DEVELOPMENT OF GIANT LIPOSOMAL FORMULATION FOR DRUG DELIVERY AND TISSUE ENGINEERING APPLICATION

A Project report submitted to National Institute of Technology, Rourkela in partial fulfillment of the requirements for the award of the degree of

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In

Biotechnology

By

BINAPANI CHOUDHURY

109BT0622

Under the guidance of

Prof. Indranil Banerjee

Department of Biotechnology and Medical Engineering

National Institute of Technology

Rourkela-769008 (ODISHA)

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CERTIFICATE

This is to certify that the Project Report entitled **"*DEVELOPMENT OF GIANT LIPOSOMAL FORMULATION FOR DRUG DELIVERY AND TISSUE ENGINEERING APPLICATION*"** submitted by Ms. BINAPANI CHOUDHURY in partial fulfillment of the requirements for the award of Bachelor of Technology in Biotechnology and Medical engineering with specialization in Biotechnology at the National Institute of Technology, Rourkela is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in the Report has not been submitted to any other University/Institute for the award of any Degree or Diploma.

Prof. Indranil Banerjee

Dept of Biotechnology and Medical Engineering

National Institute of Technology

Rourkela- 769008, INDIA.

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BINAPANI CHOUDHURY

109BT0622

B. Tech Biotechnology

Department of Biotechnology and Medical Engineering

LIST OF ABBREVIATIONS:

µl	Microliter
Chol	Cholesterol
SL	Soy lecithin
gms	Grams
HCl	Hydrochloric acid
hrs	Hours
mbar	millibar
ml	milliliter
nm	nanometer
PBS	Phosphate buffer saline
pH	Negative logarithmic of hydrogen ion concentration
rpm	Revolutions per minute
v/v	Volume/volume
w/v	Weight/volume

ABSTRACT:

This thesis delineates the development of giant liposomal formulation and application of drug loaded giant liposome encapsulated hydrogel as a theranostics formulation. Liposomes of varying composition of cholesterol and soy lecithin were prepared by ether infusion-homogenization method. Optimization of the composition was done on the basis of number of liposome formed at different lipid compostion and stirrer speed. Comparative analysis showed that among three different compositions (Chol: Soy lecithin; 1:8, 1:12, 1:16w/w) and three different stirrer speed (2000*g*, 4000*g*, 6000*g*), giant liposomes prepared from Chol: Soy lecithin 1:12 and at stirrer speed of 2000rpm could be considered as suitable candidates for the aforementioned application.

Physico-chemical characterization of the giant liposome was done by morphology (microscopy) study, vesicle size distribution profile, stability studies at different pH and temperature, efficiency of drug loading, in vitro toxicity of the drug loaded liposomes and hemocompatibility. Microscopy study confirmed giant liposomal morphology and this was further validated by vesicle size distribution profile using Image J and NI Lab View software. Stability studies at different pH and temperature revealed that giant liposomes are stable at room temperature and 4^0C temperature in PBS. In vitro toxicity of the drug loaded liposomes were done by using MTT assay method. Hemocompatibility of the liposome formulation was checked using goat blood and found to be hemocompatible which ensures (partially) its suitability for *in vivo* systemic application.

Loading of giant liposomes inside the hydrogel was done using the sodium alginate and calcium carbonate solution and 0.1 NHCl. Entrapment of giant liposome inside the hydrogel was confirmed by using fluorescence microscope. Then analysis of the mechanical properties

was performed using a P3 probe at a test speed of 1mm/sec. From the analysis it was observed there is an increase in gel strength with the incorporation of liposome.

In conclusion, giant liposome loaded hydrogel may be used as advanced pharmaceutical formulation for theranostic application.

Key words: Giant liposome, liposome encapsulated hydrogel, drug loaded liposome.

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INTRODUCTION:

Liposomes are the drug delivery systems used for the systemic administration of drugs. Liposome, a tiny vesicle made up of the same material as a cell membrane. Its membranes are composed of natural phospholipids having a head group attracted to water and a tail group which is made up of a long hydrocarbon chain. The tail group is repelled by water and also may contain mixed lipid chains containing surfactant properties. In the presence of aqueous medium, the heads are attracted to water and line up to form a surface facing the water and tails are repelled by water, and line up to form a surface away from the water same as in a cell. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer and the combined structure forms a bilayer. When phospholipid membranes are disrupted, they will reassemble themselves into tiny spheres which are smaller than a normal cell either as bilayers or monolayers. The bilayer structures are known as liposomes and the monolayer structures are known as micelles. As a drug delivery systems liposome played an important role in potent drug formulation to improve therapeutics. As a drug delivery system liposomes have many advantages such as they provide controlled drug delivery and controlled hydration and also provide sustained release and can carry both water and lipid soluble drugs. Liposomes are biodegradable, biocompatible, flexible and non ionic. It provides direct interaction of the drug with cell and can modulate the distribution of drug and also increase the therapeutic index of drugs. Liposomes also have some disadvantages such as less stability, low solubility, short half life, high production cost, and quick uptake by cells of reticulo endothelial system. We can construct liposomes containing low or high pH so that dissolved aqueous drugs will be charged in the solution. The pH neutralizes within the liposome so that protons can pass through some membranes. Then the drug will also be neutralized by allowing it to freely pass through a membrane. Liposomes prefer to deliver drug by diffusion than by direct cell fusion. Liposome drug delivery is also used to target

endocytosis events. Liposomes of a particular size range can make them viable to target natural macrophage phagocytosis. Such that in the macrophage's phagosome, liposomes will be digested by releasing its drugs. Liposomes can also used as carriers to deliver dyes to textiles, enzymes and nutritional supplements to foods and cosmetics to the skin, pesticides to plants.

Based upon structural parameters there are different types of liposomes such as medium sized unilamellar vesicles, large unilamellar vesicles (>100nm), giant unilamellar vesicles (>1µm), multivesicular vesicles (>1µm), multilamellar large vesicles (>0.5µm), oligolamellar vesicles (0.1-1µm), small unilamellar vesicles (20-100nm). Giant liposomes or giant unilamellar vesicles (GUV) of lipid memebranes with diameters greater than 1µm have been used to investigate the physical properties of membranes such as elasticity, shape change and phase separation. Giant liposomes with dimensions of living cells are useful models to: (1) study membrane curvature and elasticity under varying conditions; (2) examine interactions between lipid membranes and surfaces; (3) record the activity from reconstituted ion channels; (4) form planar lipid bilayers over micro fabricated pores; (5) create nano-fluidic networks; and (6) make micro scale bioreactors. Liposomes are often used in biological research not only because their properties are similar to those of real cell membranes, but also because they are large enough to observe and manipulate directly using optical microscope. For example, morphological transformation, microdomain information, biopolymer encapsulation, enzyme reactivity and association with reagents such as proteins, peptides, nucleic acids, etc. have all been investigated using giant liposomes from biophysical and biochemical perspectives.

REVIEW OF LITERATURE:

LIPOSOMES:

A liposome is a tiny bubble (vesicle), made out of the same material as a cell membrane. Liposomes can be filled with drugs, and used to deliver drugs for cancer and other diseases. Membranes are usually made of phospholipids, which are molecules that have a head group and a tail group. The head is attracted to water, and the tail, which is made of a long hydrocarbon chain, is repelled by water. In nature, phospholipids are found in stable membranes composed of two layers (a bilayer). In the presence of water, the heads are attracted to water and line up to form a surface facing the water. The tails are repelled by water, and line up to form a surface away from the water. In a cell, one layer of heads faces outside of the cell, attracted to the water in the environment. Another layer of heads faces inside the cell, attracted by the water inside the cell. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer, and the combined structure forms a bilayer. When membrane phospholipids are disrupted, they can reassemble themselves into tiny spheres, smaller than a normal cell, either as bilayers or monolayers. The bilayer structures are liposomes. The monolayer structures are called micelles. Liposomes are composite structures made of phospholipids and may contain small amounts of other molecules [1, 2]. In general, liposomes are defined as spherical vesicles with particle sizes ranging from 30nm to several micrometers. They consist of one or more lipid bilayers surrounding aqueous compartments, where the polar head groups are oriented towards the interior and exterior aqueous phases.

However, self-aggregation of polar lipids is not restricted to conventional bilayer structures which depend on temperature, molecular shape, and environmental and preparation conditions but may self-assemble into various kinds of colloidal particles [1, 2]. Liposomes are composed of phosphatidylcholine and cholesterol mixed with either phosphatidylserine (-ve charge) or stearylamine (+ve charge) [3]. Lecithin provides liposomes with aneutral

surface and streaylamine and phosphatidic acid components provide positive and negative surface charge respectively [4]. Cholesterol is used for formulations in order to reduce quick drug leakage [6]. Liposomes can encapsulated and effectively deliver both hydrophilic and lipophilic substances and may used as a nontoxic vehicle for insoluble drugs. It is biodegradable and hydrophilic, hydrophobic drugs and lipid insoluble drugs or DNA can encapsulated in the aqueous core within the liposome to improve their stability or bioavailability compared to free drugs or DNA [4, 5]. One of the characteristic features of liposomes is their ability to encapsulate solutions and release them to the external environment in response to temperature. Below the phase transition temperature of the phospholipid, the bilayer is solid-like and has a low permeability for the diffusion of polar molecules. Once heated above its phase transition temperature, the bilayer changes to fluid-like and its permeability can substantially increase. This property can be used for controlled release (or uptake) from liposomes in applications such as medicine or cosmetics [12].

Liposomes can be classified by either their structural properties or the basis of their preparation. The main types are listed and their characteristics are outlined in the table below.

Vesicle Types	**Abbrev**	**Diameter Size**	**Number of lipid bilayers**
Small unilamellar vesicles	SUV	Diameter of 20-100nm.	One lipid bilayer
Large unilamellar vesicles	LUV	Diameter of >100nm.	One lipid bilayer
Giant unilamellar vesicles	GUV	Diameter of >1μm.	One lipid bilayer
Multilamellar vesicles	MLV	Diameter of >0.5μm.	Five to twenty lipid bilayers
Oligolamellar vesicles	OLV	Diameter of 0.1-1μm.	Approximately five lipid bilayers
Multivesicular vesicles	MMV	Diameter of >1μm.	Multicompartmental structure

LIPOSOME IN THERAPEUTICS:

The pharmacokinetics and biodistribution of liposome encapsulated drugs are controlled by the interplay of two variables: the rate of plasma clearance of the liposome carrier and the stability of the liposome-drug association in circulation [10]. The rate of leakage or efflux of drug from liposomes should be slower than the rate of liposome clearance from blood [10]. In general successful formulation of stable liposomal drug product requires the following precautions like 1) processing with fresh, purified lipids and solvents 2) avoidance of high temperature and excessive shear forces, 3) Maintenance of low oxygen potential (Nitrogen purging), 4) Use of antioxidant or metal chelators, 5)Formulating at neutral pH and 6)Use of lyo-protectant when freeze drying [8]. Capacity to entrap ions and small and large molecules along with their low permeability presented considerable advantages as a drug delivery system. Their ability to potentiate the pharmacological efficacy of various drugs in vitro against mammalian cultured cells enhanced their prospects as a drug delivery system [9]. Liposomes can be loaded with magnetic nanoparticles for magnetic targeting and/or with colloidal gold, silver nanoparticles or fluorescent molecules for diagnostic and microscopic analysis and thermosensitive magnetoliposomes may increase the concentration of the encapsulated drugs in targeted tumor site by applying an external magnetic field [11].

Table: 2 Summary of the reports on therapeutic application of liposome

Sl.No	Therapeutic activity	Liposome used	Ref
1	Anti cancer vaccine against lung cancer	DOTAP cholesterol cationic liposome	Junfeng Zhang et al., 2013
2	Drug delivery in cancer therapy	Long-circulating liposomes	Alberto Gabizon et al., 1997
3	Antitumor vaccine against pancreatic cancer	PEGylated liposome	Cha-Eun Kim et al., 2012

4	Ultrasound-triggered therapeutic drug delivery	Paclitaxel-liposome–microbubble complex	Fei Yan et al., 2013
5	Gene delivery and vaccine therapy	Cationic liposome	Tiago Albertini Balbino et al., 2013
6	Liposome-based antigen delivery for cancer immunotherapy	pH sensitive fusogenic liposome	Eiji Yuba et al., 2013
7	Sonodynamic therapy (SDT): A novel treatment of cancer	Sonosensitizer liposome	Zhi-Jun Dai et al., 2013
8	Label free DNA sensing	Gold surface supported spherical liposome	M. Bhuvana et al., 2013
9	Inhibition of metastatic lung cancer	Liposome encapsulated all *trans* retinoic acid (ATRA)	Siddikuzzaman et al., 2012
10	Tumor growth suppression by gadolinium-neutron capture therapy	Gadolinium-entrapped liposome	Novriana Dewi et al., 2012
11	Pulmolonary delivery for local lung disorders	liposome	Michael I. Ugwoke et al.
12	Potential Diagnostic and Therapeutic Agent for Human Head-and-Neck Cancer Cells	Liposome-based Nanoprobe with Antibody	Y. Kuo et al., 2012
13	For improving brain delivery and therapeutic efficacy on brain glioma in animals	Liposome formulated with TAT-modified cholesterol	Yao Qin et al., 2011
14	Pancreatic cancer treatment	Cationic liposome	Feng Yang et al., 2011
15	Light and electron microscopy characterization of intra-articular drug delivery	Collagen–liposome entrapped chondroitin sulfate	Otilia Zarnescu et al., 2011

16	MRI monitoring of intratumoral drug delivery	Thermosensitive liposome	Tatsuaki Tagami et al., 2011
17	Metastatic breast cancer	Pegylated liposomal doxorubicin	Shailendra Verma et al., 2008
18	Ocular drug delivery	Liposome coated with low molecular weight chitosan	Ning Li et al., 2009
19	Novel drug delivery system aiming for radio-chemo-concurrent cancer therapy	Thermal neutron-sensitive liposome	Ken Akamatsu, 2009
20	Enhanced therapeutic effects in cerebral ischemia	Liposome-encapsulated citicoline	Pedro Ramos-Cabrer et al., 2011
21	Vaginal vaccine delivery against HIV-1	Liposome-based freeze-dried rods	Prem N. Gupta et al., 2010
22	High therapeutic efficacy for colon tumors	Irinotecan-loaded liposome	Yoshiyuki Hattori et al., 2009
23	Preventive against ischemia-reperfusion injury in a rat middle cerebral artery occlusion	Liposome-encapsulated luteolin	Gang Zhao et al., 2011
24	Treatment of Hepatocellular Carcinoma and Hepatic Metastases	Thermosensitive Liposomes	Mark W. Dewhirst et al., 2013
25	Intratumoral drug delivery and efficacy	Stealth thermosensitive liposomes	Li Li et al., 2013
26	Vaccine against tuberculosis of pseudo-ternary complexes	Peptide-DNA-cationic liposome	Rogério Silva Rosada et al., 2012
27	Immunological adjuvant efficacy against Newcastle disease vaccine	Glycyrrhetinic acid liposome	Xiaojuan Zhao et al., 2011

28	DNA vaccination efficiently evokes anti-HPV E6 and E7 CTL responses	Oligomannose liposome-DNA complex	Masahito Mizuuchi et al., 2012
29	Enhances Radiotherapy and Chemotherapy to Suppress Tumour Growth and Metastasis in Mice	Liposome-Encapsulated Hemoglobin	C. Murayama et al., 2011
30	Endocytosis and intracellular trafficking of for gene delivery	Cholesterol based cationic liposome	K. Doh, et al., 2007

GIANT LIPOSOMES:

Giant liposomes are large (>1µm diameter), spherical vesicles consisting of one or more phospholipid bilayers surrounding an aqueous center. They have been used for physical and biological investigations such as shape change of liposomes, elastic properties of lipid membranes, interaction of cytoskeleton proteins with membranes and dynamic structures of biomembranes [7]. Giant liposomes with dimensions of living cells are useful models to study membrane curvature and elasticity under varying conditions, to examine interactions between lipid membranes and surfaces, to record the activity from reconstituted ion channels, to form planar lipid bilayers over microfabricated pores, to create nanofluidic networks and to make microscale bioreactors [6].

LIPOSOME ENCAPSULATED HYDROGEL:

Hydrogel is a network of polymer chains that are hydrophilic. Hydrogels are highly absorbent natural or synthetic polymer and also possess a degree of flexibility very similar to natural tissue due to their significant water content. Hydrogel have the ability to absorb large amount of water. Alginate is a biocompatible and biodegradable polysaccharide obtained from sea algae, consisting of mannuronate and guluronate homopolymer blocks. While sodium alginate is water soluble, it rapidly forms a gel with multi-valent cations such as Ca2+. This property is used for the microencapsulation of various compounds including enzymes and whole cells. Alginate is also used as a wound dressing with haemostatic properties [12]. The main advantage of embedding liposomes into the alginate carrier compared to free individual liposomes is the protection of liposomes from external factors that could lead to their destabilisation and the possibility to keep a statistically significant number of liposomes in a single location rather than dispersing them throughout the host environment. Embedding of liposomes into alginate particles makes it possible to build more complex structures in the future that may contain other components such as immobilized enzymes or catalysts for facilitating chemical reactions [12]. Liposomal hydrogel as a wound dressing provides a barrier that effectively prevents the contamination of the wound and further progression of infection to deeper tissues. The drug encapsulated within the liposomes increases the drug concentration locally and decreases systemic drug concentration and hydrogels combine the features of moist wound healing with good fluid absorbance. And act as a barrier against bacteria, oxygen permeability, it is easy to handle, hence it is useful as a moist wound dressing material. Besides hydrogels by nature of transparency promote monitoring of wound healing [13]. It allows a prolonged release of drugs at the specific site, by efficient intracellular delivery of drug loaded liposomes. It is efficient also for the delivery and

modified release of protein drugs. It is of simple topic application on epithelial ulcers and it can be used on human beings, and mammalians, to treat dermatological diseases [14]. It also used as coating for biosensors, contrast agents for imaging, and scaffolds for tissue engineering [15]. Injectable hydrogels as one hydrogel type have been extensively employed as cell carriers for in vivo tissue engineering. The advantages of using injectable hydrogels lie in that they have high moldability, capable of filling irregular shaped defects; can be delivered to the in vivo environment by limited surgical invasion such as minimally invasive surgery; and are readily for cell and drug encapsulation. After being delivered in vivo, the injectable hydrogels solidify to form tissue constructs, which are exposed to the in vivo environment and therefore are capable of experiencing local biological and mechanical cues that may enhance tissue development [16].

MATERIALS AND METHODS:

Materials: Lecithin (HIMEDIA), Cholesterol (HIMEDIA), Diethyl ether (MERCK), 5-Bromo Uracil, 0.1NNaOH, 0.1NHCl, Coomassie brilliant blue G, Floral yellow (fluorescent dye) and all other chemicals needed were purchased from HIMEDIA. Every preparation was carried out with double distilled water.

Methods:

Preparation of giant liposomes and drug loaded giant liposopme:

Giant liposomes were prepared by modified ether infusion method namely ether infusion-homogenization method. Cholesterol and soya lecithin were taken at a ratio of 1:12 in a falcon tube and dissolved in diethyl ether of almost 3ml. 100ml of PBS buffer was taken in a 500 ml beaker and warmed upto 55^0C using water bath. During the process of homogenization the temperature of PBS should not decrease below 55^0 C. It should be

maintained at 55^0 C. So to maintain this temperature of PBS, the heated PBS was transferred to a plastic measuring cylinder and the cylinder was kept inside water bath. The temperature of the water bath maintained at 65^0C. After that the prepared solution was infused periodically through pipette into the preheated (at 55^0 C) PBS (pH 7.4) under continuous homogenization (to produce continuous stirring effect) for 25 min. At that temperature diethyl ether got evaporated leaving liposomes of various sizes. RPM was maintained at 2000. Similarly, for the preparation of drug loaded giant liposomes, 1 gm of 5-flurouracil was taken in a 100 ml PBS and mixed it by putting in stirrer for 15 to 20 minutes. Then liposome suspension was prepared.

Physical Characterization of Giant liposomes:

- **Morphology Studies (Microscopy studies):**

  Giant liposomes were viewed using Leica DM750 microscope and were counted manually.

- **Vesicle size distribution profile:**

  Vesicle size distribution profile was analysed by using NI labview software .

Process optimization:

(i) Giant liposomes at different RPM:

Giant liposome solutions were prepared at different RPM (2000*g*, 4000*g*, and 6000*g*) of definite lipid composition. Then the study was conducted by microscopy based manual counting of liposomes and subsequent NI Lab View based analysis.

(ii) Giant liposomes of different lipid ratio:

Giant liposome solutions of three different lipid ratios (1:8, 1:12 and 1:16) were prepared. Then the study was conducted by microscopy based manual counting of liposomes and subsequent NI Lab View based analysis.

Stability study:

(i) Stability of giant liposomes at different pH:

Giant liposome was prepared. Then the sample is divided into four groups. Control taken as uncentrifuged and to the remaining three samples, PBS buffer, 0.1NHCl, 0.1NNaOH was added. Then the study was conducted by microscopy based manual counting of liposomes and subsequent NI Lab View based analysis.

(ii) Stability of giant liposomes at different temperatures through microscopic analysis:

Aqueous stability depends on the lipid oxidation due to exposure of time so the stability studies are taken different intervals of time in 24hrs, 48 hrs and 72 hrs with respect to temperature. Readings are taken from samples at room temperature, 4^0 C and -20^0C. Then the study was conducted by microscopy based manual counting of liposomes and subsequent NI Lab View based analysis. The readings show that giant liposomal solutions are stable at 4^0C rather than room temperature and in -20^0 liposomes are least stable due to ice crystal formation which further leads to rupture of membrane.

Hemocompatibility tests:

Giant liposomes were prepared by ether infusion-homogenization method. 8ml of goat blood was taken and 10ml of 0.9% saline was added to that. Positive and negative controls were made by adding 0.5ml of 0.01N Hcl and 0.5ml of PBS respectively. Sample was 0.5ml of

giant liposomal solution added to 9ml of saline +0.5ml of diluted blood. The samples were then incubated along with positive and negative controls for 60 min. Then the samples were centrifuged along with positive and negative controls at 4000rpm for 10minutes to settle all the blood components. If there are any ruptured RBCs then its contents will be released into supernatant. Then O.D of supernatant was taken at 545nm. Percentage of haemolysis was calculated by using the formula.

% Hemolysis = 100*(O.D. of sample - O.D. of positive control) / (O.D. of negative control-O.D. of positive control)

Protein loading Studies and Dye entrapment studies:

The model protein used was BSA. 25 gm of BSA were taken in 100ml PBS. Giant liposomes were prepared using the protein solution. Loading was estimated by taking O.D at 595 nm using spectrophotometer. BSA standard curve can be taken as reference. Dye used was coomassie brilliant blue. 5 mg of coomassie brilliant blue dye were taken in 100ml of PBS and warmed up to 55^0C in a water bath. Then dye loaded liposomal suspension was prepared. Then the dye loaded liposomal suspension was centrifuged 2 times at 4000 rpm for 10 minutes and resuspended in fresh PBS. Then the study was conducted by Leica microscope.

Fluorescent Microscopy:

By adding 10mg of floral yellow in 1ml of diethyl ether 0.1% dye solution was prepared. From that 500µl was taken and mixed it with lipid solution for the preparation giant liposomes. After that samples were centrifuged 2 to 3 times for 10 minutes at 4000rpm for washing purpose. Then the pellet obtained was resuspended in 2ml of fresh PBS. Then the sample was taken in a eppendruf and carefully wrapped with a brown paper or aluminium foil in order to avoid bleaching and characterized using fluorescent microscope.

Study of in vitro toxicity of 5-fluorouracil loaded giant liposomes:

For the preparation of drug loaded giant liposomes, 1 gm of 5 flurouracil was taken in a 100 ml PBS and mixed it by putting in stirrer for 15 to 20 minutes. Then liposome suspension was prepared. It was then centrifuged and resuspended in 5ml PBS and incubated for 24hr at 37°C. The releseate containing the drug was filtered through a 0.22µm filter. HeLa cells were cultured in MEM /10% FBS and seeded in a 96 well plate at a concentration of $1x10^5$ cells/ml. The 10 µl of releasate was administered per well. The toxicity of the cells was studied for 24hr by using MTT assay method.

Preparation of giant liposome encapsulated Hydrogel:

By adding 1gm of sodium alginate in 100 ml of distilled water (1% w/v) alginate solution was prepared. From that 20 ml of alginate solution was taken in a beaker and stirred in a magnetic stirrer. During the time of stirring, 0.024 gm of calcium carbonate was added in the alginate solution. Then after 5 minutes of proper mixing 1ml of floral yellow dye loaded liposomal solution was added slowly. At last 1700µl of 0.1N HCl was added to that solution. Then fluorescence microscopy and mechanical testing of the liposome loaded hydrogel was done.

Characterization of giant liposome loaded hydrogel:

Microscopic characterization:

The morphology and distribution of the giant liposomes loaded hydrogel was studied using fluorescent microscope (Olympus IX71).

Analysis of Mechanical Property:

Gel strength, of the gels was studied using a texture analyzer (Stable Microsystems, TA-HD plus, U.K.). The test was performed using a P3 probe at a test speed of 1mm/sec.

RESULT AND DISCUSSION:

Liposomes are formed in different morphologies namely small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), multivesicular liposomes each differing in size, their internal volume and some form very large in size generally in micrometers known as giant unilamellar vesicles (GUVs). The type of liposome formed depends on method followed. For example, sonication usually results in SUVs while ether infusion and ether injection methods result in GUVs. Multilamellar vesicles can be later converted to unilamellar and liposomes of uniform size can be obtained by extrusion through filter membrane of particular pore size.

Cholesterol is commonly used as lipid for the preparation of liposomes which has highest melting point ($42^{o}C$) among all lipids present in the cell membrane and hence its function is to maintain the integrity of the cell membrane. Usually its concentration is taken in lesser compared to other lipids for the preparation of liposomes may be as to vary the membrane permeability.

Figure 1 showed the experimental set up of ether-infusion homogenization method of giant liposome synthesis. Figure 2 showed the microscopic view of giant liposomes. Figure 3 showed the microscopic view of 5-flurouracil drug loaded giant liposomes.

Fig 1: Experimental Setup for the preparation of giant liposomes.

Fig 2: Microscopic view of giant liposome at 40X scale

Fig 3: Microscopic view of 5-flurouracil drug loaded giant liposomes

Fig 4: Size distribution profile of giant liposomes and drug loaded liposomes (r is in micrometer)

OPTIMIZATION OF LIPID COMPOSITION AND STIRRER SPEED:

Giant liposomes of different lipid ratio:

Giant liposome solutions of three different lipid ratios (1:8, 1:12 and 1:16) were prepared. Then the study was conducted by microscopy based manual counting of liposomes and subsequent NI Lab View based analysis. When we compared the result of three different lipid ratios, we found more number of giant liposomes in the lipid ratio of 1:12 compared to another two.

Giant liposomes at different RPM:

Giant liposome solutions were prepared at different RPM (2000g, 4000g, and 6000g) of definite lipid composition. Then the study was conducted by microscopy based manual counting of liposomes and subsequent NI Lab View based analysis.

Fig 5: Microscopic views of liposomes formed at different RPM respectively (2000, 4000 and 6000 rpm)

When we compared the giant liposomes obtained at different RPM, we found that at 2000rpm more numbers giant liposomes were formed compared to 4000rpm and 6000rpm due to high stirrer speed.

RPM / Lipid ratio w/w	6000	4000	2000
1:8	X	X	X
1:12	X	X	✓
1:16	X	X	X

From this table we obtained that for the preparation giant liposomes best combination is the 1:12 lipid ratio and 2000 rpm stirrer speed.

STABILITY STUDIES:

Stability of giant liposomes at different pH:

Giant liposome samples were divided into four sets. Uncentrifuged samples were taken as control and remaining three sets were resuspended in 5ml PBS buffer, 0.1NHCl, 0.1NNaOH . Then the study was conducted by microscopy based manual counting of liposomes and subsequent NI Lab View based analysis.

Fig 5: Stability studies of giant liposomes at different pH: sample with PBS, sample with 0.1N NaOH and sample with 0.1N HCl respectively

Fig 6: Size distribution graph of stability studies of giant liposomes at different pH. (r is in micrometer)

From this study we are concluded that liposomes are stable in PBS and clusters of liposomes were obtained in presence of 0.1N HCl.

Stability of giant liposomes at different temperatures through microscopic analysis:

Aqueous stability of liposomes depends on the lipid oxidation. It is observed that lipid oxidation kinetics temperature sensitive. Furthermore temperature is often considered as a critical parameter for storage. To evaluate the stability of liposome prepared due stability studies are taken different intervals (24hrs, 48 hrs and 72 hrs) at three different temperature Readings are taken from samples at room temperature, 4^0 C and -20^0C. Then the study was conducted by microscopy based manual counting of liposomes and subsequent NI Lab View based analysis.

Fig 7: Temperature stability studies of liposomes at 0hr

Fig 8: Temperature stability studies of liposomes (i) of room temperature at 24 hrs, (ii) of 4^0C at 24 hrs, (iii) of -20^0C at 24 hrs, (iv) of room temperature at 48 hrs, (v) of 4^0C at 48 hrs, (vi) of -20^0C at 48 hrs, (vii) of room temperature at 72 hrs, (viii) 4^0C at 72 hrs, (ix) of -20^0C at 72 hrs

The readings show that giant liposomal solutions are stable at 4^0C rather than room temperature and in -20^0 liposomes are least stable due to ice crystal formation which further leads to rupture of membrane.

Fig 9: Reduction in liposome number

Fig 10: Size distribution graph of stability studies of giant liposomes at different temperature

It is observed in the size distribution curve there is a certain increase in the size of that liposome with incubation. This may happen due to the fusion of giant liposomes.

Hemocompatibility study:

Intravenously injected therapeutic agent or carrier system must remain and circulate in the blood before reaching the target site. So hemocompatibility of the liposome system is also important as tissue compatibility. Hemocompatibilty study was conducted using goat blood by taking 0.1N HCL as positive control which degrades blood components completely. In positive the cell debris control was whitish in colour after centrifugation and supernatant was red indicating complete hemolysis of RBCs.

Percentage hemolysis calculated using,

100*(O.D. of sample - O.D. of positive control) / (O.D. of negative control- O.D. of positive control)

Percentage of hemolysis by giant liposomes was found to be 5%.

Fig 11: Hemolysis study: Graph showing hemolysis (OD at 545nm) by positive and giant liposomes.

From the result we conclude that giant liposome can be used for systemic application. Liposome bilayer is composed of lipids should be compatible to blood components.

Protein loading Studies and Dye entrapment studies:

The model protein used was BSA and the dye used was coomassie brilliant blue. Giant liposomes were prepared using the protein solution and loading was estimated by taking O.D at 595 nm using spectrophotometer. BSA standard curve can be taken as reference. Dye loaded liposomal suspension was prepared and then the dye loaded liposomal suspension was centrifuged 2 times at 4000 rpm for 10 minutes and resuspended in fresh PBS. Then the study was conducted by Leica microscope.

Fig 12: Microscopic view of dye loaded giant liposomes

The percentage of protein loading and the percentage dye loading was calculated to be 3%.

Fluorescence microscopy of giant liposomes:

Fig 13: Fluorescence microscopy of giant liposome

Drug loading study:

1 gm of 5 flurouracil was taken in a 100 ml PBS and mixed it by putting in stirrer for 15 to 20 minutes. Then liposome suspension was prepared. It was then centrifuged and resuspended in 5ml PBS and incubated for 24hr at 37°C.

Fig 14: Microscopic view of drug loaded giant liposomes

Considering the protein loading study and dye entrapment study it was concluded that the percentage of encapsulation of drug was approximately 3%.

Study of cytotoxicity of drug loaded liposomes:

To study the cytotoxicity of drug loaded liposomes, the liposomes were added to the cell culture medium and incubated at 37^0C 5% CO_2 for 24 hours. Control was cells cultured without addition of drug loaded liposomes. After 24 hours, MTT assay was done to measure the cell proliferation. From the result, drug loaded liposomes showed cytotoxicity index of 0.26 as control was taken as 1.

Fig 15: Cytotoxic analysis of drug loaded giant liposomes

Microscopic analysis: Fluorescent images of gel showed that liposomes are evenly distributed inside the alginate gel. It is further evident from the picture that there was no agglomeration of liposomes inside the gel. Liposomes were not degraded inside the gel.

Fig 16: Fluorescence microscopy of giant liposomes loaded hydrogel

Analysis Mechanical Property:

Gel strength study gave indication about the gel strength and brittleness of the gels. These are important parameters for the development of products for topical applications. Gel strength is defined as the maximum positive force required compromising the gel integrity while the brittleness may be defined as the distance penetrated by the probe at the gel rupture. From the analysis it is observed there is an increase in gel strength with the incorporation of liposome. This is may be because of filler effect of liposomes.

Fig 17: Texture analysis graph of liposome loaded hydrogel

SUMMARY AND CONCLUSION:

In summary, our results indicate that the giant liposomes prepared from cholesterol and soy lecithin (1:12) mixture at 55^0C temperature by using ether infusion homogenization method which can be stable at room temperature and 4^0C temperature and also in PBS can be used for systemic application because liposome bilayer is composed of lipids is compatible to blood components. It can be use in drug delivery application by encapsulating the drug loaded giant liposomes inside hydrogel because giant liposome have the storage capability and hydrogel have the ability to absorb large amount of water and it is biocompatible and biodegradable. The cytotoxic index of drug loaded liposomes is very low and by the incorporation of liposome the strength of the gel increases.

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