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Engineering liposomes

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ENGINEERING LIPOSOMES

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ABSTRACT:

The purpose is to design and evaluate the liposomal formulations to overcome disadvantages such as short half-life, prone to oxidation and hydrolysis, leakage and fusion in vitro. Liposomes are prepared using different techniques like lipid hydration and extrusion, reverse phase evaporation, ethanol injection, sonication, ethanol destabilization and mannitol freeze thaw methods. The formulations made using each of these methods were evaluated based on particle size and encapsulation efficiency. The particle size was measured using Dynamic light scattering and the encapsulation efficiency was measured using fluorescence spectroscopy. Finally, the suitable formulations were tested for dye release studies using SOTAX dissolution apparatus. We have determined the advantages and disadvantages of each method using for preparation of liposomes. The formulations were selected which had an optimum particle size between 100- 200 nm and the best encapsulation efficiency was around 50-70%. The dye release studies showed a release between 25-38 days. We have developed a hybrid method for preparation of liposomes by combining lipid hydration and extrusion, sonication and mannitol freeze thaw method. The advantages from each method helped us to overcome the disadvantages of short shelf life, fusion and leakage.

INTRODUCTION:

A liposome is an artificial vesicle made from a phospholipid bilayer that can be used to deliver drug agents into specific regions of the body. Liposomes were discovered by Bangham and coworkers about 40 years ago and defined as microscopic spherical vesicles that form when phospholipids are hydrated.[2] The phospholipid bilayer is composed of a hydrophilic phosphate head and a hydrophobic lipid tail that arranges in a sphere when phospholipids come in contact with a water-based solution. Liposomes are drug delivery vehicles due to the ability of encapsulation of water soluble materials within the aqueous core and lipid soluble materials within the hydrophobic bilayer.[1]

The zeta potential is the acquired overall charge of a particle in a specific medium and is important in lipid formations because it can help predict the fate of the liposome in vivo.[1] The zeta potential can monitor any modifications on the liposome surface.[1]

The three compositions that are used to make liposomes include: dipalmitoyl phosphatidyl choline (PC)(FW 734), cholesterol (FW 387), and dipalmitoyl phosphatidyl glycerol (PG) (FW 745). The pink book suggests using 100 mg PC, 40 mg cholesterol, and 10 mg PG giving a molar ratio of 10:8:1.[3] The article Damage to liposomal lipids: protection by antioxidants and cholesterol-mediated dehydration, by Samuni et al suggests using 10:1 PC to cholesterol and no PG.[3] Neveux et al suggests having a ratio of 6:3:1 for PC:cholesterol:PG.[3]

The best method is to add the three powders in empty vials, add the chloroform and methanol solution and vortex until dissolved, and then introduce the contents into 250 mL round bottom flasks and attach to a rotary evaporator at 60 rpm and 30° C.[3] This is continued until a film is formed. The flasks are then lyophilized and HEPES buffer is

added and the round bottom flasks are vortexed again until the lipid film is removed from the walls. The suspension goes through three cycles of extrusion to get particle size of <200 nm. The liposomes are sized using the dynamic light scattering apparatus.

Entrapment capability depends upon liposome size and type, charge on the liposome surface, bilayer rigidity, method of preparation, remote loading, addition of ion pairing, and complexing agents and characteristics of the drug to be encapsulated.[4]

A major problem with injecting liposomes into the blood stream is short circulation time because of uptake by the reticuloendothelial system (RES). The get longer circulation time, the liposome can be coated with a polymer.[1] Other disadvantages of liposomes include high production cost, leakage and fusion of encapsulated drugs and molecules, oxidation and hydrolysis reactions of phospholipid, short half-life, and low solubility.[5]

Long-term stability and increased shelf-life can be achieved by lyphilization. Freezing as well as freeze-drying preserved the liposomes in a dry form. Different carbohydrates or polyalcohols, such as mannitol or glycerol are considered as cryoprotective agents to inhibit liposomal fusion or degradation during freezing procedures.[6]

To assess the entrapment of the drug a ratio of drug to lipid is determined and is compared to the preloading ratio. This is done by purification of the drug loaded liposomes by gel filtration chromatography, dialysis, or ion-exchange chromatography.[7]

Cholesterol makes the membrane less permeable by filling up holes or disruptions.[3] Studies at the Kamla Nehru Institute of Management and Technology show that as the amount of cholesterol increases the size of the liposome decreases.[3]

PEGylation changes the physical and chemical properties of the biomedical molecule, such as its conformation, electrostatic binding, and hydrophobicity, and results in an improvement in the pharmacokinetic behavior of the drug.[8]

Table 1: Approved liposomal formulations[14]

Drug	Product name	Type	Lipid composition	Route of administration	Approved treatment
Amphotericin B	Ambisome	Liposome	HSPC<comma> DSPG and cholesterol	Intravenous	Sever fungal infections
Doxorubicin	Myocet	Liposome	EPC and cholesterol	Intravenous	Metastatic breast cancer
Doxil	PEGylated liposome	HSPC<comma> cholesterol and DSPE-PEG2000	Intravenous	Kaposi's sarcoma<comma> ovarian and breast cancer	[5] and [13]
Lipo-dox	PEGylated liposome	DSPC<comma> cholesterol and DSPE-PEG2000	Intravenous	Kaposi's sarcoma<comma> ovarian and breast cancer	[14]
Daunorubicin	DaunoXome	Liposome	DSPC and cholesterol	Intravenous	Blood cancer
Verteporfin	Visudyne	Liposome	EPG and DMPC	Intravenous	Age-related molecular degeneration
Cytarabine	Depocyt	Liposome	DOPC<comma> DPPG<comma> cholesterol and triolein	Spinal	Neoplastic meningitis and lymphomatous meningitis
Morphine sulfate	DepoDur	Liposome	DOPC<comma> DPPG<comma> cholesterol and triolein	Epidural	Pain
Vincristine sulfate	Marqibo	Liposome	Egg sphingomyelin and cholesterol	Intravenous	Acute lymphoblastic leukemia

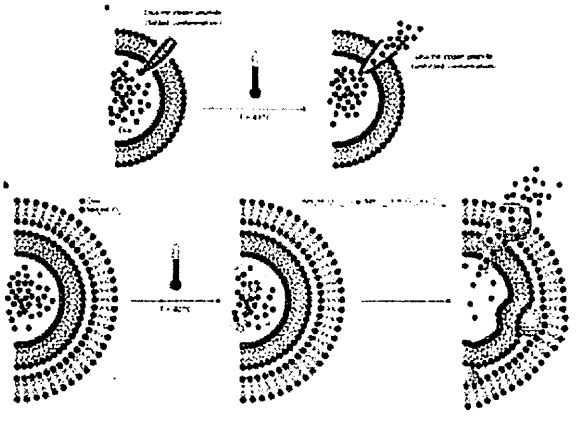
The addition of cholesterol in the gel phase will increase the fluidity, fill the gaps, and prevent the liposome from leaking.

Liposomes can be classified into different sizes. They can vary from sizes very small (0.025 μm) to large (2.5 μm) vesicles.[9] Liposomes can have one or bilayer membranes. The number of bilayers and the size of the liposome affect the amount of drug encapsulation in the liposomes. The vesicle size helps determine the circulation

half-life of liposomes. Other categories in which liposomes can be classified are multiamellar vesicles (MLV) and unilamellar vesicles. The unilamellar vesicles can go more in depth and can be classified as large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV).[10] The MLV have an onion structure, while unilamellar liposomes have a single phospholipid bilayer sphere enclosing the aqueous solution.

There is a temperature based actuation mechanism for liposomal drug delivery. The design of the nanoscale stimuli-responsive systems make them able to control drug biodistribution in response to specific stimuli.[17]

Figure 1: Temperature-based actuation mechanisms for liposomal drug delivery.



a, The temperature-triggered unfolding of a leucine zipper peptide inserted in the membrane of a doxorubicin (Dox)-carrying liposome opens a channel through which the drug is released. b, Drug-permeable pores can also be created by the...

Antioxidants such as vitamin E and Tempol are used in liposomes to protect them against oxidative damage during long-term storage.[3] Antioxidants also help protect liposomal membrane from radiation damage.[11]

The encapsulation efficiency of the liposome is heavily influenced by its lamellarity. The lamellarity also influences the efflux rate of the encapsulated material, as well as the fate of the drug after cellular intake.[12] The lamellarity of a liposome can be

determined by ^{31}P-NMR, but the result might not be correct depending on the experimental settings and the shape of the liposomes.[12] Other ways to determine the lamellarity of liposomes are trapped volume measurement, quasielastic light scattering (QELS) and freeze-fracture electron microscopy.[13]

A major obstacle in treating ocular diseases is accessibility of drugs due to the corneal barrier, aqueous barrier, and inner and outer blood-retinal barrier. The design of liposomes and nanoparticles seeks to improve penetration. Intravitreal injections of target liposomes has become a promising approach for ocular disease therapy.[15]

Cryoprotectants used in increasing the shelf life of liposomes include DMSO and glycerol. Ice crystals form as cells freeze and these crystals can puncture the plasma membrane, leading to the death of the cell. Cryoprotectants protect the cell by partially solubilizing the membrane of the cell so it is less likely to puncture and interrupting the crystal lattice of the cell so less crystals form.[16]

The fate of the liposome is to get degraded in the body so that the encapsulated drugs in the lipid and aqueous layer can be released. The presence of PEG on the liposomal carrier extends the blood-circulation time while decreasing the phagocyte system uptake (stealth liposome).[18]

The complement-dependent release of liposomal contents is one of the dominant factors in determining the biological fate of liposomes. Serum components that inhibit the phagocytosis of pathogens or particles, called dysopsonins, have been identified. Human serum albumin and IgA have dysopsonic properties. If the liposome is engineered with human serum albumin and IgA then the liposome won't be recognized by the body and so the phagocytosis of the liposome will not occur. This will reduce the chance of the

liposome being recognized and degraded by the body so the liposome will be able to reach its destination tissue.[19]

OBJECTIVE:

The objective is to design and evaluate the liposomal formulations to overcome disadvantages such as short half-life, prone to oxidation and hydrolysis, leakage and fusion in vitro.

METHODS:

A) Preparation of liposomes

- Liposomes prepared by lipid hydration method:

Fluorescein, a model dye, was mixed with different combinations of phospholipids and cholesterol to obtain a total lipid concentrations of 10 mM in chloroform- methanol mixture in ratio of 2:1. The mixture was rotovaped to get a thin film on the surface of the round bottom flask. The film was further flushed with argon for complete drying. Now, the lipid film was hydrated with phosphate buffer overnight. Finally the milky suspension was extruded using 0.4 and 0.2 μm polycarbonate filters. The final solution was lyophilized till further use.

- Liposomes prepared by reverse phase evaporation method:

Total lecithin- cholesterol mass of 200 mg was used in molar ratios of 7:2 and 7:4. Both the compounds were dissolved in 2:1 chloroform-methanol mixture. The solvents were evaporated off using a rotovap under vacuum at 40oC. The lipid film was re-dissolved in ether to produce reverse phase vesicles. 20 mg of drug was dissolved in acetone and 6 ml of PBS (pH 7.4). The system was sonicated for 4 min in a bath sonicator. The organic phase was then evaporated using a rotovap. The liposomes were allowed to equilibrate at

room temperature and then 10 ml PBS was added to liposome suspension, which was refrigerated overnight.

- Liposomes by Freeze thaw method

EPC, DPPE, DPPG and cholesterol were mixed at various molar ratios in a round bottom flask. To this, 2 ml of chloroform was added to make a uniform organic phase. The solvent was argon dried to get a uniform film of lipid layer. This lipid layer was hydrated using the drug solution in 0.32 M mannitol to get a final phospholipid concentration of 10 mg/ml. The dispersion was sonicated for 5 min in a bath sonicator. Now, the solution was frozen at -70°C for 30 min and then thawed to 40°C. The process was repeated at least 3 times and the final sample was lyophilized.

- Liposomes by ethanol destabilization method:

PC: cholesterol in the molar ration of 70:30 were dissolved in 0.4 ml ethanol and slowly injected into 0.6 ml of pH 4 citrate buffer. This mixture was extruded through 200 nm filters (3 passes). The protein solution was slowly added while vortexing. The ethanolic dispersion was incubated at 40°C for 1 hr, dialyzed for 2 hr against citrate buffer and twice against HBS to remove excess ethanol.

B) Separation of free drug:

Free unentrapped drug was separated from the liposomes by centrifugation at 17000 rpm for 1 hr at 4oC. The pellets formed were washed with distilled water twice and then re-suspended, centrifuged again for 1 hr.

C) Encapsulation efficiency:

An aliquot of liposomes was taken and treated with Triton X 100 to break the lipid layer and release the drug. The concentration of the drug released on adding the surfactant was

measured using fluorescence spectrophotometry.

$$\% \text{ drug encapsulated} = \frac{(F1-F2)}{(F3-F2)} \times 100$$

F1 – Intensity from liposome solution after treatment with Triton X

F2 – Intensity from liposome solution before treatment with Triton X

F3 – Intensity from drug solution

D) Particle size measurement using DLS:

The particle size was measured by Dynamic light scattering (DLS) on a Brookhaven BI-200SM Research Goniometer and Laser Light Scattering System (5mW He–Ne laser, λ=632 nm) using CONTIN software. Cumulant analysis was used to obtain the particle size distribution from the correlograms generated by the software. The temperature was fixed at 25 °C.

E) Characterization using Negative staining transmission electron microscopy:

Briefly, a drop of a water-diluted suspension of the liposomes (about 0.05 mg/mL) was placed on a 200-mesh formvar copper grid, allowed to adsorb and the surplus was removed by filter paper. A drop of 2% (w/v) aqueous solution of uranyl acetate was added and left in contact with the sample for 5 minutes. The surplus water was removed and the sample was dried at room conditions before the vesicles were imaged with a TEM operating at an acceleration voltage of 200 KV

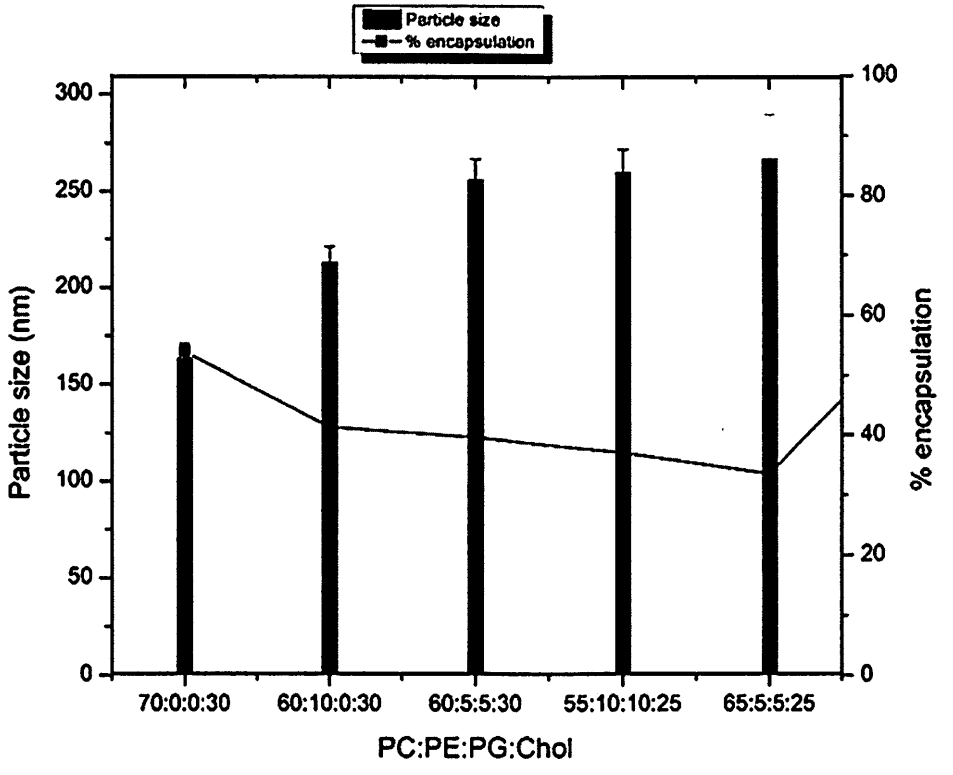
F) Drug release studies using liposome solutions:

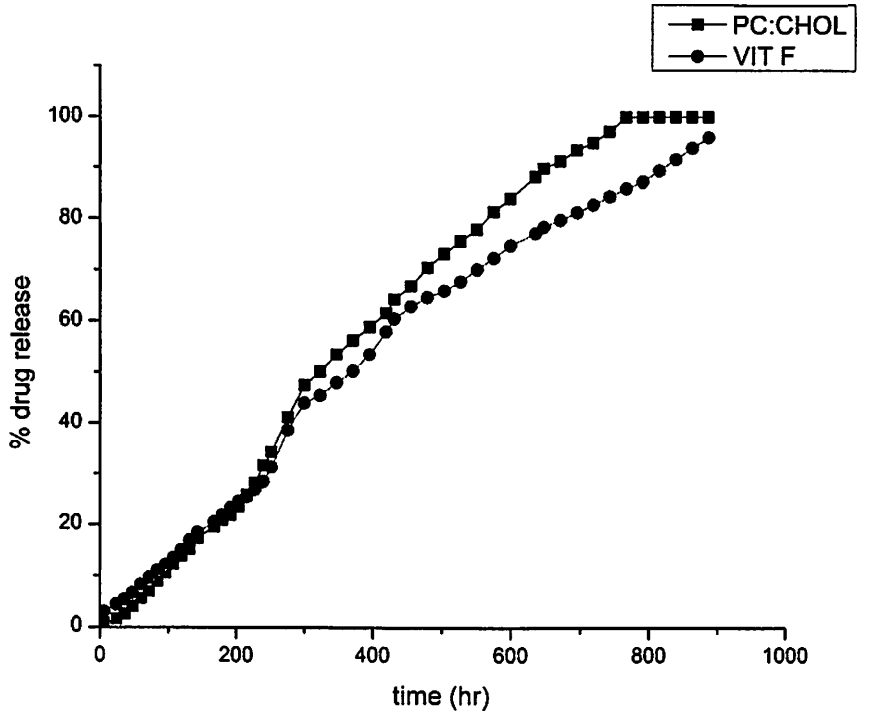
Drug release studies were performed using USP 4 dissolution apparatus (SOTAX Corporation). The flow rate was maintained at 0.5 ml/min. Aliquots were removed at regular intervals and the concentration of the drug released was measured using

fluorescence spectrophotometry.

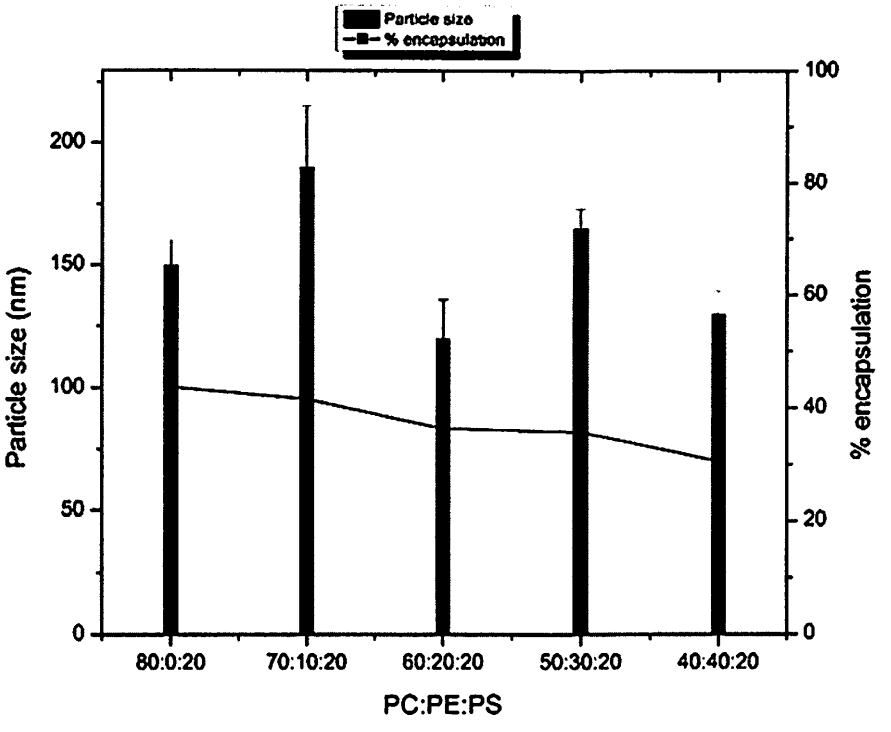
RESULTS:

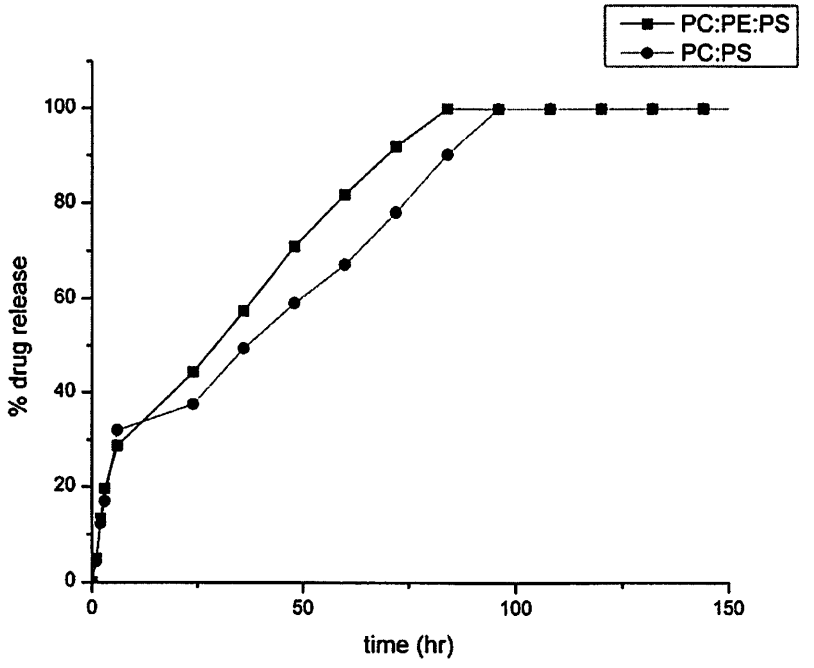
Lipid hydration and extrusion method:



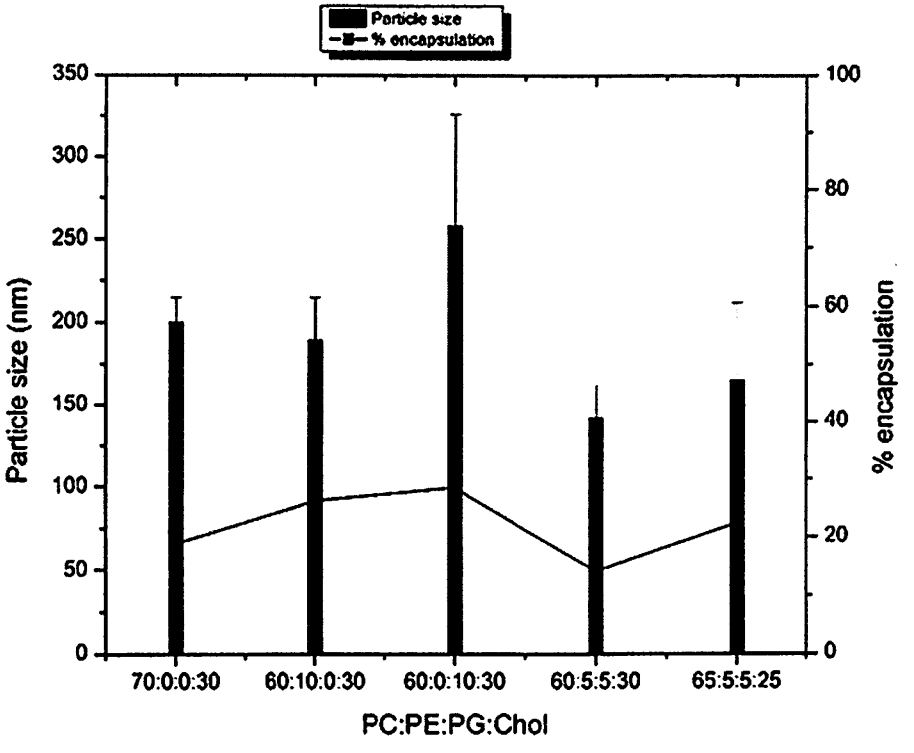


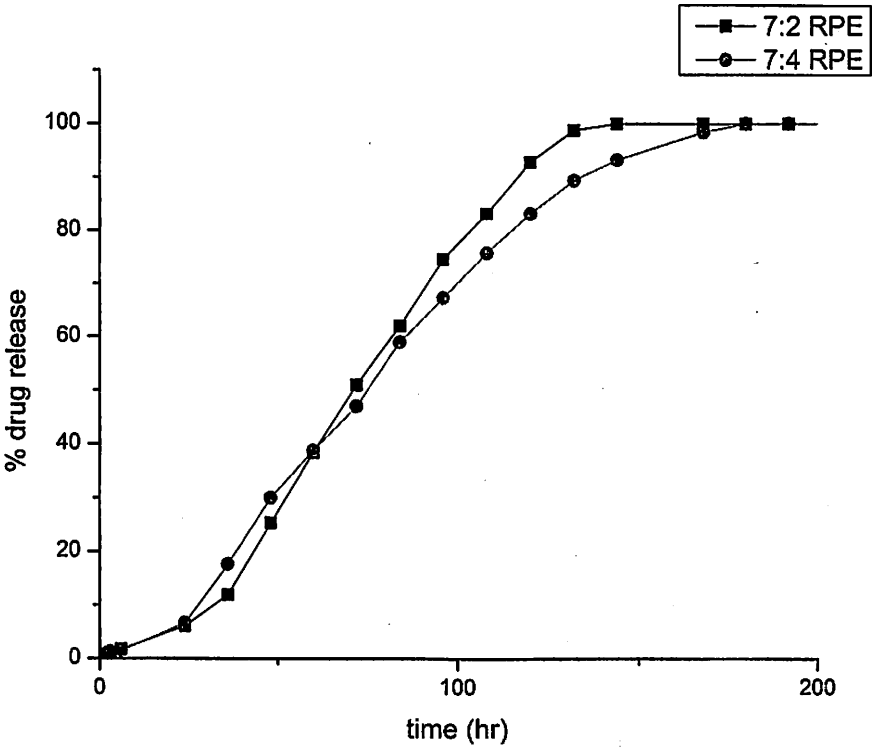
Sonication method:



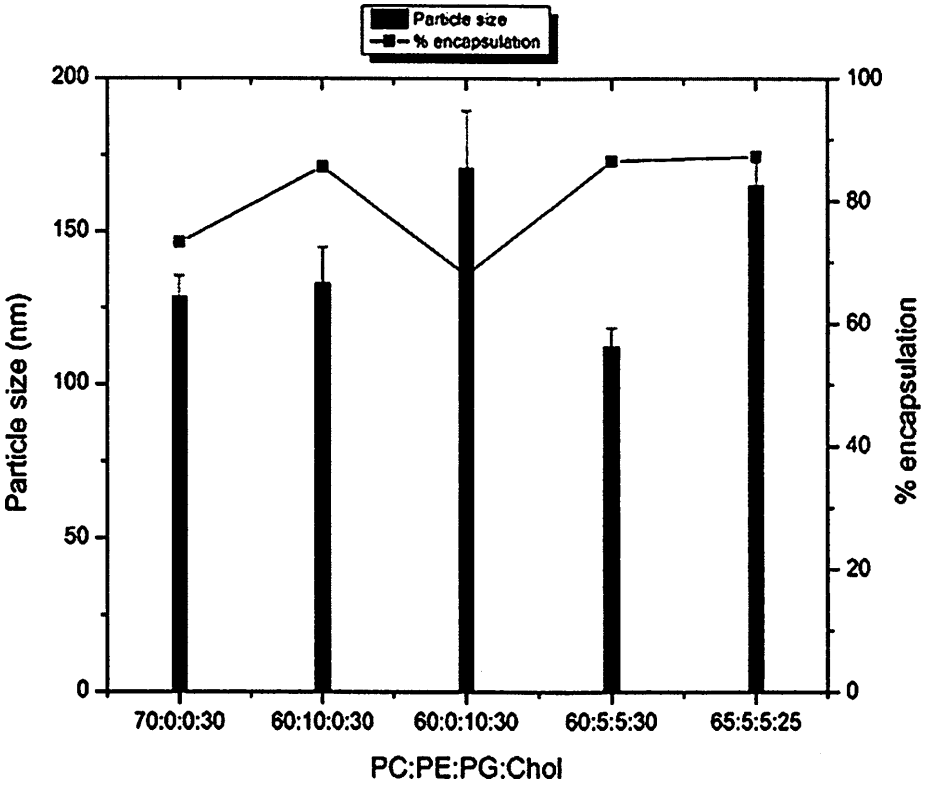


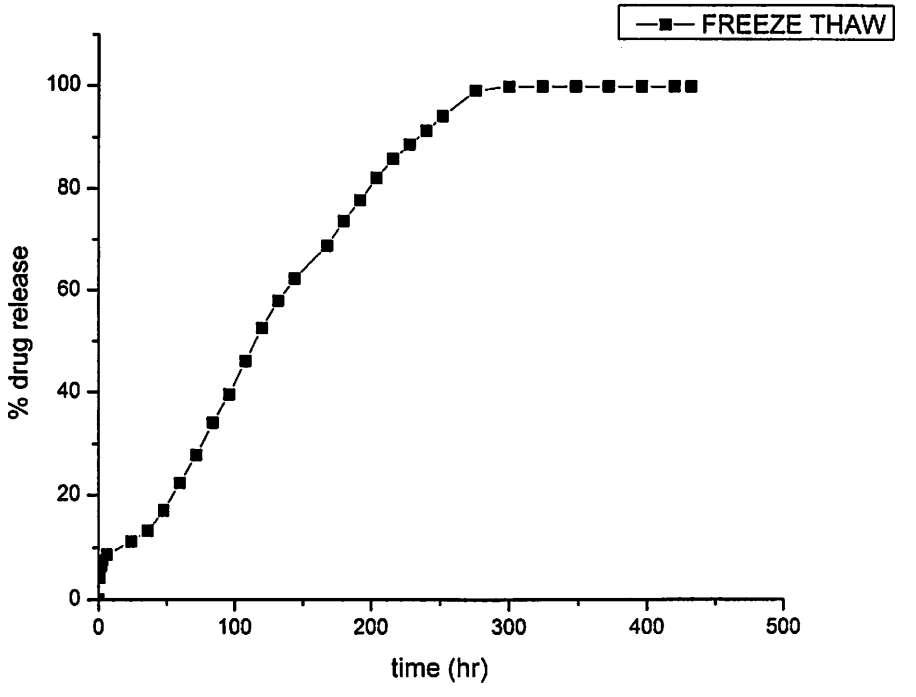
Reverse phase evaporation method:



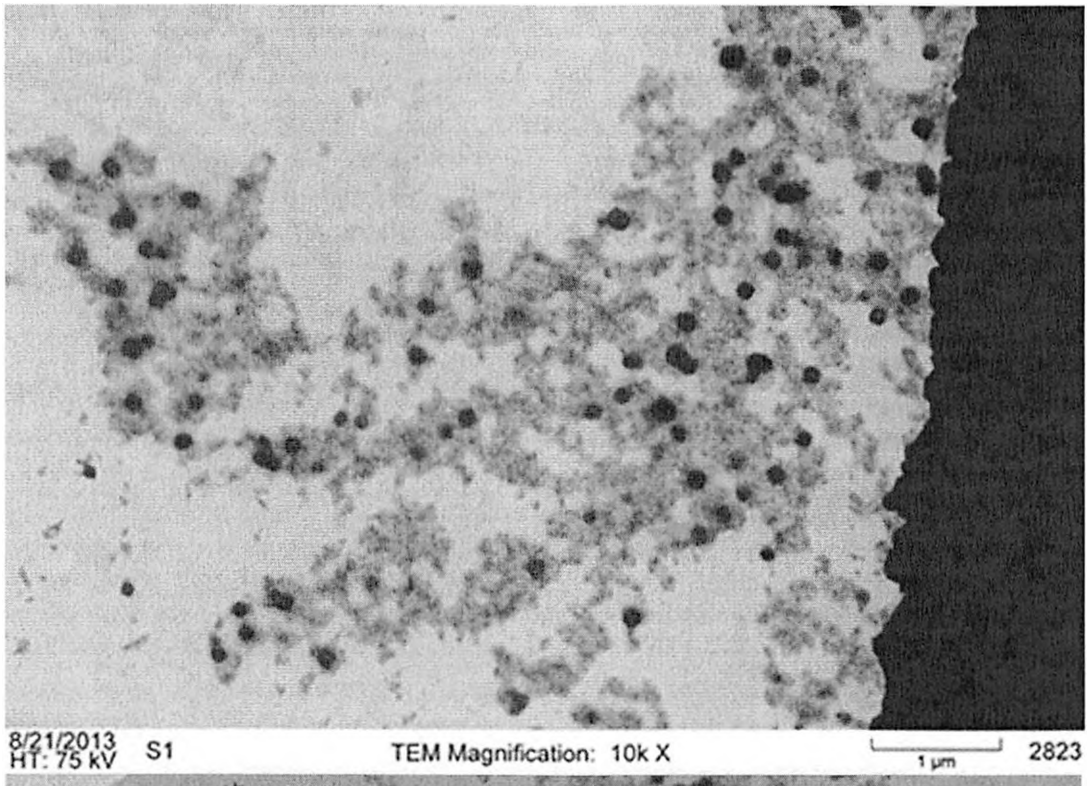


Freeze thaw method:





Electron microscopy images of the best formulations: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HT: 75 kV
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TEM Magnification: 10k X
1 µm
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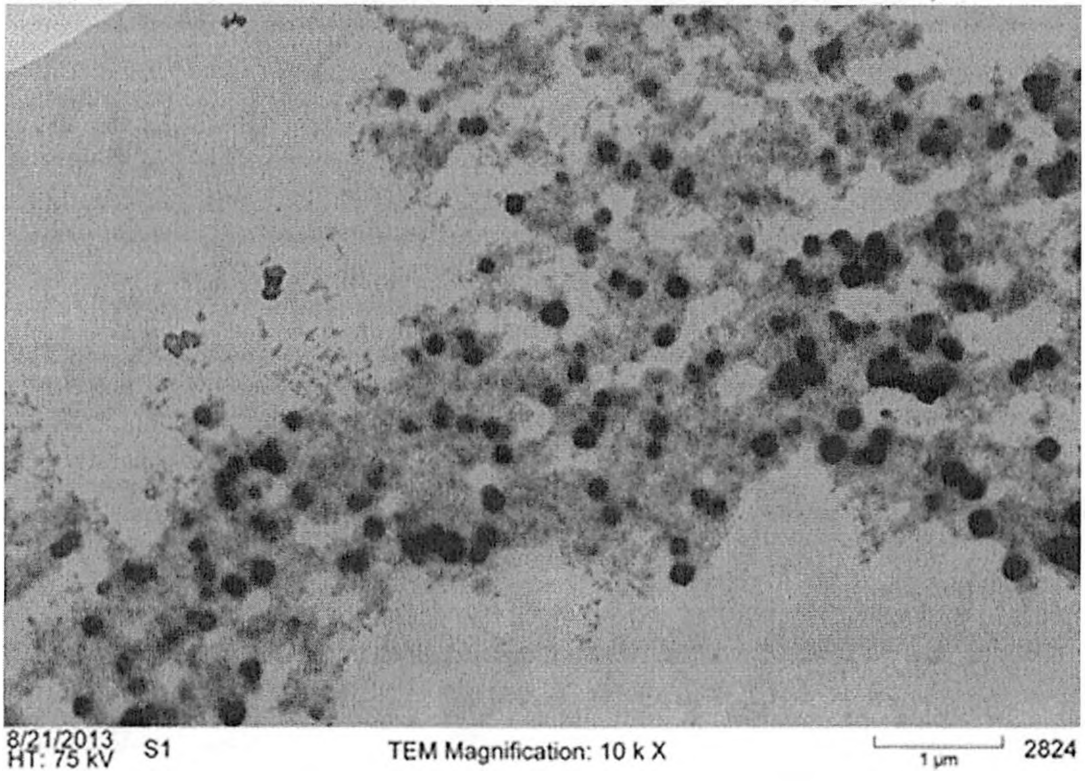
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HT: 75 kV
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TEM Magnification: 10 k X
1 µm
2824

From our preliminary studies, we found the best formulations. Lipid hydration and extrusion method gave moderate encapsulation and optimum particle size. But, the problem was that the liposomes start to fuse. In case of mannitol freeze thaw method, the particle size was small and also the liposomes showed very good encapsulation. The problem with this method is that the liposomes leak the solute at a very fast rate due to osmotic imbalance inside and outside the liposomes. So, we used a modified method where we combined both the lipid hydration and extrusion as well as the mannitol freeze thaw method. We were able to get good particle size as well as very good encapsulation. In the future, we are going to use this method for encapsulating drugs and studying their potential as ocular drug delivery systems.

DISCUSSION:

One of the major problems with liposomes is leakage and this was overcome by determining the appropriate concentration of cholesterol. Cholesterol makes the membrane less permeable by filling up holes or disruptions. Another major problem is stability and a very short shelf life. This can be overcome by freeze drying in the presence of a cryo-protectant. Different carbohydrates, such as mannitol and trehalose were considered as cryo-protective agents to inhibit liposomal fusion or degradation during freezing procedures. These agents also create an osmotic balance further decreasing the leakage of drug from the liposomes.

The applications for liposomes has been targeted drug delivery during which the goal is to prolong, localize, and target a protected drug with a diseased tissue. Advantages of this include a reduction in the frequency of dosage taken by the patient. Newer applications include delivery of dyes to textiles, pesticides to plants, nutritional

supplements to foods, and cosmetics to skin. With our technology, we have developed more stable liposomes which can be used for a sustained release over a longer period of time.

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