological semi-permeable membrane that separates the cell from the outside environment. It is selectively permeable allowing the entry of only some ions and molecules. Its main function is to protect cell from foreigners. Cell membrane is made up of lipid bilayer and some embedded protein.

According to fluid mosaic model biological membranes can be considered as a two dimensional fluid in which protein and lipid dissolve easily. The lipid bilayer that forms the basis of the cell membrane actually forms a 2-D liquid; the cell membrane also contains a lot of protein which gives a support structure.

Lipid bilayer is formed through the process of self-assembly. The cell membrane consists mainly of a thin layer of amphipathic phospholipids which spontaneously arrange themselves so that the hydrophobic "tail" regions are isolated from the surrounding polar liquids, causing the more hydrophilic "head" regions to associate with non-polar regions of the resulting bilayer. This forms a continuous spherical lipid bilayer. All kind of forces such as van der walls, electrostatic and hydrogen bond play a factor in membrane formation. But the most important factor is the hydrophobic interactions.

Lipid bilayer is generally impenetrable for ions and polar molecules. The arrangement of lipid bilayer is such that polar solutes are prevented from entering it, while hydrophobic molecules can pass freely. This gives the cell membrane the ability to be selectively permeable towards transmembrane protein complexes.

1.3 RBC Cryopreservation

RBCs are biconcave blood cells, which possess hemoglobin (Hb), Membrane contain equimolar quantities of phospholipids ,unestrified cholesterol with small amounts of glycolipids and free fatty acids,52% of RBC membrane is comprised of membrane proteins, these protein provides the structural support to the membrane. Accidents and certain diseases causing anaemia require immediate transfusion of RBC’s. Red blood cells are made in the bone marrow. They live for about 120 days in the bloodstream and then die. Lack of RBC’s

can leads to oxygen depletion in brain and can lead to coma as well as death. Thus it is very important to preserve RBC, it can be preserved at 42 degree centigrade for 40 days or else it can be cryopreserved with the addition of certain cryoprotectants.

Cryopreservation is done to preserve cells in low temperature, at such low temperature all biological activities are stopped and cells do not die. When the cells are being cryopreserved ice is formed which is harmful for the cells. To avoid intra and extracellular ice cryoprotectants are added. Cryoprotectants are of two types permeating and non-permeating. Once the cells and the cryoprotectants have been combined and dispensed into vials, the next step is to cool the suspension. The rate of cooling is important since it affects the rate of formation and size of ice crystals, as well as the solution effects that occur during freezing. Different types of cells may require different cooling rates; however a uniform cooling rate of 1°C per minute from ambient temperature is effective for a wide variety of cells. After the samples are taken out from liquid nitrogen the cells are thawed. Different thawing protocols are used depending upon the type of cell. Generally rapid thawing is best in order to get maximum viability of the recovered cells. After thawing the supernatant is removed and the cells are resuspended in fresh media to remove residual cryoprotective agent. Post-thaw viability tests are used to determine the effectiveness of the procedure.

CHAPTER 2: LITERATURE REVIEW

a) Samad A. et.al, 2007-Liposomal drug delivery systems: an Update review

Liposomes are small spherical vesciles, which can uptake potent drugs and deliver than at the required site for therapeutic benefits. The therapeutic index for drugs are usually small, in such cases encasing them a in protective liposome provides better target delivery and accumulation at target site. The liposomes are characterized depending on chemical, physical and biological parameters. This mode of drug delivery is more effective and safe for administration of antiviral, antimicrobial, antifungal drugs. The new developments in this field are specific cell binding properties of drug carrying liposome to a target cell. Anticancer drugs like doxuribicin, mioxtrone have already been loaded on to sheath liposomes.

b) Mohammad Riaz, 1996-Liposme preparation method

Liposome vesicles have a central aqueous core which is enclosed by phospholipid bilayers. Molecules of low molecular weight to high molecular weight all have been incorporated within liposomes. The water soluble components are present in the aqueous core while the lipid soluble components get entrapped in the lipid bilayers. Liposome encapsulated drugs can be administered by various routes: intravenous, oral, local application, ocular. Liposomes are classified based on size, preparation method and type. Liposomes are prepared by several methods namely mechanical method, solvent evaporation method and detergent removal method.

c) Uhumwangho MU et. al., 2005-Current trends in the production and biomedical application of liposomes: a review

This paper reviews methods for preparation of liposome, their stability, biodistribuation and their uses as drug carrier. The conventional method of preparation, prepares mutilamellar vesicles but other methods are used to produce small unilamellar vesicle for better tissue detection. There are other methods for preparation of large unilamellar vesicle; each method

has its own advantages and disadvantages. Liposomal encapsulation of drugs increases their pharmacokinetic properties in comparison to liposomal free drugs.

d) Torchilin V.P- Recent advances with liposomes as pharmaceutical carriers

Liposomes have been in recent demand due to their potential as great pharmaceutical carriers. There have recent developments of drug encapsulation process in liposomes; liposomes are also being used for gene delivery. For further success in development of this field, promising trends should be identified and exploited.

e) Seema Sood, 1999- Characterization of liposome manufacturing using extrusion

The process parameters of extrusion where changed to study the effect on liposome and its properties. The size characterisation was done by Dynamic Light Scattering, Capillary Hydrodynamic and Zimm Plots. Extrusion history did not affect mean size but it did affect mean size.

f) Martin Francis et.al, 1998-The challenge of liposomes in gene therapy

Recently, liposomes are also being used as gene delivery systems: most methods use intratumoral, subcutaneous and other local delivery. Stealth liposomes (coated with polyethylene glycol to camouflage the liposome and evade detection by the immune system) have a remarkable longevity in body fluids, have negligible toxicity with respect to their lipid components, reduce the toxicity of the encapsulated drug, and can deliver efficiently their doxorubicin payload (DOXIL) or cis-platin to definite targets. This paper proposes that these stealth liposomes, could find future applications to deliver plasmid DNA with therapeutic genes.

g) Beroard J.C et.al- A note on sugar determination by anthrone method

Anthrone is used a cellular assay for colorimetric determination of carbohydrate. It reacts with the carbohydrate to produce a purple pink complex. The rated and extent of colour development with anthrone test differs with different sugar having similar structure. Anthrone has an advantage over phenol and orcinol procedure because it is applicable to 80% of ethanol extracts and even reacts with fructose at 50 degree centigrade.

h) Satpathy G.R et. al,2004-Loading red blood cells with trehalose: a step towards biostabilization .

High viability of RBCs preservation is of utmost importance for blood transfusion and clinical medicine. Trehalose, a well known disachharide, which can survive dehydration, can help in RBC's preservation. Trehalose is can be loaded onto RBCs by the phospholipid phase transition and osmotic imbalance, resulting in intracellular trehalose concentrations of about 40 mM. Trehalose acts as cryoprotectants, increasing osmotic protection of RBCs.

i) Holovati J.L et.al,2008-Effects of trehalose-loaded liposomes on red blood cell response to freezing and post-thaw membrane quality

Liposomes are being tested for intracellular delivery of trehalose to mammalian cells. Liposome-treated RBCs were resuspended in either physiological saline, 0.3 M trehalose or liposome solution, then cooled with slow, medium and fast cooling rates and storage in liquid nitrogen, followed by a 37 degree C thawing step. The cell viability test a postthawing shows that the recovery of RBCs frozen in liposome solution and trehalose solution was significantly higher than that of RBCs frozen in NaCl solution.

j) Farrugia A. et. al,1993-Cryopreservation of red blood cells: effect of freezing on red cell quality and residual lymphocyte immunogenicity

This paper investigates treatment with glycerol/washing as a potential substitute for freeze-thawing in the production of leucocyte. The standard procedure of treatment with glycerol, freezing, thawing, washing was compared with a similar procedure in which freezing was omitted. Compared with red cells subjected to the standard freeze-thaw technique, red cells undergoing the non-freezing procedure and suspended in additive solutions had significantly better biochemical preservation.

k) Lopez M. F. et. al 1984- Levels of trehalose and glycogen in Frankia sp. HFPArI3 (Actinomycetales)

The levels of soluble trehalose were compared in Frankia sp. Ar13 under various conditions in batch culture. Levels reach a peak of 10-20% cell dry weight depending on cultural conditions, then drop to a minimum of 1-2% cell dry weight. A similar pattern is observed

whether cells are grown in a medium containing NH_4CL or in a medium lacking nitrogen substrates; however, the total levels of trehalose are higher in the latter. When cells were inoculated into a medium lacking nitrogen to induce fixation of atmospheric nitrogen, trehalose levels reached their peak in 4 day after which the level dropped.

l) Quan G. et. al. 2007- Intracellular sugars improve survival of human red blood cells cryopreserved at -80 degrees C in the presence of polyvinyl pyrrolidone and human serum albumin

This study focuses on the effect of intracellular trehalose or glucose on human red blood cells cryopreserved in the presence of a polymer. Red blood cells were cryopreserved for 48 h-72 h at -80 degrees C. The percent hemolysis induced by intracellular trehalose was less than that induced by extracellular trehalose, but the difference was not significant. Cryopreservation can increase the percentage of cells with exposed phosphatidylserine (PS), but the ability of trehalose to maintain PS normal distribution is higher than that of glucose. Furthermore, intracellular sugars can protect membrane integrity of cryopreserved red blood cells, although a small portion of cells appeared spherocytic or echinocytic shape.

m) Chen Y. et. al.- 2006- Trehalose loading red blood cells and freeze-drying preservation

This study was aimed to investigate the effect of trehalose on lyophilized RBCs. The RBCs were observed by light and scanning electron microscopy, the hemolysis rate of loaded RBCs was detected by using Drabkin's kit, the intracellular trehalose levels were measured by anthrone method. The results showed that the intracellular trehalose concentration was 36.56 +/- 7.95 mmol/L, the microscopic images of trehalose-loaded RBCs showed the membrane integrity, the hemolysis rate in trehalose-loaded RBCs was 15.663 +/- 3.848%, while hemolysis rate in controlled RBC was 5.03 +/- 1.85%.

CHAPTER 3: MATERIALS AND METHODS

3.1-Chemicals and Reagents used

1. Lecithin- HIMEDIA
2. Cholesterol- HIMEDIA
3. Chloroform-Rankem
4. Methanol- Lobal chemie
5. Trehalose - HIMEDIA
6. Drabkin's reagent –Crest biosystems
7. Anthone-
8. PBS
    - NaCl- HIMEDIA
    - KCl- Qualigen
    - Na_2HPO_4- Qualigen
    - K_2HPO_4- Lobal
9. ADSOL solution
    - NaCl- HIMEDIA
    - Dextrose- Rankem
    - Adenine- Lobal chemie
    - Mannitol- Lobal chemie
10. CPDA solution
    - Trisodium citrate- SRL
    - Citric acid- Rankem
    - Dextrose- Rankem
    - Adenine- Lobal chemie
11. Blood collection by the adult donor of laboratory

3.2-Apparatus and important Wearing

Glass wares like measuring cylinder, beakers, small petri plates, test tubes flasks etc and plastic wares like syringe, falcon tubes, and eppendof tube.

INSTRUMENT

1. Bath sonicator- APE
2. Microscope- Olympus *i*NEA
3. Vortexer- SPINIX
4. Centrifuge
5. Rotatory vacuum evaporator- IKA RV 10
6. Controlled rate freezer- PLANER Kryo 560-16
7. Spectrophotometer- SYSTRONICS Double Beam Spectrophotometer 2203

8. Nano Zetasizer- NANO ZS
9. Water bath- REMI
10. Micro centrifuge
11. Fourier Transform Infrared Spectroscopy- Perkin Elmer , SQ 300S

3.3-Methods used

1. Bath sonicator method of preparation of liposomes

- A solution was made by adding 0.02g of cholesterol and 0.24g of lecithin to 3ml of n-hexane, to this solution 12 ml PBS was added and was vortexed for 30-35 minutes. This solution was kept in water bath for 5 minutes at 40 degree Celsius. Another solution was produced by using the above procedure which was vortexed in a water bath for 8 minutes at 60 degree Celsius.
- Three different solutions of oil and water were made. One solution has 20% (v/v) ATDL in water, another has 40% (v/v) and the other has 60 %(v/v).All tubes are vortexed for half an hour and centrifuged at 2000 rpm for 5 minutes. After centrifugation oil, water and tissue is separated. The separated water is sonicated in bath sonicator for 9mins at 40 degree Celsius.

2. Ether infusion method of preparation of liposomes

A solution was made up of 2 ml ATDL and 3ml of diethyl ether. 80ml PBS was kept in a 100 ml beaker in water bath at 60 degree Celsius (preheated). This was homogenized at 200 rpm and the solution was added to it 1 drop at a time. After the entire solution is added the homogenizer is kept on for about 10 minutes.

3. Lipid film hydration by rotatory vacuum evaporator method of preparation of liposomes.

A solution is made of 6 ml chloroform and 3 ml methanol to which 0.72g of lecithin and 0.06 of cholesterol is added. The solution is vortexed for 30-40 minutes till the lecithin and cholesterol is fully dissolved. This solution is added to round bottom flask of the rotatory vacuum evaporator. The temperature of the water bath attached to the rotatory vacuum evaporator is raised to 66 degree Celsius. The solution is rotated at 25 rpm till the all solvent gets evaporated and a thin lipid film is visible. 36 ml of PBS is added to the round bottom flask and the flask was vigorously vortexed till the entire film is dissolved in the aqueous

medium.

4. Folch method (Folch et al., J Biological Chemistry 1957, 226, 497) for the preparation of liposomes from adipose tissue

The tissue is homogenized with chloroform/methanol in 2:1 ratio, to a final volume 20 times the volume of the tissue sample .After dispersion, the whole mixture is allowed to stand for 5 minute at room temperature after that the mixture is agitated for 15-20 min in an orbital shaker at room temperature. The mixture is either filtrated or centrifuged (again and again) to recover the liquid phase. The solvent is washed with 0.2 volumes (4 ml for 20 ml) of water or 0.1ml HCl solution. After vortexing for some time, the mixture is centrifuged at 2000 rpmto separate the two phases. Upper phase is removed. After centrifugation and removing of the upper phase, the lower chloroform phase that contains lipids is evaporated under vacuum in a rotary evaporator. The temperature of the water bath attached to the rotatory vacuum evaporator is raised to 66 degree Celsius. The solution is rotated at 25 rpm till the all solvent gets evaporated and thin lipid film is visible. 36 ml of PBS is added to the round bottom flask and the flask is vigorously vortexed till the entire film is dissolved in the aqueous medium.

5. Preparation of liposomes loaded with trehalose

Two sets of liposome are prepared by the rotary vacuum evaporation method, one with lecithin cholesterol and the other with ATDL. These liposomes are loaded with trehalose buffer (300mM trehalose). The liposomes are concentrated by centrifuging at 3500 rpm for 10 minutes and the supernatant is disposed.

FIGURE 3.1: IKA rotatory vacuum evaporator RV 10

6. Size characterisation of liposomes:

A Zetasizer was used to measure particle and molecule size by Dynamic Light Scattering and to measure Zeta Potential. Dynamic Light Scattering is used to measure particle and molecule size. DLS measures the diffusion of particles moving under Brownian motion, and converts this to size and size distribution using the Stokes-Einstein relationship.

7. Anthrone test for analysis of trehalose uptake by the liposomes

PRINCIPAL-

In this test carbohydrates are dehydrated by to form conc. H_2SO_4 to form furfural which in turn complexes with anthrone to form a bluish green complex which can be measured colorimetrically at 620 nm by using a spectrophotometer.

PROCEDURE-

- 8 test tubes are obtained and labeled.
- 50µl of water is added to test tube 1 for reagent blank.
- Trehalose standards 2, 4, 6, 8, 10 mg of pure trehalose in test tubes 2 to 6.
- 50µl of liposome made from lecithin cholesterol is added to test tube 7
- 50µl of liposome made from ATDL is added to test tube 8
- To each test tube 2.9ml of anthrone reagent is added which was prepared by adding 0.2% anthrone in 100ml of conc. H_2SO_4 and vortexed for 30 seconds
- Test tubes and removed and placed in cold water for 10 minutes
- Absorbance is checked against blank at 620nm.

8. Cryopreservation of RBCs with liposomes loaded with trehalose

The blood is collected from healthy human adult volunteers (7 ml) in CPDA solution and stored at 4 degree Celsius for 1-4 hours. 0.5 ml of blood is added 6 different cryovials .Then to each test tube different solutions are added.

Cryovial 1- whole blood (1ml)

Cryovial 2- 0.5ml PBS

Cryovial 3- 0.5ml liposome lecithin cholesterol

Cryovial 4- 0.5ml liposome ATDL

Cryovial 5- 0.5 ml liposome (lecithin) loaded with trehalose

Cryovial 6- 0.5ml liposome (ATDL) loaded with trehalose

All the cryovials are incubated at 4 degree Celsius for 1-4 hours. The samples are cryopreserved by a controlled rate freezer by freezing the sample from 0 degree Celsius to -10 degree Celsius at 1degree/min and then from -10 degrees to -100 degree at 10 degree Celsius/min.

After the cyrovials are cooled till -100 degree Celsius they are plunged into liquid nitrogen at -196 degree and stored there overnight.

9. Percentage Hemolysis using Drabkin's Reagent

PRINCIPLE-

Hemoglobin is converted to methemoglobine by potassium ferricynde in the sample. Then a complex cynamethmoglobin is formed when methenoglobin further reacts with potassium cyanide. The complex is very stable and denotes a peculiar color whose intensity is directly proportional to concentration of plasma hemoglobin. The actual happening in hemolysis is that the RBC gets broken and starts releasing hemoglobin. Therefore, we can measure hemolysis by measuring the concentration of hemoglobin in the cell suspension, after centrifuging all blood samples at 500 rpm by a microcentrifuge. Then we measure their absorbance at 540 nm of their supernatant.

PROCEDURE- For hemoglobin assay 20µl of blood is added to 5ml of Drabkin's solution. The sample is incubated at room temperature for 5 -10 mins and the absorbance were taken at 540 nm in a spectrophotometer. A calibration curve is first determined from hemoglobin standard that has 0.6mg/ml. The hemoglobin concentration in the samples is determined by the standard curve and the percentage hemolysis is determined by

Percentage hemolysis = (OD_{540nm} of sample)/ ($OD_{540\ nm}$ of whole blood) * 100

10. Fourier transform infrared spectroscopy (FTIR) of liposomes- the intensity of liposomes and liposomes loaded with trehalose is measured over a narrow range of wavelength at a time by FTIR machine (Perkin Elmer, SQ 300S).

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1-Liposome

		Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm): 772.8	Peak 1:	912.5	83.1	233.8
PdI: 0.649	Peak 2:	163.5	15.3	27.49
Intercept: 0.949	Peak 3:	5560	1.6	8.632e-5
Result quality : Refer to quality report				

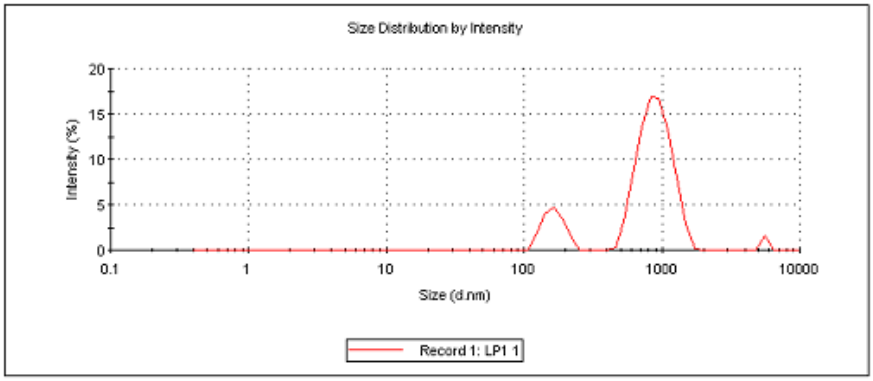


FIGURE 4.1- Average size characterisation of the liposome prepared by Sonication

		Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm): 284.4	Peak 1:	224.5	66.6	75.32
PdI: 0.431	Peak 2:	1959	33.4	782.9
Intercept: 0.948	Peak 3:	0.000	0.0	0.000
Result quality : Good				

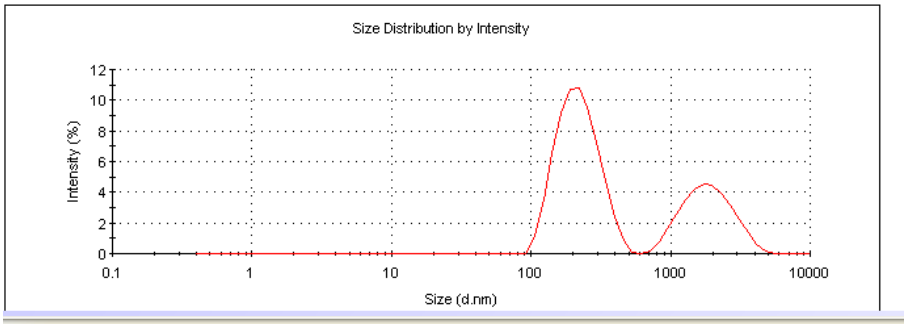
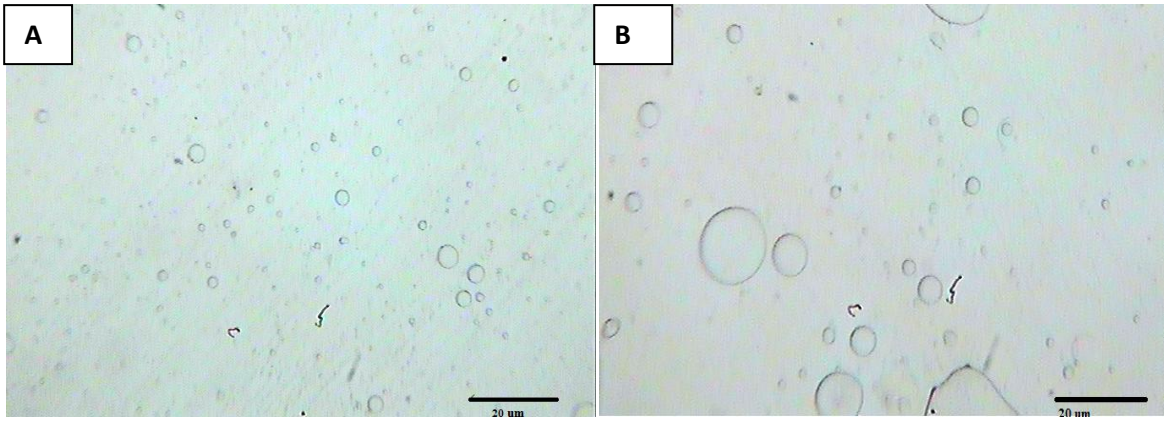


FIGURE 4.2- Average size characterisation of the liposome (ATDL) prepared by Sonication

The average size of liposomes made up of ATDL by sonication method is 284.4nm (diameter) and of the liposomes made up of lecithin is 772.8nm. The adipose tissue liposomes are smaller in size.



A:FIGURE 4.3- Liposome formed from ATDL by ether infusion method

B:FIGURE 4.4- Liposome prepared by lecithin cholesterol by rotatory vacuum evaporator

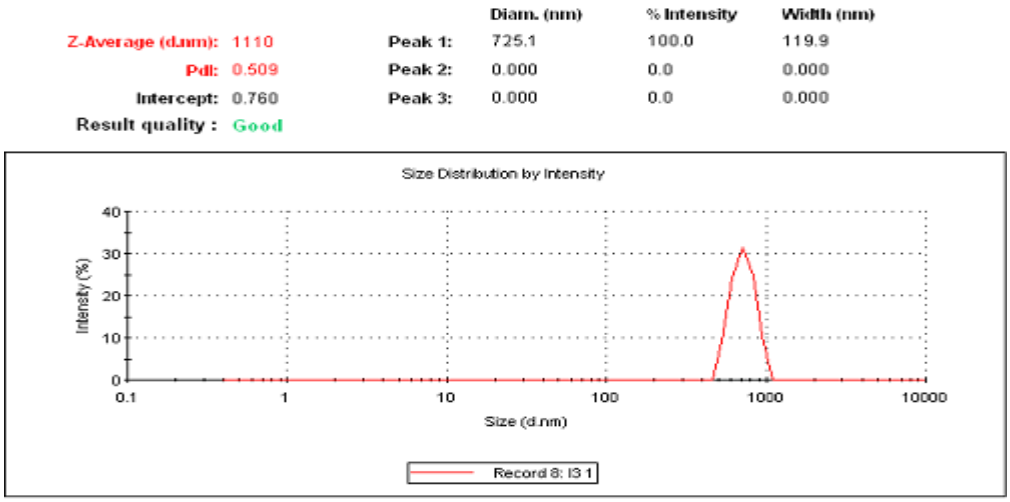
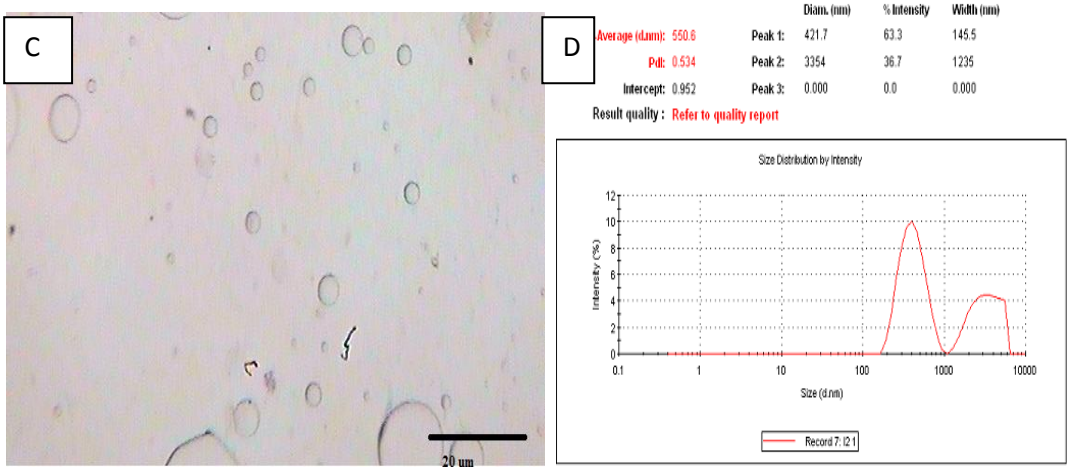
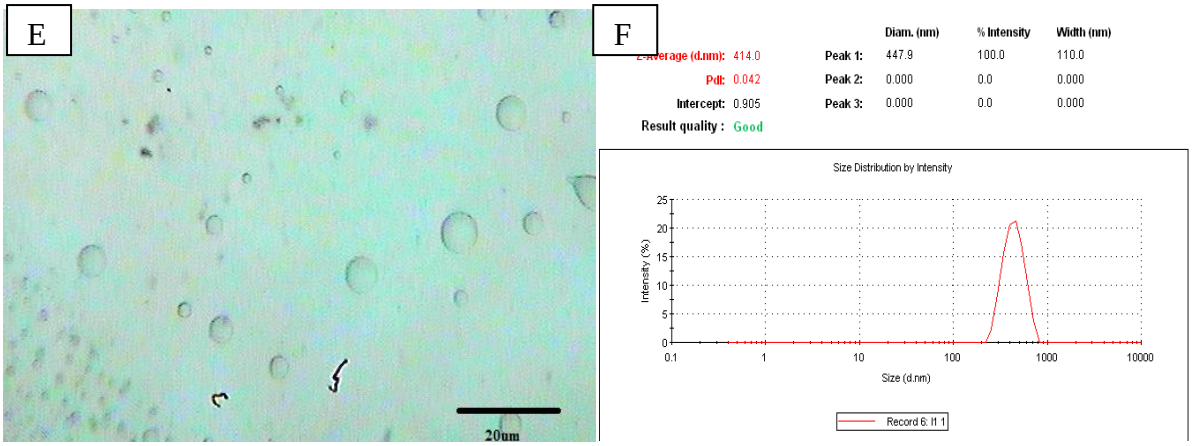


FIGURE 4.5- Average size characterisation of the liposome prepared by lecithin cholesterol by rotatory vacuum evaporator



C-FIGURE 4.6- Liposome prepared by lecithin cholesterol by rotatory vacuum evaporator and sonicated for 280s

D-FIGURE 4.7- Average size characterisation of the liposome prepared by lecithin cholesterol by rotatory vacuum evaporator and sonicated for 280s



E-FIGURE 4.8- Liposome prepared by ATDL by rotatory vacuum evaporator

F-FIGURE 4.9- Average size characterisation of liposome prepared by ATDL by rotatory vacuum evaporator

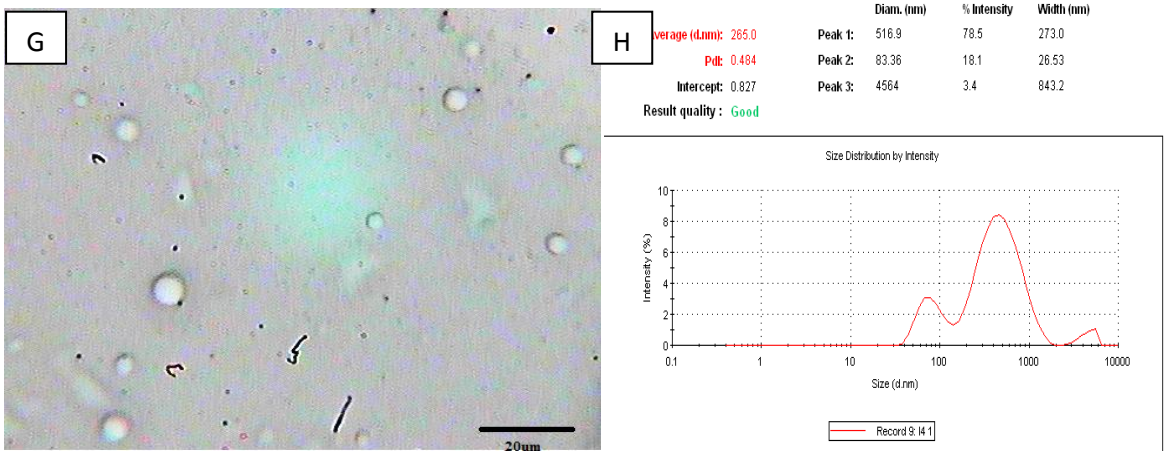


FIGURE 4.10- Liposome prepared by ATDL by rotatory vacuum evaporator and sonicated for 280 seconds

FIGURE 4.11- Average size characterisation of liposome prepared by ATDL by rotatory vacuum evaporator and sonicated for 280 seconds

The liposomes produced by rotatory vacuum evaporator are larger than the ones produced with sonication method, the average diameter of liposome from lecithin are larger (1100nm) in size than the liposomes from ATDL (474nm). The same trend is observed when the liposomes are sonicated for 280 seconds the average diameter of liposomes produced by average diameter of liposome from lecithin is 550nm and 265nm.

4.2-Anthrone test for percentage trehalose encapsulation

Tube	Sample	Content	Anthrone	Absorbance at 620nm
1	Blank		2.9ml	
2	Standard	(50µl) 2mg	2.9ml	0.049
3	Standard	(50µl) 4mg	2.9ml	0.145
4	Standard	(50µl) 6mg	2.9ml	0.125
5	Standard	(50µl) 8mg	2.9ml	0.381
6	Standard	(50µl) 10mg	2.9ml	0.424
7	Liposome(lecithin)	(50µl)	2.9ml	0.81
8	Liposome (ATDL)	(50µl)	2.9ml	0.32

Table 2: Table for anthrone test

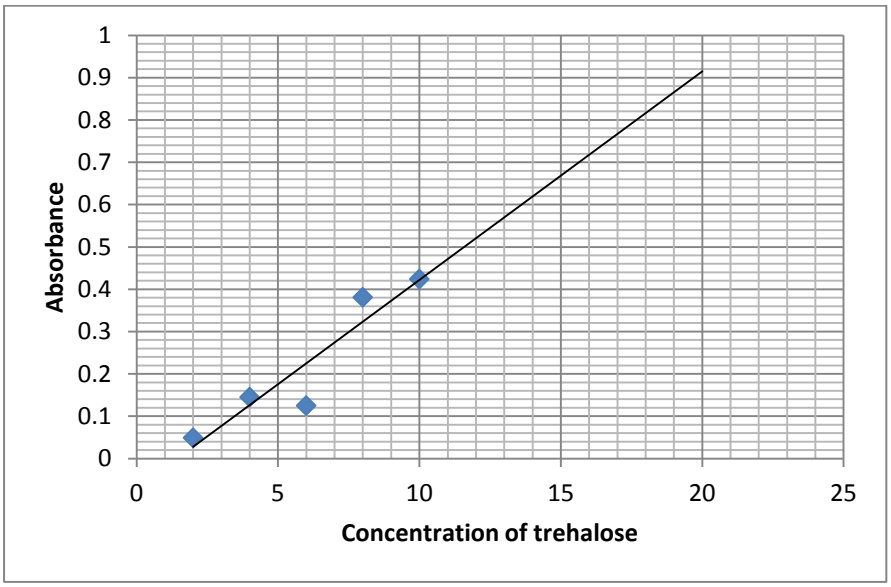


FIGURE 4.12: Standard graph of concentration of trehalose

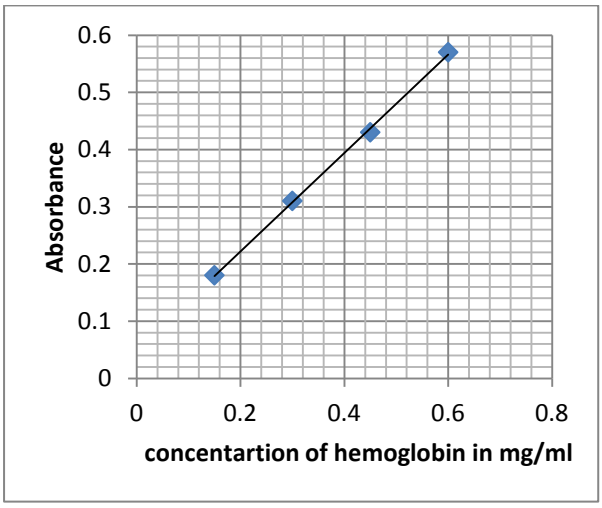
The concentration of the standard curve starts with 2mg/50microlitre till 20mg/50µl. The concentration for liposome (lecithin-cholesterol) came out as 18mg/50µl which is 60mg/1ml. The concentration for liposome (ADL) came out as 8mg/50µl which is 160mg/1ml. The amount of trehalose put in the buffer was 300mm which was 11.6g/1ml or 1160mg/1ml. So percentage trehalose encapsulated by liposome (lecithin-cholesterol) is 31.03% or and percentage trehalose encapsulated by liposome (ADL) is 13.79%

4.3-Drabkin's test for percentage hemolysis

A standard calibration curve is made by using haemoglobin standard.

Stock solution (0.6mg/ml)	Drabkin's reagent	Concentration	Absorbance at 540 nm
0	4ml	0 (blank)	0.00
4ml	0ml	0.6mg/ml	0.57
3ml	1ml	0.45mg/ml	0.43
2ml	2ml	0.3mg/ml	0.31
1ml	2ml	0.15mg/ml	0.18

Table 3: Table for Drabkin's test



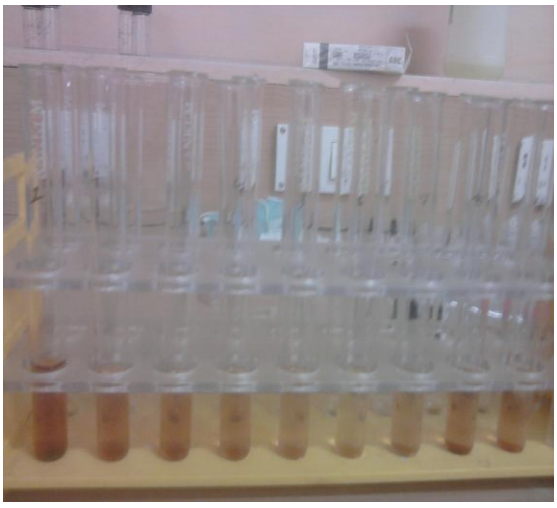


FIGURE 4.13: Calibration graph for hemoglobin.

FIGURE 4.14: Different color intensity shown by Drabkin's reagent due the presence of hemoglobin.

The percentage hemoglobin in the sample is calculated by measuring absorbance at 540nm with the help of spectrophotometer. The amount of hemoglobin in each sample in supernatant determines the amount of cell lysis that has occurred. The percentage of hemolysis is calculated for each sample.

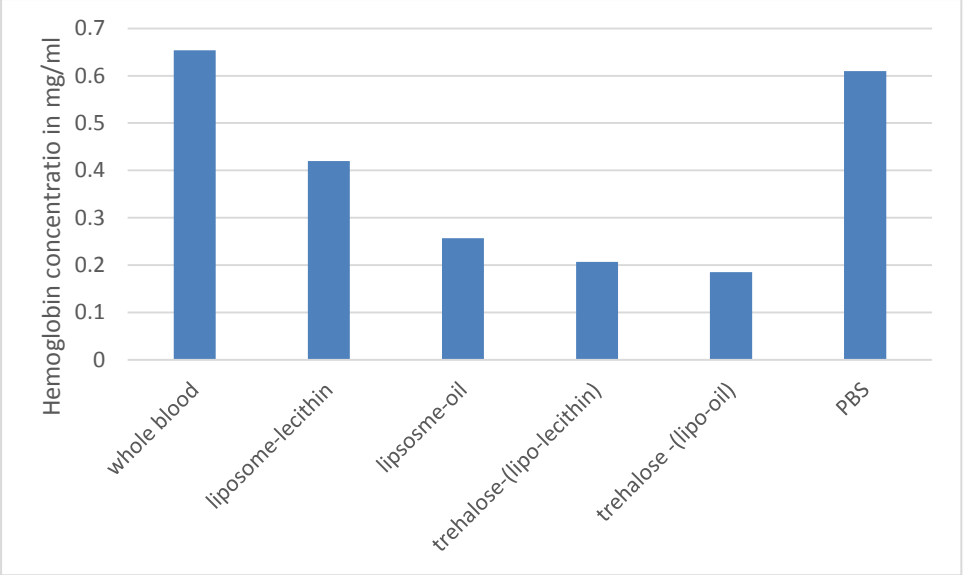


FIGURE 4.15: Hemoglobin concentration in mg/ml after cryopreservation

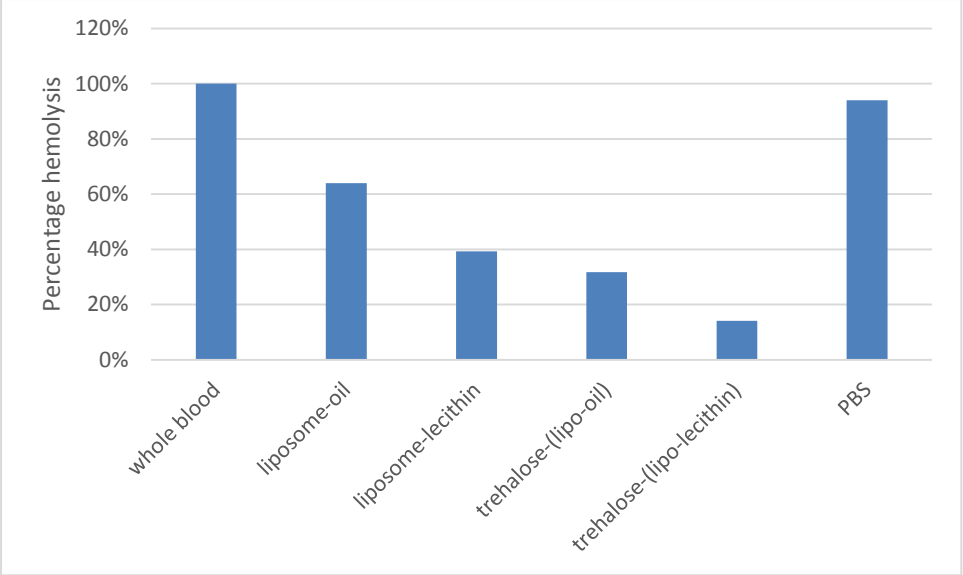


FIGURE 4.16: Percentage hemolysis of samples

It can be inferred from the results that liposomes themselves have some cryoprotective properties and even when they are not loaded with cryoprotectants. The liposomes made of lecithin cholesterol loaded with trehalose has the highest % survival of RBCs followed by liposomes made of ATDL loaded with trehalose, liposomes made of lecithin and cholesterol and liposomes made of ATDL.

4.4 – Fourier Transform Infrared Spectroscopy of liposomes

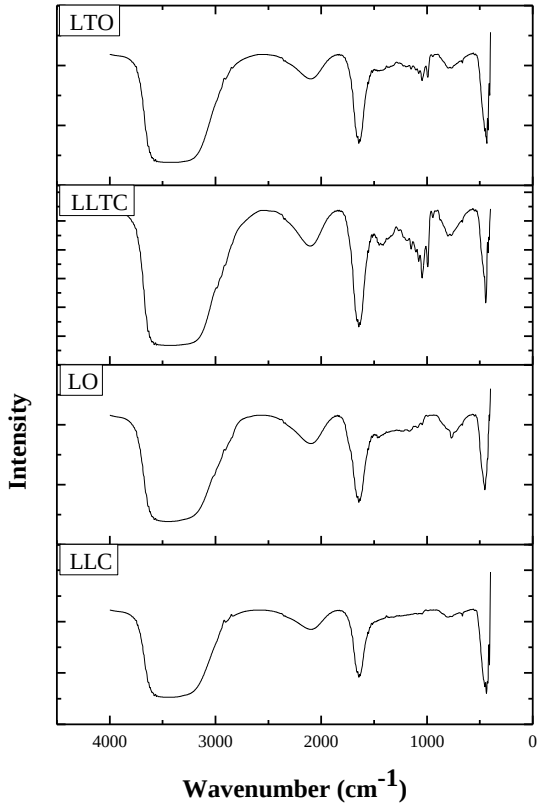


Figure 4.17: Intensity vs. wavenumber graph of liposomes made of lecithin cholesterol and oil and liposomes loaded with trehalose made of lecithin cholesterol and ATDL

LTO refers to liposome (ATDL) loaded with trehalose, LTLC refers to liposome (lecithin) loaded with trehalose while LO is liposome made of ATDL and LLC is liposome from lecithin –cholesterol. All the samples have peaks at 3011 and 1160 which denotes the presence of lecithin. All the samples also have peaks at 3399 and 1670 which denotes the presence of cholesterol. The first two samples also have peaks at also has peaks at 1041.46 and 991.45 which point towards the presence of trehalose in those sample. The 3550 - 3200 peaks denote Alcohol/Phenol O-H Stretch, Alkynyl C=C Stretch is because of peaks in the region 2260 - 2100 and Amide C=O Stretch is given by the 1690 – 1630 peaks.

CHAPTER 5: CONCLUSION

The adipose tissue derived lipids form liposomes in with the help of thin film hydration method. These liposomes are smaller in size than conventional liposomes. The uptake of trehalose, which is a water soluble drug, in case of liposomes made up of ATDL is less than the conventional liposomes. Liposomes, no matter what the source is have a cryoprotective effect on the RBC even when they are not loaded with any cryoprotectant. The conventionally produced liposomes loaded with trehalose have very high cryoprotective properties (viability-85% RBCs) but at the same time the cryoprotective properties of ATDL liposomes loaded with trehalose is also quite significant (viability-70% RBCs). The liposomes formed by adipose tissue derived lipids may have higher lipid content so encapsulation of lipid soluble drug or dye will be higher. Further tests leading to the encapsulation lipid soluble drugs in liposomes will bring out conclusive results.

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