CHAPTER 3. FORMULATION AND DEVELOPMENT

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3. INTRODUCTION

Liposomes have been widely considered as potential drug delivery systems ever since the published observation of Bangham and co-workers [1]. Liposomes are colloidal vesicles ranging from few nanometers to several micrometers in diameter with one or more lipid bilayers surrounding aqueous compartments [2, 3]. They are prepared from natural or synthetic phospholipids and cholesterol, however, other lipids or derivatives can also be incorporated as needed [3, 4]. Liposomes are biodegradable and biocompatible, non-toxic, non-immunogenic [5]. They can entrap a wide variety of therapeutic drugs [6-8] and genetic material. Hydrophilic drugs can be entrapped in aqueous compartments of liposomes, whereas hydrophobic drugs incorporated in their lipid bilayer [5]. A few liposomal formulations are in clinical practice and some in preclinical trials.

Many methods are exist for preparing liposomes and loading them with foreign substances of interest, most of which methods involve forming the liposome vesicles within an aqueous carrier lipid containing said substances distributed therein. During liposome formation, a portion of said carrier liquid becomes entrapped within the vesicles, together of course, with a small amount of the desired substances to be encapsulated. This technique is called “passive entrapment”. The efficiency of loading liposomes with passively entrapped aqueous phases is often quite low because it strongly depends on the nature of the carrier phase and, practically, the concentration of the substance dissolved therein which may affect the yield of liposome formation. However, for drug delivery purpose, the loading efficiency (which is generally defined as the weight of material entrapped over the total weight of material involved in entrapment) is usually not critical because the non-entrapped material can generally be recovered and reused afterwards; hence, the important factor is rather the ratio of useful entrapped material versus the weight of the lipids used for entrapment, i.e., the lipids involved in forming the liposomes membrane.

The ratio of the weight of encapsulated material over the weight of encapsulating lipids is in direct relation with the so-called captured volume, i.e. the volume of the aqueous phase entrapped in the liposome core per weight of liposome lipids (µl/mg) [9]. In classical passive entrapment described by Bangham *et al.* (1965) [1] the aqueous phase containing the compound of interest is put into contact with a film of dried phospholipids deposited on the walls of a reaction vessel. Multilamellar vesicles (MLVs) are formed spontaneously when phospholipids are hydrated into aqueous solution with mechanical agitation due to swelling of the lipids. Large unilamellar vesicles (LUVs) can be prepared from MLVs, for example by extrusion. The most common way to manufacture small unilamellar vesicles (SUVs) is to sonicate MLVs with probe sonicator.

The captured volume of MLVs is low, typically near 2 to 4µl/mg of lipids. By sonication, the MLVs can be converted to small unilamellar vesicles whose captured volume is even smaller, e.g., near 0.5-14µl/mg. The Reverse Phase Evaporation (REV) method described by Szoka and Papahdjopoulos *et al.* (1978) [10] in which a solution of lipids in water insoluble organic solvent is emulsified in an aqueous carrier phase and the organic solvent is subsequently removed under reduced pressure gave liposomes with captured volume of 8 to 15µl/mg of lipids.

Improved passive entrapment has been achieved by subjecting liposomes to successive dehydration and rehydration treatment, or freezing and thawing; dehydration was carried out by evaporation or freeze-drying [11]. The other method described [12] wherein, liposomes were prepared by sonication are mixed in aqueous solution with the solute to be encapsulated. Further attempts to increase the amount of the drugs entrapped in liposomes by using higher concentrations thereof in the carrier liquid reduced the captured volume and had a detrimental effect on captured volumes.

In order to improve the anti-cancer drug delivery efficiency and to reduce the toxic effects associated with anticancer drugs various alternative dosage forms have been developed, such as microparticulate lipoidal vesicles (liposomes) [13, 14], cyclodextrins [15], polymeric nanoparticles [16], micelles [17], solid lipid nanoparticles (SLN) [18] and nanostructured lipid carriers (NLC) [19]. Among these forms, liposomes, NLC and SLN belong to lipid-based nanocarriers which have such favourable characteristics as: (a) improved drug dispersibility; (b) enhanced drug solubilisation; (c) enhanced drug transmembrane transport capability and (d) increased therapeutic efficacy and reduced toxicity. In the present study, liposomes composed of mixture of phospholipids (DPPC and DSPG), as combination of more than one lipid can increase the drug loading, were developed. We studied the effect of various process and formulation variables during the preparation of gemcitabine conventional liposomes and optimized them for maximum gemcitabine loading in minimum quantity of phospholipids.

The delivery of liposomes to the appropriate site, however, is still being investigated. For this purpose, both active targeting and passive targeting are considered. Conventional liposomes are tend to be trapped by the reticuloendothelial system (RES) such as liver and spleen before encountering the target. On the contrary, passive targeting, especially targeting to tumor tissues, could be achieved by reducing the RES trapping, since the vasculature in the tumor tissues is leaky enough to extravasate liposomes and circulating liposomes may accumulate passively in tumor tissues [20]. The development of liposomes containing lipid derivatives of PEG or saturated phospholipids such as DSPC with

cholesterol has made targeted liposomal therapy more feasible by reducing the uptake by the RES system and there by prolonging the circulation time [21].

Particularly, PEG is useful because of its ease of preparation, relatively low cost, controllability of molecular weight and linkability to lipids or proteins including the antibody by a variety of methods. The presence of PEG reduces binding of serum proteins, i.e. opsonins marking the liposome for clearance by macrophages.

As a polymer for in vivo use, it should exhibit certain minimum properties, such as biocompatibility, biodegradability, non-immunogenicity and non-toxicity. Besides these advantages, it can be obtained under GMP conditions and it is FDA approved. The major role to play for PEG in bioconjugation for pharmaceutical and biotechnological use are giving stealth effect to biomolecule or carrier systems by shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the RES, and preventing recognition and degradation by proteolytic enzymes, increased body residence time, modification of organ disposition, drug penetration by endocytosis and new possibilities of drug targeting. In addition to these properties, PEG facilitates conjugation by providing the functional groups required for conjugation. Now PEG derivatives are becoming available in a variety of activated and highly reactive end functional groups which need a minimum number of steps for conjugation. In a recent scenario more and more peptide and other macromolecules are delivered as a PEGylated form to overcome pharmacokinetic associated problems. Successful protein biopharmaceuticals include PEGylated interferons (PEGasys® and Intron®), PEGylated growth hormone receptor antagonist (Somavert®), PEGasparaginase (Oncospar®), adenosine deaminase (ADAGEN®), and granulocyte colony stimulating factor (Neulasta®) [22].

Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells. Several type of ligands have been used for this purpose, including antibodies or antibody fragments, vitamins, glycoproteins, peptides (RGD-sequences), and oligonucleotide aptamers. Among the different approaches of active targeting, liposomes conjugated with RGD peptide as a targeting ligand and a lipid vesicle as a carrier for both hydrophilic and hydrophobic drugs, is a fascinating prospect in cancer therapy. Arginine-glycine-aspartic (RGD), is a cell adhesion motif displayed on many extracellular matrix (ECM) and plasma proteins [23]. Since RGD was first identified as specific binding sites for fibronectin (FN) and the FN receptor [24]. RGD plays an important role in cell recognition and cell adhesion, it has been used into tumor therapy and tissue engineering by recombinant means and some chemical methods. Hynes *et al.* (1987) [25] had reported that the membrane proteins associated with ECM glycoprotein receptors on the cell surface were called integrins, which

were members of the adhesion receptors. The binding of integrins to theirs ligands were dependent on divalent cations to mediate cell-cell and cell-matrix adhesion. Thus, integrins constituted cell adhesion receptors not only for cell-matrix adhesion but also for signaling bidirectionally across the membrane. The large heterodimeric cell surface receptors-integrins were found in many animal species ranging from sponges to mammals [26]. They were involved in fundamental cellular processes such as attachment migration, proliferation, differentiation, and survival. Integrins also contributed to the initiation and progression of many biological diseases such as angiogenesis, thrombosis, inflammation, osteoporosis neoplasia, tumor metastasis and gene expression [27]. RGD-based ligands for integrins are studied in pathology and pharmacology. Furthermore, the RGD-integrin system is exploited to target cell recognition and internalization, which is applied to man-made constructs by mimicking the pathogens. This system enables the study of many aspects (such as diagnostics, therapeutics and the regenerating of transplanted tissue. RGD modified drugs and imaging agents have been investigated and developed by conjugation of the RGD-peptides with a carrier device. The carrier device has been equipped with drug molecules or reporter molecules. RGD-peptides and RGD-mimetics have also been applied to modify liposomes, polymers and peptides by chemical means to improve the biological effects of therapeutic agents. Additionally, RGD-peptides were utilized in gene delivery by viral and non-viral vectors [28]. The surface modification technology with fixed RGD peptides has promoted the application of integrin-mediated cell adhesion to develop tissue engineering, especially for biomaterials [29]. Zhang *et al.* (2010) [30] had prepared RGD-modified liposomes encapsulated with combretastatin A-4 (CA-4) and doxorubicin. He reported the release rate of Dox was proved to be much slower than that of CA-4 in vitro. Additionally, flow cytometry and laser confocal scanning microscopy clearly showed that RGD-modification promoted intracellular uptake of liposomal drugs by B16/B16F10 melanoma tumor cells and human umbilical vein endothelial cells (HUVECs). Cytotoxicity assay showed that the IC50 of RGD-modified liposomes was lower than that of the corresponding unmodified liposomes. Vyas, S. P *et al.* (2004) [31] had formulated the Cyclic RGD peptide anchored sterically stabilized liposomes (RGD-SL) for selective and preferential presentation of carrier contents at angiogenic endothelial cells overexpressing a_vb_3 integrins on and around tumor tissue and thus for assessing their targetabilty. Liposomes were prepared using distearoylphosphatidylcholine (DSPC), cholesterol and Distearoylphosphatidyl-ethanolamine-polyethyleneglycol–RGD peptide conjugate (DSPE–PEG–RGD). The in-vitro endothelial cell binding of liposomes exhibited 7-fold higher binding of RGDSL to HUVEC in comparison to the SL. Spontaneous lung metastasis and angiogenesis assays showed that RGD peptide anchored liposomes were significantly

effective in the prevention of lung metastasis and angiogenesis compared to free 5-FU and SL. In the tumor regression study carried out in B16F10 tumor bearing BALB/c mice showed that cyclic RGD peptide anchored sterically stabilized liposomes bearing 5-FU were significantly active against primary tumor and metastasis than the non-targeted sterically stabilized liposomes and free drug. Thus, the author reported cyclic RGD peptide anchored sterically stabilized liposomes as a potential carrier to target cancer chemotherapeutics.

Freeze-drying of nanocarriers is not an easy process and requires a comprehensive expertise and understanding of the process. However, one may find that most of papers published in this field studied the freeze-drying of nanocarriers by trial and error, i.e. by trying different conditions of freeze-drying and selecting the best after the analysis of freeze- dried product.

Freeze drying is a complex process because it consists of simultaneous heat and mass transfer. During the primary drying period, the sublimation kinetics are controlled either by heat transfer flux from the shelf and from the surrounding toward the ice sublimation front inside the vial or by water vapour mass transfer through the dried layer. Various methods have been developed to increase the heat and mass transfer rates during freeze drying. Out of these, the most common and effective method is the annealing of the frozen sample before freeze drying. The annealing of the sample leads to increase in the ice crystal size and its percentage distribution and hence increase in heat [32]. Recently, Daoussi *et al.* [33] have reported that the presence of organic solvents such as tertiary butanol in the formulation increases the primary drying rate as compared to the pure water- based system. Nevertheless, the freeze-drying process generates various stresses during freezing and drying steps. The freezing protocol and drying conditions have a significant impact on the quality parameters of the final product [34, 35].

It is now well known that various stages of lyophilization are based on very sound physical, chemical and engineering principles and can be controlled to the extent that the outcome of a given process performed on a given product can often be estimated to be within fairly close tolerance, without the need for trial-and-error experimentation [36]. Even more important, stable freeze-dried nanoparticles can be designed by matching an optimum nanoparticle formulation with its associated optimum drying process cycle. In order to design an optimum nanoparticles freeze-drying process, process development scientists need to know the critical properties of the optimized formulation and how to apply this information to process design. The critical formulation properties include the glass transition temperature of the frozen sample (Tg′), the collapse temperature of the formulation (Tc), the stability of the nanoparticles and their encapsulated drug, and also the properties of the excipients used. The collapse temperature is the maximum allowable

product temperature during primary drying [37]. Freeze-dried product loses macroscopic structure and collapses during primary drying if it is heated to above the temperature of collapse (Tc).

Freeze-drying as a drying method has many applications for nanoparticles technology. The literature contains many examples of such applications. The main use of freeze-drying is for improving long term nanoparticles stability. The transformation of colloidal suspension into solid form has the advantage of preventing particles aggregation, also the degradation of polymer forming nanocarriers and the leakage of encapsulated drug out of nanocarriers. Furthermore, freeze- drying could be transformed into another solid dosage form intended for different administration routes (parenteral, oral, nasal, or pulmonary).

In our research we used lipid derivative of PEG (DSPE-mPEG_{2000}) to provide stealth effect to the liposomes. In addition; we also grafted the RGD peptide to make the liposomes a functionalized nanocarriers for targeted drug delivery to the tumor site. The finally optimized formulation(s) was lyophilized to avoid the dug leakage and to impart the stability to the formulation. Thus, we hypothesize that the lyophilized PEGylated liposomes, as a carrier of anticancer drug, conjugated with RGD peptide, as a targeting ligand, will have the following benefits.

1. Avoid macrophages uptake and increase the blood circulation time leading to passive accumulation of drug loaded liposomes in solid tumors.
2. Kill angiogenic blood vessels and, indirectly, the tumor cells that these vessels support
3. Penetrate into the tumor interstitial space and function as a sustained release system, resulting in direct cancer cell kill, including cytotoxicity against cells that are at the tumor periphery and are independent of the tumor vasculature.

Thus, this combined strategy has the potential to overcome some major limitations of conventional chemotherapy.

3.1 Materials and Methods

Gemcitabine Hydrochloride (GEM) (HPLC purity >99%) was a gift from Sun Pharma, Vadodara, India. Hydrogenated Soya Phosphatidylcholine (HSPC), Dipalmitoyal phosphotidylcholine (DPPC), Disteroyal phosphotydalglyserole DSPG), Dipalmitoyal phosphotydalglyserol (DPPG), Disteroyal phosphotydalethanol amine megloyal polyethylene glycol (DSPE-mPEG_{2000}) and Cholesterol (Chol) were obtained as gift sample from Lipoid GmbH, Germany. All other chemicals and solvents used were of analytical grade (S.D Fine Chemicals, Mumbai, India) and were confirmed for purity before use.

3.1.1 Preparation of liposomes by thin film hydration method

Various types of formulations containing different lipids as described in **Table 3.1** were developed. All formulations were optimized based on entrapment efficiency of liposomes. Liposomes were prepared by thin film hydration method [38] and described below.

Table 3.1 Various Liposomal Formulations with Their Composition

Batch no.	Liposome	Composition
GEM-4	HSPC	HSPC : Cholesterol
GEM-5		HSPC : DPPG : cholesterol
GEM-6		HSPC : DSPG : cholesterol
GEM-7	DPPC	DPPC : Cholesterol
GEM-8		DPPC: DPPG : cholesterol
GEM-9		DPPC : DSPG: cholesterol
GEM-10	DMPC	DMPC : Cholesterol
GEM-11		DMPC : DPPG : cholesterol
GEM-12		DMPC : DSPG : cholesterol

The liposomes composed of various lipid composition as given in **Table 3.1** and anticancer drug gemcitabine HCl were prepared by Thin Film Hydration Technique as described by Bangham *et al.* (1965) [1].

Formation of Thin Lipid Film: The lipid phase composed of various lipid composition as given in **Table 3.1** were weighed accurately and dissolved in chloroform and methanol solvent mixture (2:1 ratio, v/v). The round bottom flask was then attached to the rotary evaporator (BUCHI Rotavapor R-200), evacuated and rotated at 100 rpm in a thermo stated water bath maintained at 45±2 °C. The process was allowed to continue until all the solvent had evaporated and a dry thin lipid film had deposited on the walls of the flask. The flask was rotated under vacuum for additional 30 minutes after the dry residue was first appeared. Subsequently, the flask was kept overnight under vacuum to remove the residual solvents.

Hydration of Lipid Film: The dry lipid film was hydrated with GEM solution (5mg/mL) in double distilled water (3mL) at above glass transition temperature (50±1 ^{0}C) for 60 minutes.

Liposome Size Reduction: Particle size of liposomes was reduced using successively passing through 1, 0.4, 0.2 and 0.1 µm polycarbonate membranes (Whatman, USA) using high-pressure extruder (Avestin, USA). Polyethylene drain disk (Whatman, USA) was used to support the polycarbonate membrane and hence to potentiate the extrusion process. Prepared liposomal dispersion was used to determine the average mean particle size, and

zeta potential. The unused liposomes were stored in a glass container at 2-8°C till further processing.

GEM liposomes dispersion were added to the centrifuge tube and centrifuged at 70000 x g for 4 hr at 4°C to separate the unloaded drug. The supernatant was analysed to calculate the % of unentrapped gemcitabine content. Pellet was vortexed with sufficient quantity of methanol and was subsequently spun at 14,000 rpm at 4°C for 10 min to calculate the % of entrapped gemcitabine content.

3.2 Formulation Optimization of Gemcitabine liposomes

GEM liposomal formulations were optimized using 3^3 full factorial design by varying the Drug: lipid molar ratio (1:5, 1:10 and 1:15), DSPG molar % (1, 2 and 3) and hydration volume (1.5mL, 3mL and 5mL) at three different levels such as low (-1) middle (0) and high (1) (Table 3.2) for higher drug content and lower particle size by keeping all other process. The prepared batches were evaluated for drug content and particle size, as dependent variables, Table 3.2 summarizes the experimental runs and the factor combinations employed, along with the translation of their coded levels into the units used in the study.

Table 3.2 Independent variables and their corresponding levels

Variables	**Levels**		
	-1	**0**	**1**
X1 (Drug: Lipid Ratio)	1:5	1:10	1:15
X2 (DSPG level mol %)	1	2	3
X3 (Hydration Volume)	1.5 ml	3 ml	5 ml

3.2.1 Optimization of Process and Formulation Variables

In above given methods, various process and formulation parameters were involved and all were optimized to achieve best suited formulation for GEM incorporation.

3.2.2 Optimization of Cholesterol levels

At optimized formulation and process variables the liposomes containing different concentrations of cholesterol were prepared to determine the effect of cholesterol concentration on mean particle size and % drug content and loaded drug retention character. Liposomal formulations prepared with different concentrations of cholesterol were transferred to nitrogen purged screw capped glass vials (in triplicate) and stored at 2-8^0C for a period of two weeks. The liposomal dispersion analysed for mean particle size.

3.2.3 Preparation of PEGylated and Functionalised (RGD Grafting) liposomes

Optimal formulation containing DPPC, DSPG and cholesterol was further improved by incorporation of (1mol%, 3mol% and 5mol %) mPEG2000-DSPE (PEGylated) and tumor cells targeting cyclic RGD peptide-polymer conjugate; (1mol%, 3mol% and 5mol %) RGD-mPEG2000-DSPE (Functionalised) was also added along with above listed lipids in the initial phase during thin film formation to incorporate mPEG2000-DSPE and RGD into the liposomes. Prepared PEGylated and functionalised liposomes were evaluated for mean particle size and zeta potential and % GEM content.

3.2.4 Lyophilization of GEM Liposomes

Prepared liposomes were lyophilized to impart physical stability to the liposomes. Various types of cryoprotectants are used at different ratio to optimize the lyophilization and to preserve particle size during freeze drying. Liposomes were diluted with water, containing optimized amount of cryoprotectant, upto 1.0 mL and filled into the 2 mL glass vial (Schott, USA) having 13 mm neck diameter. Vials were half stoppered with grey bromo butyl slotted rubber stoppers (Helvoet, Belgium) and kept on the shelf of lyophilizer (Virtis-Advantage plus, USA). Liposomes were freezed upto -40°C and dried under vacuum for next 44 hr. Complete lyocycle describing freezing time, primary and secondary drying time, ramp (R) and hold (H) duration, vacuum level are given below in **Table 3.3**.

Table 3.3 Thermal Cycling for Lyophilization of formulation

Thermal Treatment					
	Temperature (^{0}C)	Time (min)	R/H		Temperature (^{0}C)
Step 1	5	40	H	Freeze	-40
Step 2	-40	100	R	Additional Freeze	0
Step 3	-40	300	H	Condenser	-40
Step 4	0	0	H	Vacuum	200
Step 5	0	0	H		
Step 6	0	0	H		
Step 7	0	0	H		
Step 8	0	0	H		
Step 9	0	0	H		
Step 10	0	0	H		
Step 11	0	0	H		
Step 12	0	0	H		

Primary Drying				
	Temperature (^{0}C)	Time (min)	Vacuum	R/H
Step 1	-40	60	200	H
Step 2	-30	50	100	R
Step 3	-30	100	100	H
Step 4	-20	50	100	R
Step 5	-20	300	100	H
Step 6	-10	60	100	R
Step 7	-10	600	100	H
Step 8	10	200	100	R
Step 9	10	600	100	H
Step 10	20	50	100	R
Step 11	20	200	100	H
Step 12	0	0	0	H
Post Heat	25	900	200	

3.2.5 Entrapment Efficiency in Liposomes

Gemcitabine entrapped within the liposomes was estimated after removing the un-entraped drug by centrifugation at 70000 x g for 4 hr at 4°C. The supernatant was analysed to calculate the % of unentrapped gemcitabine content. The settled pellet of liposomes was treated with chloroform: methanol (2:1) to extract the loaded drug and lipids, suitably diluted and analysed using UV-visible spectrophotometer (Shimadzu 1601, Japan) at 268 nm against blank (methanol) containing lipid concentration similar to formulations tested). The concentration of drug was calculated from the standard calibration curve. The percentage gemcitabine loaded in the liposomes was determined using the following formula.

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Quantity of drug encapsulated}}{\text{Total quantity of drug added}} \times 100$$

3.2.6 Particle Size and Zeta Potential Analysis

The mean particle size (z-average) and polydispersity index (PDI) of the liposomes were analysed by photon correlation spectroscopy (PCS) using Malvern Zetasizer Nano (NanoZS, Malvern Instruments, UK). 0.2mL of liposomal suspension was diluted to 2.0mL with distilled water and measured after equilibration time of 2 minutes. The Zetasizer Nano is

operating with a 4mW He-Ne-Laser at 633nm and non-invasive back-scatter technique (NIBS) at a constant temperature of 25 ^{0}C. The measurements were conducted in the manual mode using 20 sub runs of 10 seconds. The size distribution by intensity and volume was calculated from the correlation function using the multiple narrow mode of the Dispersion Technology Software version 4.0 (Malvern, Herrenberg, Germany). Thereby, the resulting size distributions show the hydrodynamic diameter. The average particle size and PDI was calculated after performing the experiments in triplicate. The PDI of 0.0 represents a homogenous particle population while 1.0 indicates a heterogeneous size distribution of liposomes.

The zeta potential of the liposomal suspensions prepared was measured by microelectrophoresis using Malvern Zetasizer NanoZS (Malvern Instrument, U.K.). 0.2mL of the liposomal suspension was diluted to 2.0mL with distilled water for zeta potential analysis. Zetasizer NanoZS offers the highest ever sensitivity, accuracy and resolution of zeta potential. The instrument works on the principal of Brownian motion and measured the light by Phase Analysis Light Scattering (PALS). The zeta potential was measured at 25 ^{0}C using standard sample cell. The measurements were performed in triplicate.

3.2.7 Residual Water Content

The residual water content of lyophilized liposomes was determined by Karl-Fischer titration [39]. Commercially available pyridine free reagent was used for analysis. The reagent was standardized with addition and determination of known quantity of water (250 mg). Firstly, 40 mL of methanol was added into the titration vessel and titrated with the reagent to determine the amount of water present in the samples. Following this, samples were added and water content was determined.

3.2.8 Cryo-Transmission Electron Microscopy (Cryo-TEM)

Morphology, lamellarity and size of the liposomes was studied using Cryo-TEM (TECNAI G2 Spirit BioTWIN, FEI – Netherlands) operating at 200 kV with resolution of 0.27 nm and magnifications of the order of 750,000X. In order to perform Cryo-TEM observations, hydrophobic carbon grid was initially converted to hydrophilic nature by using Glow Discharge (Emitech K100X, Quoram Technologies, UK), on which 10 µl of liposomal suspension was evenly dispersed and the sample along with grid was cryo-freezed in Liquid Ethane at –180^{o}C. Cryo-freezed grid was transferred to cryo-holder maintained at -175^{o}C using Liquid Nitrogen storage box. The cryo-holder was then inserted in the microscope for imaging the sample. Combination of bright field imaging at increasing

magnification and of diffraction modes was used to reveal the form, lamellarity and globule size of the liposomes.

3.2.9 Statistical Analysis

All the experiments were performed in triplicates unless otherwise specified. Statistical analysis of data was performed using an ANOVA and Student-t test. GraphPad Prism (version 5, USA) was used for all analyses and P value < 0.05 was considered significant.

3.3 Results & Discussion

3.3.1 Selection of method for preparation of Gemcitabine HCl loaded liposomes

The Gemcitabine HCl loaded liposomes were prepared by the conventional methods which were reported in the literature for liposomes preparation. The drug loading was initially tried with both Active as well as Passive loading techniques. The details of the method was given below. In order to select the drug loading and liposomes preparation method primary HSPC and CH were used as a lipids in liposome preparation. The method selection was based on the entrapment efficiency in the liposomes. The methods tried were as below.

a) Passive loading by Thin Film Hydration Method.
b) Passive Loading by Ethanol Injection Method.
c) Active Loading (pH gradient) by Ethanol Injection Method.

3.3.1.1 Passive loading by Thin Film Hydration Method

Procedure: Multilamellar vesicles comprising HSPC: Cholesterol (7:3 molar ratio) with entrapped Gemcitabine HCl were prepared by Thin Film hydration (TFH) technique. The lipids were dissolved in a mixture of chloroform and methanol (2:1) in a 100 ml round bottom flask. The solvent was evaporated in the rotary flask evaporator under vacuum. The thin dry lipid film thus formed was hydrated using distilled water containing required amount of Gemcitabine HCl (5mg/mL) at 58 ± 3°C i.e. above phase transition temperature of lipid (Tg).

3.3.1.2 Passive Loading by Ethanol Injection Method

The lipids HSPC: Cholesterol (7:3 molar ratio) were dissolved into the minimum amount of the ethanol and injected into the preheated (58 ± 3°C) required amount of Gemcitabine HCl solution (5mg/mL)and allow them for hydration for 90 minute.

The liposomal dispersion prepared by above two methods was subjected to size reduction. The extruded liposomes were then allowed to stand undisturbed for about 60 min for

annealing and subjected to removal of the unentrapped drug and to calculate entrapment efficiency (EE).

3.3.1.3 Active Loading (pH gradient) by Ethanol Injection Method.

The lipids HSPC: Cholesterol (7:3 molar ratio) were dissolved into the minimum amount of the ethanol and injected into the preheated (58 ± 3°C) 250 mM of Ammonium sulphat solution adjust the pH of hydration medium at 2 with 0.1 N HCl. The liposomal dispersion prepared by was subjected to size reduction. The extruded liposomes were then allowed to stand undisturbed for about 60 min for annealing. Then external Ammonium sulphat was removed by diffusion against 10% sucrose solution for overnight. The drug loading was performed by hydrating the liposome suspension at 58 ± 3°C for 3hr at pH 6 (adjusted with 0.1 N NaOH) with distilled water containing required amount gemcitabine HCl (5mg/mL) and subjected to removal of the unentrapped drug and to calculate percent drug entrapment (PDE).

Table 3.4 Selection of method for preparation of liposomes

Batch No.	**Different methods**	**PDE ± SD (n=3)**
GEM-1	**Passive loading by thin film hydration method**	**40.50 ± 0.21**
GEM-2	Passive loading by ethanol injection method	32.20 ± 0.26
GEM-3	Active loading by ethanol injection method	33.66 ± 0.35

The drug entrapment by thin film hydration and ethanol injection was found to be 40.50 ± 0.21 and 32.20 ± 0.26 respectively (Table 3.4). However, the PDE for active and passive drug loading was 32.20 ± 0.26 and 33.66 ± 0.35 respectively (Table 3.4). Amongst the methods tried for liposome preparation the PDE for the thin film hydration with passive drug loading was higher. The improved drug entrapment attributed to CH may be because of improved stability of liposomal membrane during hydration [40].

However, the active drug loading didn't showed any significant improvement in PDE. Hence, based on the results of PDE, prevalence, ease of handling and processing time, the passive loading by thin film hydration was employed for further formulation development and optimization.

3.3.2 Preparation of liposomes by thin film hydration method

3.3.2.1 Optimization of the process parameter

Process parameter optimization such as vacuum conditions for dry film formation, hydration time, and speed of rotation of flask were optimized for desired results. The effect of one variable was studied at a time keeping other variables constant. From the results following conclusions were drawn:

Vacuum Applied: The vacuum required for solvent evaporation to form a uniform thin film was raised from 400 mm Hg to 650 mm Hg. The low vacuum (400 mm Hg) was found to be insufficient for the complete removal of the solvent. The presence of residual solvent may lead to physical destabilization of liposomes by interfering with the co-operative hydrophobic interactions among the phospholipid methylene groups that hold the structure together [41]. The vacuum of 600 mm of Hg for 60 min was found to be optimum for complete evaporation of solvent and producing more translucent and thin lipid film. However, for complete solvent removal of residual solvent (post film formation) the flask was purged with nitrogen for 4 hr. higher vacuum (650 mm Hg) resulted in rapid evaporation of the solvent system leading to crystallization and hence resulted in poor orientation of liposomes. This was in agreement with the findings of Martin et al (1990)[41] that differential solubility of amphiphilic components of bilayer and drug in organic solvents were often encountered and must be taken into consideration in order to avoid crystallization of a single component during solvent-stripping operations.

Speed of rotation: The speed of rotation of flask was increased from 50 rpm to 150 rpm. Rotation of 50 rpm resulted in thick incompletely dried film and presence of residual solvents. While at 150 rpm speed, a dry film with varying thickness was produced with a thicker film at periphery and thinner film at the center. A speed of 100 rpm was found to be adequate to give thin, uniform and completely dry film. Hence, 100 rpm speed of rotation of flask was selected to be optimum for liposomal preparations.

Hydration time: The lipid film was hydrated from 30 min to 2 hr before size reduction. An optimal hydration time was required for complete conversion of planner bilayers to spherical liposomes. Lower hydration time led to a non-uniform shape and size of the liposomes and also the un-hydrated part posed difficulty in size reduction. The hydration time beyond 1 hr resulted in no further improvement. Hence, 1 hr hydration time was found to be optimum for all preparations.

Table 3.5 Selection of Process Parameters for Liposomes Preparation

COMPOSITION OF SOLVENT SYSTEM	
Solvent	***Observation***
Chloroform: Methanol (2:1)	Suitable
SOLVENT EVAPORATION TIME	
Time (min)	***Observation***
45 min	Not proper hydration
60 min	Suitable (Solvent is completely removed)
90 min	No further improvement
SPEED OF ROTATION	
Time (min)	***Observation***
45 min	Not proper hydration
60 min	Suitable (Solvent is completely removed)
90 min	No further improvement
HYDRATION TIME	
Time (min)	***Observation***
30 min	Not properly hydrated
60 min	Suitable hydration
90 min	No further improvement but decrease in PDE
VACUUM APPLIED	
vacuum (mm of Hg)	***Observation***
400	Flecking during hydration
500	Flecking during hydration
600	Uniform film and uniform liposomal dispersion
650	Un-uniform film

3.3.3 Formulation Optimization

3.3.3.1 Optimization of the lipid composition

The liposomes composed of various lipids were used to encapsulate drug. The lipids such as HSPC, DPPC and DMPC were primary lipid for liposomes preparation and gemcitabine HCl encapsulation. Combinations of lipids were tried to encapsulate gemcitabine HCl as given in **Table 3.6.** Optimization of the lipid composition for Gemcitabine HCl loaded liposome were done on the basis of drug entrapment. Different lipid compositions were tried and optimized for maximum drug entrapment within minimum amount of lipid. The

liposomes were prepared by using the thin flim hydration method. The liposomes were prepared at drug:lipid ratio of 1: 10 and the drug loading were done at 5mg/mL concentration. The cholesterol were used due to its membrane rigidizing capacity [42, 43]. All the process perameters were constant for all batches.

The drug entrapment were found to be highest for DPPC based liposomes followed by HSPC and DMPC based liposomes. In addition, the drug entrapment for the liposomes composed of combination of DSPG with HSPC, DPPC and DMPC based liposomes were also higher than the combination of DPPG with HSPC, DPPC and DMPC based lipid structures **(Table 3.6)**. Overall, amongst the various lipid combination tried the PDE of the liposomes composed of DPPC and DSPG were found to be the highest.

Table 3.6 Selection of the lipids

Batch no	Liposome	Composition	PDE ± SD (n=3)
GEM-4	HSPC	HSPC : Cholesterol (7:3)	40.50 ± 0.21
GEM-5		HSPC : DPPG : cholesterol (6:2:2)	43.05±0.32
GEM-6		HSPC : DSPG : cholesterol (6:2:2)	55.41±0.15
GEM-7	DPPC	DPPC : Cholesterol (7:3)	48.12±0.30
GEM-8		DPPC: DPPG : cholesterol (6:2:2)	50.59±0.41
GEM-9		**DPPC : DSPG: cholesterol (6:2:2)**	**62.06±0.52**
GEM-10	DMPC	DMPC : Cholesterol (7:3)	30.54±0.51
GEM-11		DMPC : DPPG : cholesterol (6:2:2)	34.14±0.22
GEM-12		DMPC : DSPG : cholesterol (6:2:2)	48.01±0.89

Phospholipid was selected as trapping efficiency of liposomes increases with increase in fatty acid carbon chain length from C_{12} (Dilauroyl Phosphatidylcholine) to C_{16} (Dipalmitoyl phosphatidylcholine). DPPC is a natural phospholipid and is most common components of biological membranes. DPPC has low transition temperature (Tc) of 41 ^{0}C at physiological pH. Liposomes composed of DPPC results in fusion of liposomes with cell plasma membrane which may further lead to endocytosis by the cell having the endocytic activity [44]. The use of more than one phospholipid in the preparation of liposomes will increase the drug loading and liposomes stabilization [45, 46]. Hence, we selected DSPG phospholipids in combination with DPPC. The use DSPG, negatively charged phospholipid, will enhance the suspension stability by inducing negative charge over liposomes and it also reduces the liposomal size to nanometer. In our experiments hydration of phospholipid was carried out using double distilled water, instead of any buffer, because the presence of ionic solute in the hydration media can interact with negatively charged

liposome and might alter the physical character of liposomes (increase in particle size and decrease in zeta potential) during preparation and storage. Indu Javeri et al. (2013) [47] invented DTX liposomes composed of sodium oleate, L-α-phosphatidyl choline (soya), and containing no cholesterol. They prepared liposomes loaded with DTX (5mg/mL) and having size less than 100nm. The presence of cholesterol has one of the most important roles in the maintenance of membrane bilayer stability and long circulation time *in vivo* [48-50]. In the absence of cholesterol, conventional liposomes are destabilized by high density lipoprotein (HDL) particles [51, 52] and release their components, which upon readily eliminated from the circulation. Hence we used cholesterol as one of the major components of the liposomes. The chemical and *in vivo* stability of liposomes prepared with saturated phospholipids was more as compared to liposomes prepared with unsaturated phospholipids [53]. The phospholipid component also plays a prominent role in the maintenance of high plasma levels of liposomes. DPPC/Cholesterol have higher $T_{1/2}$ values in the circulation compared with more fluid liposomes containing unsaturated eggPC [50]. To be most effective, the phosphatidyl choline component must have a phase transition that is significantly above 37°C. The gel-to-liquid-crystalline phase transition (T_g) for eggPC is below 37°C, whereas DPPC has a T_g value of only a few degrees above body temperature (42°C). Thus, at 37°C, DPPC containing liposomes have a considerably more rigid membrane bilayer that resists penetration of serum opsonins than do eggPC containing formulations. It is no surprise, then, that these liposomes tend to be the most stable in the circulation and display the longest circulation lifetimes. Hence, in the present study the further liposomal formulation development were done using combination of DPPC and DSPG as these phospholipids have T_g above body temperature and also this combination would enhance the gemcitabine loading.

Using 3^3 factorial design as shown in **Table 3.7**, 27 batches of GEM loaded liposomes were prepared varying three independent variables. The % GEM content and mean particle size are recorded as dependent variable in **Table 3.7**. Our objective of this factorial design was to optimize basic parameters like drug to lipid ratio, DSPG ratio and hydration volume. The batches were prepared at all the optimized process variables given in **Table 3.7.** The increase in drug: lipid ratio shows increase in drug loading and mean particle size. At 1:10 drug to lipid ratio we observed maximum GEM loading and minimum particle size. With increase in drug: lipid ratio beyond this level (i.e. 1:15) we observed similar amount of drug loading but increased mean particle size than 1:10. Hence, we considered drug to lipid ratio of 1:10 as optimal condition. The DSPG level was optimized (2 mol %) for maximum drug content by preparing liposomes at different levels of DSPG by varying the DPPC level while keeping cholesterol concentration as constant. The decrease in GEM loading was observed

when the DSPG ratio was 1 mol % as compared to 2 and 3 mol %. There was no significant improvement in the drug content was observed at 2 and 3 mol % DSPG levels. This might be due to high concentration of DSPG (having T_g of 55 ^{0}C) and that might require hydration and annealing temperature more than what we used (50±2^{0}C) as compared to DPPC having T_g of just 42^{0}C. The hydration volume of 3-5mL was considered as optimal. We observed increase in GEM loading and decrease in mean particle size with increase in hydration volume from 1.5mL to 5mL and in between 3-5mL we observed similar results. The decrease in GEM loading and increase in mean particle size at hydration volume of 1.5mL might be due to insufficient water which was unable to completely hydrate the total phospholipids. As expected, the presence of negatively charged phospholipid (DPPG) decreased the size during size reduction to nanometer and also enhanced the suspension stability at 4 ^{0}C.

The % GEM loading of 62.06 ± 1.52% and mean particle size of 126±3nm (PDI: 0.242±0.022) was observed at drug to lipid ration of 1:10, DSPG level of 2 mol% and hydration volume of 3mL.

Table 3.7 3^3 Full factorial design consisting of experiments for the study of three experimental factors in coded and actual levels with experimental results

Formulation	Actual value variables			Response value	
	X1	X2	X3	% Drug content	Mean particle size with PDI
GEML-1	1:5	1	1.5 mL	30.61±2.41	113±3(0.380±0.085)
GEML -2	1:5	1	3 mL	41.81±1.44	103±2(0.326±0.062)
GEML -3	1:5	1	5 mL	43.72±1.38	99±1(0.339±0.044)
GEML -4	1:5	2	1.5 mL	28.63±3.22	101±5(0.247±0.011)
GEML-5	1:5	2	3 mL	36.75±2.31	108±3(0.243±0.009)
GEML-6	1:5	2	5 mL	37.7±1.15	106±2(0.257±0.019)
GEML-7	1:5	3	1.5 mL	26.87±3.21	113±5(0.316±0.044)
GEML-8	1:5	3	3 mL	33.11±2.51	108±3(0.303±0.019)
GEML-9	1:5	3	5 mL	32.66±2.40	110±2(0.321±0.022)
GEML-10	1:10	1	1.5 mL	41.42±2.60	99±2(0.235±0.008)
GEML-11	1:10	1	3 mL	55.77±2.40	104±1(0.258±0.015)
GEML-12	1:10	1	5 mL	57.42±1.61	103±2(0.282±0.013)
GEML-13	1:10	2	1.5 mL	57.31±4.73	118±3(0.291±0.012)
GEML-14	**1:10**	**2**	**3 mL**	**62.06±1.52**	**126±3(0.242±0.022)**
GEML-15	1:10	2	5 mL	64.01±3.31	104±2(0.277±0.022)
GEML-16	1:10	3	1.5 mL	58.16±4.55	115±1(0.248±0.006)
GEML-17	1:10	3	3 mL	62.12±3.71	109±3(0.257±0.014)
GEML-18	1:10	3	5 mL	61.11±2.91	105±2(0.261±0.014)
GEML-19	1:15	1	1.5 mL	50.83±1.62	118±6(0.414±0.011)
GEML-20	1:15	1	3 mL	51.84±1.48	125±3(0.332±0.019)
GEML-21	1:15	1	5 mL	52.01±2.62	128±5(0.424±0.011)
GEML-22	1:15	2	1.5 mL	54.04±5.11	130±3(0.251±0.021)
GEML-23	1:15	2	3 mL	55.08±1.20	134±3(0.261±0.018)
GEML-24	1:15	2	5 mL	56.21±1.65	138±2(0.367±0.014)
GEML-25	1:15	3	1.5 mL	58.41±2.41	151±8(0.461±0.031)
GEML-26	1:15	3	3 mL	60.50±1.42	163±5(0.381±0.021)
GEML-27	1:15	3	5 mL	60.40±1.80	168±3(0.341±0.018)

Values are Mean±SD, n=3. GEML: Gemcitabine Liposomes

3.3.3.2 Optimization of drug loading level

Different drug loading levels at optimized drug : lipid ratio (1:10) were tried (**Table 3.8)** and optimized. The drug loading levels from 5mg/ml to 10mg/ml were tried and optimized for maximum drug entrapment. The drug level was optimized (5 mg/ml) for maximum drug content by preparing liposomes at different levels of drug loading at optimized drug: lipid ratio. There was no significant improvement was observed in the drug content at 5mg/ml - 10mg/ml drug loading (**Table 3.8)**. This might be due to the saturation in the drug loading capacity of the lipid vesicles which did not allowed further entrapment of the drug solution. In addition, at the higher drug loading drug content was not improved much whereas the process ability was reduced as the liposomal gel was formed. This physical change might be due to higher solid content in the formulation.

Table 3.8 Optimization of drug loading level

Optimized Drug : Lipid ratio	Different Drug loading level	PDE ± SD (n=3)
1:10	**5mg/ml**	**62.06±0.52**
	7mg/ml	63.21 ± 0.45
	10mg/ml	Liposome gel is formed

3.3.3.3 Optimization of cholesterol concentration

GEM liposomes containing different concentrations of cholesterol **(Table 3.9)** were prepared and analysed for % GEM loading, mean particle size. The maximum % GEM loading (62.06 ± 1.52%) and minimum mean particle size (126±3nm) was observed at cholesterol concentration of 1 mol%. As the cholesterol concentration was increased from 1 mol% to 4 mol% the decrease in % GEM loading and increase in mean particle size was observed.

After 2 weeks of storage at 2-8 °C all formulations were analysed for mean particle size and % GEM content in order to determine the effect of cholesterol concentration on % GEM and mean particle size retention behaviour of liposomes. The formulation with lowest cholesterol content (1 mol%) having lowest mean particle size and highest % drug content showed more leakage of loaded drug and increased mean particle size as compared to other formulations. This increased leakage might be attributed to the low membrane rigidity at low cholesterol content [43, 54]. Similarly, the formulation with highest cholesterol content (4 mol%) having highest mean particle size and lowest GEM content also failed to retain the same like formulation with lowest cholesterol concentration. This

might be due to increased rigidity of bilayer membrane with increased cholesterol concentration that leads to more leakage of drug from the liposomes. At cholesterol concentration of 2 mol%, we observed about 62.06±1.52 of % GEM loading and mean particle size of 126±3nm at day 1. After 2 weeks of storage at 2-8 °C the drug content of this formulation was found to be maximum (56.81±2.20%) and mean particle size was minimum (150±5nm) as compared to other formulations. Hence, the cholesterol concentration of 2 mol% was considered optimal because at this cholesterol concentration the formulation showed minimum drug leakage (10±2%) as compared to other concentrations.

Table 3.9 Effect of cholesterol concentration on % drug loading and mean particle size

Formulation	Cholesterol concentration (mol %)	Formulations at day 1		Formulations after 2 weeks	
		% Drug Content	Mean size with PDI	% Drug content	Mean size with PDI
GEML -14.1	1	61.26±2.04	107±4nm (0.259±0.008)	38.31±3.85	183±8nm (0.368±0.016)
GEML -14.2	**2**	**62.06±1.52**	**126±3nm (0.242±0.022)**	**56.81±2.20**	**150±5nm (0.429±0.045)**
GEML -14.3	3	58.61±1.80	140±6nm (0.275±0.024)	35.85±2.86	190±2nm (0.288±0.011)
GEML - 14.4	4	54.20±2.41	155±2nm (0.346±0.022)	33.88±4.31	215±4nm (0.383±0.021)

Values are Mean±SD, n=3. PDI: Polydispersity Index

3.3.3.4 Preparation of PEGylated and Functionalized liposomes

The PEGylated liposomes were prepared using DSPE-mPEG$_{2000}$ at different mol% by pre-insertion technique. The mean particle size and % GEM content of formulation were analysed and represented in **Table 3.10**. Upon increasing the DSPE-mPEG$_{2000}$ and DSPE-mPEG$_{2000}$ –RGD concentration the GEM loading increases slightly but not significantly. This might be due to increase in total lipid content after inclusion of DSPE-mPEG$_{2000}$ and DSPE-mPEG$_{2000}$ –RGD. Although the mean particle size remains unchanged at 1 and 3 mol%, we observed increased in mean particle size at 5mol%. The zeta potential of prepared PEGylated and functionalized liposomes were found to be slightly increased (-55.2±5.2mV) as compared to conventional liposomes (-43.6±4.9mV). The DSPE-mPEG$_{2000}$ and DSPE-mPEG$_{2000}$–RGD concentration on the liposome surface was optimized based on *in vitro* studies.

Table 3.10 Effect of DSPE-mPEG$_{2000}$ and DSPE-mPEG$_{2000}$ –RGD concentration on % drug content and mean particle size

DSPE-mPEG$_{2000}$ Concentration	% GEM Content	MPS(nm) with PDI
CLs	62.06±1.52	126±3 (0.242±0.022)
PLs-1 mol %	63.24±4.43	126±2 (0.262±0.0070)
PLs-3 mol %	65.10±2.43	131±1 (0.249±0.0240)
PLs-5 mol %	66.35±2.63	142±6 (0.228±0.0403)
PLs-RGD-1 mol %	60.24±2.10	128±5 (0.247±0.0500)
PLs--RGD 3 mol %	59.10±3.04	135±4 (0.262±0.0350)
PLs--RGD 5 mol %	63.35±1.54	140±2 (0.217±0.0430)

Values are Mean±SD, n=3. GEM: Gemcitabine; MPS: Mean particle size; CLs: conventional liposomes; PLs: PEGylated liposomes.

3.3.3.5 Lyophilization of GEM Liposomes

The liposomal formulations were stabilized by lyophilization. As liposomes are prone to increase in size with complexation and hence, on aging also size may increase. Thus, to provide physical stability lyophilization was carried out. Different cryoprotectants at various concentrations were added to the liposomal dispersion. The role of Cryoprotectant was to act as a bulking agent and hence, to provide physical structure to the lyophilized cake and secondly to preserve the particle size of the liposomes during thermal treatment i.e. freezing step of lyophilization. During freezing there is an ice formation from water molecules and this ice may rupture the morphology of prepared liposomes. Cryoprotectant helps to stabilize the system by providing the protection against developed local effects during freezing and also prevent increase in local concentration of the precipitated solid during freezing. These all collectively stabilize the nanomaterial in its much possible original form. The lyophilized formulations were tested for particle size, zeta potential and physical appearance. Lactose, mannitol and sucrose were used at three different concentrations i.e. 20 g/mL, 40 mg/mL, 60 mg/mL in the final formulation (RGD- liposomes (3%). Results for the lyophilization optimization are summarised in **Table 3.11.**

Table 3.11 Optimization of Lyophilization

Cryoprotectants	Concentration (mg/mL)	Before lyophilization		After lyophilization	
		particle size (nm)	Zeta potential (mV)	particle size (nm)	Zeta potential (mV)
Lactose	20	135±4	-55.2±5.2	280±7	-55.32±1.25
Sucrose				176±6	-53.84±0.60
Mannitol				242±10	-52.47±0.78
Lactose	40			244±7	-55.52±0.55
Sucrose				152±8	-52.03±0.25
Mannitol				230±9	-53.26±0.91
Lactose	60			269±7	-54.32±1.27
Sucrose				158±6	-51.64±0.79
Mannitol				220±9	-51.59±1.18

Cryoprotectants	Concentration (mg/mL)	Lyophilized cake Integrity	Reconstitution time	Water Content (%w/w)
Lactose	20	Poor	25	1.11±0.10
Sucrose		Poor	30	1.23±0.05
Mannitol		Poor	25	1.41±0.04
Lactose	40	Good	30	1.32±0.04
Sucrose		Good	35	1.78±0.03
Mannitol		Good	40	1.85±0.09
Lactose	60	Good	60	3.14±0.04
Sucrose		Good	75	3.20±0.14
Mannitol		Good	65	3.15±0.11

3.3.3.6 Effect on Particle size and zeta potential

Lactose and mannitol did not preserve the particle size of liposomes. At all concentration these two sugars failed to maintain the particle size below 200 nm. Sucrose did perform the task by maintaining the size of liposomes at 40 mg/mL and 60 mg/mL concentrations. At

all concentrations the maintenance of particle size by these cryoprotectants followed below given order: Lactose < Mannitol < Sucrose. At all concentrations of used cryoprotectants, the zeta potential value did not change significantly. This result suggest the liposomal dispersion stability and hence preservation particle aggregation after lyophilization.

3.3.3.7 Physical Integrity and Re-dispersion

Lower concentration of cryoprotectant i.e. 20 mg/mL did not form physical good cake. Lyophilized material was not in an intact form and poor quality of cake was formed. However, at higher concentrations, 40 mg/mL and 60 mg/mL, this problem was solved. Less than 40 sec were required to reconstitute the lyophilized formulations with all types of cryoprotectant. However, at higher concentration 60 mg/mL, more than 60 sec were required for the reconstitution. Water content at 20 mg/mL and 40 mg/mL concentrations were found below 2% w/w. As sucrose preserved the particle size within narrow range as compared to non-lyophilized liposomes, the concentration having minimum particle size, good cake property and good redispersion property was selected. Taking collectively these results, 40 mg/mL of sucrose as a cryoprotectant was chosen.

3.3.3.8 Entrapment Efficiency

All formulations were subjected to study entrapment of gemcitabine encapsulated within liposomes. Optimized formulations were also subjected to ultracentrifuge method to determine GEM entrapment by direct analysis of liposomal fraction only, because free drug was removed from the supernatant after centrifugation. Results are summarized in **Table 3.12.** Maximum around 65% of entrapment was achieved in all the optimized formulations. RGD grafting did not affect the entrapment efficacy and difference between with and without RGD grafting was insignificant.

Table 3.12 Drug Content of various formulation

Formulations	% GEM Content
CLs	62.06±1.52
PLs	65.10±2.43
PLs-RGD-1 mol %	60.24±2.10
PLs--RGD 3 mol %	59.10±3.04
PLs--RGD 5 mol %	63.35±1.54

Values are Mean±SD, n=3. GEM: Gemcitabine; MPS: Mean particle size; CLs: conventional liposomes; PLs: PEGylated liposomes.

3.3.3.9 Particle Size and Zeta Potential Analysis

Particle size of the liposomes was mainly dependent on the rigidity of liposomes. The particle size ($p<0.05$) of CLs was drastically increased after pegaylation and due to attachment of the RGD on the surface of the liposomes. However there was no significant effect found on Zeta potential after pegaylation and RGD grafting. **Table 3.13** describe change in particle size and zeta potential of developed liposomes. **Figure 3.1** and **Figure 3.2** show particle size and zeta potential reports of the RGD-grated optimized liposomes (3%) batch.

Table 3.13 Mean particle size and zeta potential various liposomal formulations

Formulations	MPS(nm)	Zeta potential (mV)
CLs	126±3	-55.32±1.25
PLs	131±1	-53.84±0.60
PLs-RGD-1 mol %	128±5	-52.47±0.78
PLs--RGD 3 mol %	135±4	-55.52±0.55
PLs--RGD 5 mol %	140±2	-52.03±0.25

Values are Mean±SD, n=3. GEM: Gemcitabine; MPS: Mean particle size; CLs: conventional liposomes; PLs: PEGylated liposomes.

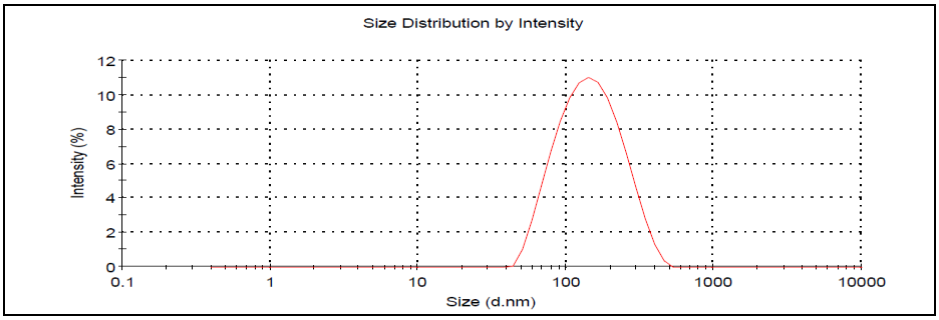

Figure 3.1 Particle size reports of the RGD-grated optimized liposomes

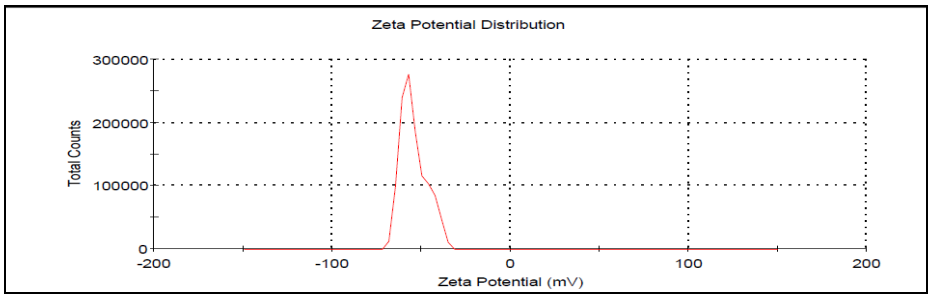

Figure 3.2 Zeta potential reports of the RGD-grated optimized liposomes

3.3.3.10 Residual Water Content

Water content was well characterised by Karl fisher titration method. All lyophilized samples such as conventional, peylated and RGD-grafted liposomes were found to contain below 2% of moisture after lyophilization. Residual water contents of lyophilized formulations are given below in **Table 3.14.**

Table 3.14 Residual Water Content of Various Lyophilized Products

Formulations	% Water Content
CLs	1.51±0.18
PLs	1.43±0.09
PLs-RGD-1 mol %	1.41±0.03
PLs--RGD 3 mol %	1.32±0.09
PLs--RGD 5 mol %	1.58±0.13

Values are Mean±SD, n=3. GEM: Gemcitabine; MPS: Mean particle size; CLs: conventional liposomes; PLs: PEGylated liposomes.

3.3.3.11 Transmission Electron Microscopy

Images obtained by TEM revealed that prepared liposomes are spherical in shape as shown in **Figure 3.3**. All vesicles are unilamellar in structure and having particle size below 200 nm. This range can also help in EPR effect for tumor internalization of nano materials [55]. Bilayer thickness was also measured and found to be in-between 5-10 nm in size.

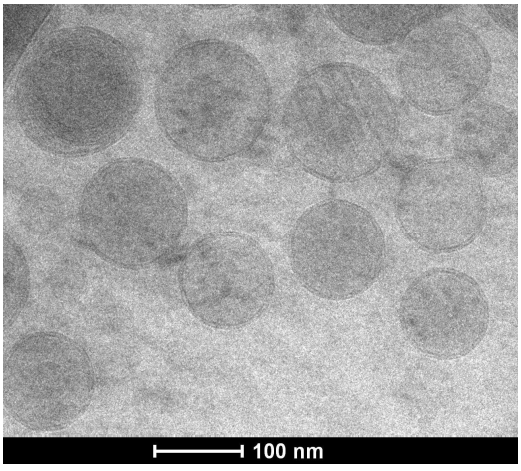

Figure 3 3 CryoTEM image of RGD grafted optimized liposomes.

3.4 Conclusion

Amongst the methods tried for liposome preparation the PDE for the thin film hydration with passive drug loading was higher. The improved drug entrapment attributed to CH may be because of improved stability of liposomal membrane during hydration. However, the active drug loading didn't showed any significant improvement in PDE. Hence, based on the results of PDE, prevalence, ease of handling and processing time, the passive loading by thin film hydration was employed for further formulation development and optimization.

Process parameter optimization such as vacuum conditions for dry film formation, hydration time, and speed of rotation of flask were optimized for desired results. The vacuum of 600 mm of Hg for 60 min was found to be optimum for complete evaporation of solvent and producing more translucent and thin lipid film. However, for complete solvent removal of residual solvent (post film formation) the flask was purged with nitrogen for 4 hr. higher vacuum (650 mm Hg) resulted in rapid evaporation of the solvent system leading to crystallization and hence resulted in poor orientation of liposomes. A speed of 100 rpm was found to be adequate to give thin, uniform and completely dry film. Hence, 100 rpm speed of rotation of flask was selected to be optimum for liposomal preparations. The hydration time beyond 1 hr resulted in no further improvement. Hence, 1 hr hydration time was found to be optimum for all preparations. Optimization of the lipid composition for Gemcitabine HCl loaded liposome were done on the basis of drug entrapment. Different lipid compositions were tried and optimized for maximum drug entrapment within minimum amount of lipid. The liposomes were prepared by using the thin flim hydration method.

The drug entrapment were found to be highest for DPPC based liposomes followed by HSPC and DMPC based liposomes. In the present study the further liposomal formulation development were done using combination of DPPC and DSPG as these phospholipids have T_g above body temperature and also this combination would enhance the gemcitabine loading. As expected, the presence of negatively charged phospholipid (DPPG) decreased the size during size reduction to nanometer and also enhanced the suspension stability at 4 ^{0}C. The drug loading levels from 5mg/ml to 10mg/ml were tried and optimized for maximum drug entrapment. The maximum % GEM loading (62.06 ± 1.52%) and minimum mean particle size (126±3nm) was observed at cholesterol concentration of 1 mol%. As the cholesterol concentration was increased from 1 mol% to 4 mol% the decrease in % GEM loading and increase in mean particle size was observed. The DSPE-mPEG$_{2000}$ and DSPE-mPEG$_{2000}$–RGD concentration on the liposome surface was optimized based on *in vitro* studies.

Result suggest the liposomal dispersion stability and hence preservation particle aggregation after lyophilisation. Taking collectively these results, 40 mg/mL of sucrose as a cryoprotectant was chosen.

Maximum around 65% of entrapment was achieved in all the optimized formulations. RGD grafting did not affect the entrapment efficacy and difference between with and without RGD grafting was insignificant. The particle size ($p<0.05$) of CLs was drastically increased after pegaylation and due to attachment of the RGD on the surface of the liposomes. However there was no significant effect found on Zeta potential after pegaylation and RGD grafting. All vesicles are unilamellar in structure and having particle size below 200 nm. This range can also help in EPR effect for tumor internalization of nano materials.

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