INTERFACIAL KINETIC AND MECHANISTIC STUDIES ON DENDRIMER-LIPOSOME INTERACTIONS

A Thesis submitted to the University of North Bengal

For the award of
Doctor of Philosophy
in Chemistry

By
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M. Sc. in Chemistry

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March 2018

DECLARATION

I declare that the thesis entitled "**INTERFACIAL KINETIC AND MECHANISTIC STUDIES ON DENDRIMER-LIPOSOME INTERACTIONS**" has been prepared by me under the guidance of Dr. Amiya Kumar Panda.

No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that **Mr. Biplab Roy** has prepared the thesis entitled "**INTERFACIAL KINETIC AND MECHANISTIC STUDIES ON DENDRIMER-LIPOSOME INTERACTIONS**", for the award of PhD degree from the University of North Bengal, under my guidance. He has carried out the work at the Department of Chemistry, University of North Bengal.

No parts of this thesis have formed the basis for the award of any degree or fellowship previously.

Amiya Kumar Panda

Date:

URKUND

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Instances where selected sources appear:

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Signature of the Supervisor

Signature of the HOD, Chemistry

Dedicated

to

My Parents

&

Brothers

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This thesis is the upshot of a long laborious passage in which I have been encouraged and maintained by many peoples who actually make my dream possible. It is really pleasure moment for me to express my full gratitude for them.

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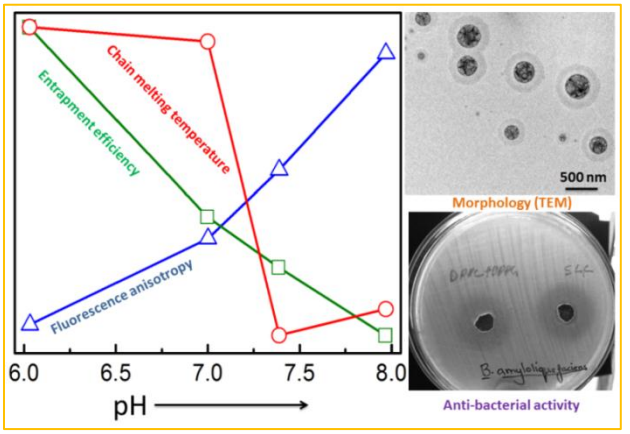
ABSTRACT

The research work auspicious in this thesis entitled "**INTERFACIAL, KINETIC AND MECHANISTIC STUDIES ON DENDRIMER-LIPOSOME INTERACTIONS**" is primarily focused on the two different important aspects; one is to prepare stable liposomes and to study the different kinds of physicochemical properties of these types of liposome and another is to study the interaction with these types of stable liposomes with different generation of PAMAM dendrimer. The first part of the thesis is completely focused on to prepare the stable liposome and investigate the effects of pH, temperature and lipid composition on various physicochemical properties of liposomes. Antibacterial activities were assessed using a gram positive bacteria *Bacillus amyloliquefaciens*. A second part of this thesis is devoted to the interaction of different generations of PAMAM dendrimers with the lipid bilayer interface. The lipid anionic membranes (liposomes) were used to model bio membranes, whereby the compositions of the liposomes were varied together with the chemical nature of the anionic lipids (in combination with DPPC). The type and strength of the interaction was dependent on charge and size of the liposomes as well as the dendrimer generation. The impact of dendrimer concentration and generation on four different kinds of liposomes was investigated using a combination of various physicochemical properties. Finally the dendrimer-liposome complexes, also known as dendriosomes, were explored in terms of its toxicity in healthy human blood cell lymphocyte as well as human red blood cells. Although there are different reports on the interaction studies between dendrimers and liposomes, to the best of the knowledge, no comprehensive and systematic studies have been carried out previously in order to assess the impact of dendrimer generation, concentration as well as the variation of the liposome type.

Chapter I describes about the effects of pH, temperature and lipid composition on various physicochemical properties of liposomes. Four different liposomes with soy phosphatidylcholine (SPC), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidyl glycerol (DPPG) as well as a 7:3 (M/M) mixture of DPPC+DPPG alongwith 30 mol% cholesterol were studied for this purpose. Antibacterial activities were assessed using a gram positive bacteria *Bacillus amyloliquefaciens*. Although there are scattered reports on the effect of pH on physicochemical studies on liposomes, however, no comprehensive studies have been made to understand the

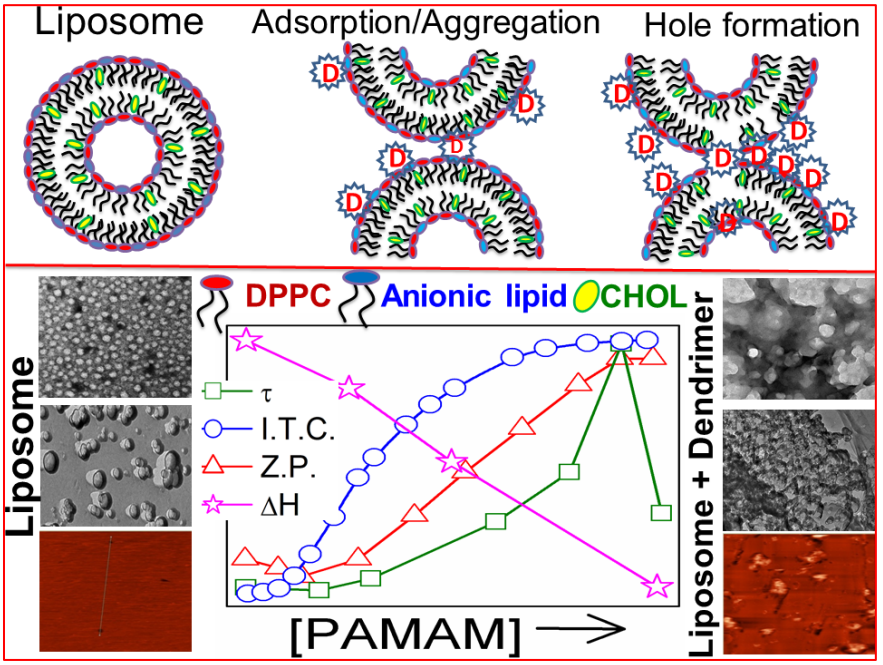
combined effect of pH as well as the charge on liposomes. Besides, exploration on the curcumin loaded liposome as potential antibacterial agent is not so common in the literature.



Chapter II reported the interaction of different generation poly(amidoamine) (PAMAM) dendrimers and combinations of liposomes. Second, fourth and sixth (2G, 4G, and 6G) generation PAMAM dendrimers were used, which are cationic under normal conditions. Liposomes comprised of soy lecithin + cholesterol (SLC+CHOL) (negative surface charge), DPPC+CHOL (positive surface charge), DPPG+CHOL (negative) and a biologically simulated mixture of DPPC + DPPG (7:3) + CHOL (negative) were used as model bilayers. Silica was used as a negatively charged hard sphere model to make a comparative study. Absorbance (turbidity) at 420 nm, dynamic light scattering, zeta potential measurements on liposome and finally atomic force microscope (AFM) measurements on solid supported bilayers (by vesicle fusion on freshly cleave mica) were performed to study the interactions. Maxima in absorbance and size of liposome was observed upon PAMAM addition. Charge reversal happened with the progressive addition of dendrimer. Interaction between PAMAM with liposome were found to be driven predominantly electrostatic. PAMAM activity was found to be generation dependent as 6G >4G>2G in terms of overall dendrimer concentration. But, interestingly, the order gets reverse when PAMAM activity was considered in terms of total end group concentrations. AFM studies reveal the rupture of bilayer structure upon addition of dendrimer.

In **Chapter III,** interaction of liposomes carrying net negative charges with cationic polyamidoamine dendrimers (PAMAM) of different generations were investigated by combined size, zeta potential, turbidity, electron microscopy, atomic force microscopy, fluorescence

spectroscopy and calorimetric studies. Stability of the liposomes comprising 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) + dihexadecyl phosphate, DPPC+1, 2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, DPPC+1, 2-dipalmitoyl-*sn*-glycero-3-phosphate and DPPC+1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanol were checked through their size and zeta potential with the variation of time. Existence of lipid bilayer and subsequent adsorption of dendrimer onto the liposome surfaces were evidenced. Interaction between the dendrimers and liposomes were electrostatic in nature, as evidenced through the charge neutralization of liposomes and its subsequent reversal with increasing dendrimer concentration. Extent of dendrimer-liposome interaction followed the sequence: generation 5 > 4 > 3 in addition to the head group charge, moiety and hydrocarbon chain length of the lipids. Fluorescence anisotropy and differential scanning calorimetry (DSC) studies suggest the fluidization of the bilayer although the surface rigidity was enhanced by the added dendrimers. Thermodynamic parameters of interaction processes were evaluated by isothermal titration and differential scanning colorimetric studies; the binding processes were exothermic in nature. Enthalpy of the transition of the chain melting of lipids decreased systematically with increasing dendrimer concentration and generation. Dendrimer-liposome aggregates were non-toxic to healthy human blood cell lymphocyte as well as in human RBCs suggesting the potential of such aggregates as drug delivery systems against microbial diseases.



In **Chapter IV**, the mutual miscibility and stability of the mixed monolayers of zwitterionic phospholipid, dipalmitoylphosphatidylcholine (DPPC) with negatively charged phospholipids (dihexadecyl phosphate (DHP), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPP) and 1,2-dipalmitoyl-sn-glycero-3-phospho ethanol (DPPEth) were investigated at the air-buffer interface. Interaction between the positively charged dendrimer with the monolayers has been studied in detail using surface pressure-area isotherms. Thermodynamic analysis indicates miscibility of the binary mixtures when spread at the air/buffer interface with synergistic interaction between the components. The surface pressure-area isotherms the binary monolayers of DPPC and negatively charged lipids at the air-water interface showed maximum deviation for DPPC : anionic lipid at 7:3 M/M ratio mixed monolayer was more stable than the monolayers individual components. Based on the regular solution theory, the miscibility and stability of the two components in the monolayer were analyzed in terms of compression modulus (C_s^{-1}) and excess Gibbs free energy ($\triangle G^0$) and these physiochemical parameters dependent on phospholipids composition. Stable liposomes were formulated by the binary mixture in 7:3 molar ratio of DPPC with negatively charged phospholipids. Subsequently adsorbed monolayers were generated through vesicles disruption technique. Effects of polyamidoamine (cationic) dendrimers on the adsorption kinetics at the vesicles were followed. Bylayer disintegration and subsequent interfacial adsorption of lipids were followed up through the surface pressure. Time analysis bylayer disintegration kinetics was governed by the lipid head groups, chain length as well as the dendrimer generation an concentration.

The thesis then follows comprehensive summary and conclusion followed by the cited references and off-prints of the published journal articles.

PREFACE

Naturally occurring cell membranes are generally negatively charged. Different kinds of anionic phospholipids have been used to form model membranes with the intention to understand membrane function. A variety of drug delivery systems or vehicles including polymer microcapsules, liposomes, polymer conjugates, and nano particles are in pre-clinical and clinical development to improve drug bioavailability. One class of biomaterials that can serve as drug carriers or, more generally as a platform for theranostics, and that have the potential to overcome these limitations is "dendrimer". Dendrimers are well-defined synthetic hyper branched macromolecules which possess high number of active termini that defines their properties and functions. As a result of perfect branching, dendrimers have the highest number of terminal functionalities of any polymeric material at a given molecular weight and are monodisperse. Comparing the features of dendrimers with those of linear polymers, it is well established that the dendrimer architecture presents several advantages for drug delivery. The dendrimers usually cross cell barriers by endocytosis, thus they are entrapped in endosomes and only a small amount of the active drug is able to reach the intracellular target. The translocation mediated by PAMAM dendrimers may be very useful in the delivery of drugs; although the mechanism of the spontaneous translocation is not yet well understood. The translocation of PAMAM dendrimers through lipid bilayers could motivate changes in the bilayer, which need to be taken into account in designing the drug delivery system. To understand how a PAMAM dendrimer mediates the cell membrane crossing, it is necessary to understand its interactions with lipid bilayers. Liposomes are outstanding model systems for biological experiments because of their simple and membrane-like composition, easy preparation, biodegradability, biocompatibility and acceptable stability over time. Calorimetric studies on liposome and dendrimers, can lead to the evaluation of the thermodynamic parameters of the interaction process. Different modes of interactions between dendrimers and liposomes are reported in the literature. Dendrimers can either pass through the lipid bilayer or form dendrimer-lipid aggregates. Some dendrimers can interact with lipids by hydrophobic interactions between the lipid acyl chains and the hydrophobic dendrimer interior. The strength of the interaction mainly depends on the size and charge of the molecule. Ultimately the formation of dendrimer-liposome aggregates or complex will be used as new drug delivery systems.

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Appendix A : List of Research Publications

Publications related to thesis

1. Roy, B.; Panda, A. K.; Parimi,S.; Ametov,I.; Barnes,T.; Prestidge, C.A.; Physico-chemical Studies on the Interaction of Dendrimers with Lipid Bilaylers. 1. Effect of Dendriemer Generation and Liposome Surface Charge. Journal of Oleo Science 2014, 65, (11) 1185-1193.
2. **Roy, B**.; Guha, P.; Bhattarai, R.; Nahak, P.; Karmakar, G.; Chettri, P.; Panda, A. K., Influence of Lipid Composition, pH, and Temperature on Physicochemical Properties of Liposomes with Curcumin as Model Drug. Journal of Oleo Science 2016, 65, (5), 399-411.
3. **Roy, B**.; Guha, P.; Nahak, P.; Karmakar, G.; Mandal, A.K.; Maiti, S.; Bykov, A.G.; Akentiev,A.V.; Noskov, B.A. Tsuchiya, K.; Torigoe, K.; Panda, A. K., Biophysical Correlates on the Composition, Functionality and Structure of Dendrimer-Liposome Aggregates. *Langmuir* (communicated and under revision)
4. **Roy, B**.; Guha, P.; Nahak, P.; Karmakar, G.; Panda, A. K., Effect of cationic dendrimer on the physicohemistry of solvent spread and adsorbed membrane mimetic lipid monolayer. (Manuscript is under preparation)

Other publications

1. Guha, P.; **Roy, B**.; Karmakar, G.; Nahak, P.; Koirala, S.; Sapkota, M.; Misono, T.; Torigoe, K.; Panda, A. K., Ion-Pair Amphiphile: A Neoteric Substitute That Modulates the Physicochemical Properties of Biomimetic Membranes. J. Phys. Chem. B 2015, 119, 4251-4262.
2. Koirala, S.; **Roy, B.;** Guha, P.; Bhattarai, R.; Sapkota, M.; Nahak, P.; Karmakar, G.; Mandal, A. K.; Kumar, A.; Panda, A. K., Effect of double tailed cationic surfactants on the physicochemical behavior of hybrid vesicles. RSC Advances 2016, 6, (17), 13786-13796.
3. Bhattarai, R.; **Roy, B**.; Guha, P.; Bista, A.; Bhadra, A.; Karmakar, G.; Nahak, P.; Chettri, P.; Panda, A. K., Spectroscopic Investigation on the Interaction of Curcumin with Phosphatidylcholine Liposomes. Journal of Surface Science Technology 2015, 31 (1-2), 1-9.

4. Bhattarai, R.; Sutradhar, T.; **Roy, B.;** Guha, P.; Chettri, P.; Mandal, A.K.; Bykov, A.G.; Akentiev, A.V.; Noskov, A.V.; Panda, A.K. Double-Tailed Cystine Derivatives as Novel Substitutes of Phospholipids with Special Reference to Liposomes. J. Phys. Chem. DOI: 10.1021/acs.jpcb.6b06413
5. Nahak, P.; Karmakar, G.; **Roy, B**.; Guha, P.; Sapkota, M.; Koirala, S.; Chang, C.-H.; Panda, A. K., Physicochemical studies on local anaesthetic loaded second generation nanolipid carriers. RSC Advances 2015, 5, (33), 26061-26070.
6. Nahak, P.; Karmakar, G.; Chettri, P.; **Roy, B**.; Guha, P.; Besra, S.E.; Soren, A.; Bykov, A.G.; Akentiev, A.V.; Noskov, A.V.; Panda, A. K. Influence of Lipid Core Material on Physicochemical Characteristics of an Ursolic Acid-Loaded Nanostructured Lipid Carrier: An Attempt To Enhance Anticancer Activity. *Langmuir*. 2016, 32, 9816−9825.
7. Karmakar, G.; Nahak, P.; Guha, P.; **Roy, B.**; Chettri, P.; Sapkota, M.; Koirala, S.; Misono, T.; Torigoe, K.; Ghosh, S.; Panda, A. K., Effects of Fatty Acids on the Interfacial and Solution Behavior of Mixed Lipidic Aggregates Called Solid Lipid Nanoparticles. Journal of Oleo Science 2016, 65, (5), 419-430.
8. Karmakar, G.; Nahak, P.; Guha, P.; **Roy, B**.; Chettri, P.; Sapkota, M.; Koirala, S.; Misono, T.; Torigoe, K.; Ghosh, S.; Panda, A. K., Effects of Fatty Acids on the Interfacial and Solution Behavior of Mixed Lipidic Aggregates Called Solid Lipid Nanoparticles. Journal of Oleo Science 2016, 65, (5), 419-430.
9. Sapkota, M.; Karmakar, G.; Nahak, P.; Guha, P.; **Roy, B**.; Koirala, S.; Chettri, P.; Das, K.; Misono, T.; Torigoe, K.; Panda, A. K., Effect of polymer charge on the formation and stability of anti-inflammatory drug loaded nanostructured lipid carriers: physicochemical approach. RSC Advances 2015, 5, (81), 65697-65709.
10. Bhadra, A.; Karmakar,G.; Nahak, P.; Chettri, P.; **Roy, B.;** Guha, P.; Mandal, K. A.; Nath, K. R.; Pnda, A. K., Impact of detergents on the physiochemical behavior of itraconazole loaded nanostructured lipid carriers. Colloids surfaces A; 2017, 516, (5), 63
11. Karmakar, G.; Nahak, P.; Chettri, P.; Guha, P.; **Roy, B**.; Sapkota, M.; Koirala, S.; Misono, T.; Torigoe, K.; Ghosh, S.; Panda, A. K., Effects of Fatty Acids on the Interfacial and Solution Behavior of Mixed Lipidic Aggregates Called Solid Lipid Nanoparticles. Journal of Oleo Science 2016, 65, (5), 419-430.

12. Guha, P.; **Roy, B**.; Nahak, P.; Karmakar, G.; Chang, C. H.; Bikov, A. G.; Akentiev, A. B.; Noskov, B. A.; Mandal, A. K.; Kumar, A.; Hassan, P. A.; Aswal, V. K.; Misono, T.; Torigoe, K.; Panda, A. K., Exploring the dual impact of hydrocarbon chainlength and the role of piroxicam a conventional NSAID on soylecithin/ion pair amphiphiles mediated hybrid vesicles for brain – Tumors targeted drug delivery. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*.. doi.org/10.1016/j.colsurfa.2018.03.025 (Just accepted)
13. Karmakar, G.; Nahak, P.; **Roy, B**.; Guha, P.; Tsuchiya, K.; Torigoe, K.; Nath, R. K.; Panda, A. K., Use of ion pair amphiphile as an alternative of natural phospholipids in enhancing the stability and anticancer activity of oleanolic acid loaded nanostructured lipid carriers. *Colloids and Surfaces A: Physicochemical and Engineering Aspects***.** doi.org/10.1016/j.colsurfa.2018.02.039. (Just accepted)
14. Karmakar, G.; Nahak, P.; Chettri, P.; **Roy, B**.; Guha, P.; K. Tsuchiya; Torigoe, K.;. · Kumar. A.; Nath, R.K.; Bhowmik. S.; De. U. C.; Nag, K.; Panda, A. K. Physicochemical Characterization of Chrysin-Derivative-Loaded Nanostructured Lipid Carriers with Special Reference to Anticancer Activity. J. Surfact. Deterg. 2018. (Just accepted)
15. Karmakar, G.; Nahak, P.; Guha, P.; **Roy, B**.; Nath, R.K.; Panda, A. K. Role of PEG 2000 in the surface modification and physicochemical characteristics of pyrazinamide loaded nanostructured lipid carriers. Journal of Chemical Sciences. (Just accepted)

Appendix B : List of Oral and Poster Presentations

Oral Presentations

1. "Indo- UK International Workshop On Advanced Materials And Their Applications In Nanotechnology" at BITS Pilani KK Birla Goa campus during May 17- 19, 2014.
2. "North East Regional Seminar on Trends in Colloid and Interface Science" held in North Eastern Hill University, Shillong on November 27-28, 2014.
3. 17th National Conference on Surfactants, Emulsions and Biocolloids" held in Pt. Ravishankar Shukla University, Raipur on November 4-6, 2015 (**This presentation has been adjusted for an award**)
4. 22nd West Bengal State Science and Technology Congress 2015. Organized by Department of Science and technology Govt. of West Bengal and University of North Bengal. February 28 and March 1, 2015.

Poster Presentations

1. 5th Asian Conference on Colloids and Interface Science. Organized by the Asian Society for colloid and surface science and Department of Chemistry University of North Bengal. November 20 to 23, 2013
2. Frontiers in Chemistry-2015, Founded by University Grant Commission and SAP (DRS-III), Organised by Department of Chemistry, University of North Bengal.
3. 19th CRSI National Symposium in Chemistry. Organized by Department of Chemistry University of North Bengal. 14 to 16 July, 2016.
4. "International Conference on Challenges in Drug Discovery and Delivery" held at Birla Institute of Technology and science, Pilani, March 2–4, 2017.

DPPC	1, 2 - dipalmitoyl-snglycero -3-phosphocholin
DPPG	1, 2 - dipalmitoyl-snglycero -3phosphogylcerol
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3- phosphocholin
DPH	1,6- diphenyl – 1, 3, 5- hexatriene
7HC	7- hydroxycumarin
Na_2HPO_4, $12H_2O$	Disodium hydrogen phosphate
LUV	Large unilamellar vesicles
MUV	Multilamellar vesicles
ITC	Isothermal Titration Calometry
CUR	Curcumin
SLC/SPC	Soylecithin
TS	Tristrarin
FA	Fatty acid
LA	Lauric acid
MA	Myristic acid
PA	Palmatic acid
SA	Stearic acid
Tween 60	Polyoxy ethylene(20) sorbital monostearate
CP	Cetylpalmitate
TP	Tripalmitine
OA	Oleic acid
DLS	Dynamic light scattering
DSC	Dfferential scanning calorimetry
TEM	Transmission electron microscopy
FF-TEM	Frezed fractured electron microscopy
AFM	Atomic force electron microscopy
OLA	Oleionic acid
DL%	Drug loading capacity
EE%	Entrapment efficiency

PDI	Plydispersity index
ZP	Zeta potential
SEM	Scanning electron microscopy
T_m	Phase transition temperature
$\Delta T_{1/2}$	Half peak width
ΔH	Change in enthalpy
ΔC_p	Change in heat capacity
SLN	Solid lipid nanoparticles
NLC	Nanostructured lipid carriers
DHP	dihexadecyl phosphate
DPPEth	1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanol
DMPG	1, 2-dimyristoyl-*sn*-glycero-3-phosphoglycerol
DPP	1, 2-dipalmitoyl-*sn*-glycero-3-phosphate
PAMAM	polyamidoamine
CUR	Curcumin
AFM	Atomic force microscope
CPP	Critical packing parameter

INTRODUCTION

The development of different drug delivery systems have conveyed significant innovations in molecular biology and other fields of biological sciences leading to unique prospect for delivering nanoparticles of various dimensions to biological systems. In recent years, substantial considerations have been bred in formulating nano colloidal systems for drug or gene delivery applications.[1] Dendrimers are one such type of nanoparticle, having a high degree of branching, low polydispersity, nanometer size range and controllable architecture.[2] Polyamidoamine (PAMAM) dendrimers, as used in this research study, have demonstrated various biopharmaceutical applications such as antibacterial agents, antiviral drugs, as well as drug or gene delivery vehicle. Potential application of dendrimers as drug delivery vehicle and therapeutic agent require critical understanding of their interaction with the liposome bilayer. However, the molecular and physical complexity of cell membranes have limited the detailed kinetic and mechanistic investigations of dendrimer interactions with living cells.

On the other hand, it should be noted that liposomes have already been applied as cell models for investigating drug transport from unilamellar liposomes when the latter were employed as drug delivery systems.[3] Many reports have covered the interactions between dendrimers and biological membranes. [4] But the main objective of the present work is to study the interaction between dendrimer and liposome and to prepare new drug delivery systems of dendrimer-liposome complex. Although there are different reports on the interaction studies between dendrimers and liposomes,[5, 6, 7] however, limited comprehensive and systematic studies have been carried out previously in order to assess the impact of dendrimer generation, concentration as well as the variation of the liposome type.

1. Amphiphiles:

Amphiphiles are the class of compounds having special structural features as it possess the hydrophilic (water loving) and hydrophobic part (fat loving) shown in Figure 1.[8] The hydrophilic portion generally consists ions (anionic or cationic or uncharged polar groups) whereas lipophilic parts are generally long chain hydrocarbon, such in the form of $CH_3(CH_2)_n$, with $n > 10$.

Carboxylate (COO^-), sulphate (SO_4^-), sulfonate (SO_3^-), phosphate (PO_4^-), etc., are the anionic and amine (NH_4^+) is the cationic group attach to lipophilic hydrocarbon chain. Alcohols are the class of polar uncharged group often attached with long chain hydrocarbon, such as diacyl glycerol. In aqueous medium they spontaneously form self-asembled structure.

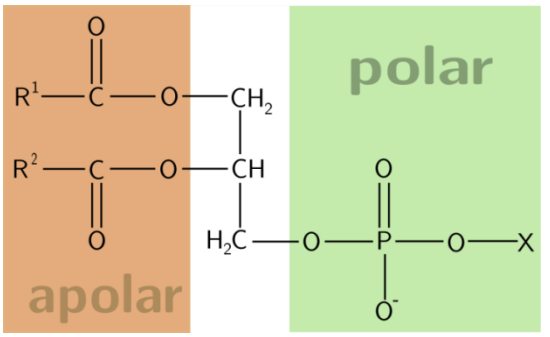


Figure 1: General structure of an amphiphile.

Because of the hydrophilic-lipophilic behavior, it has diverse range of applications: in detergent, paint, pharmaceutical industries to food, etc. The variety of self-assembled structures make the amphiphiles to be useful in large number of areas.[9,10] Throughout the present work, mixed lipid bilayer is the most enlighten/highlighted aggregate structure. Naturally occurring anionic and zwitterionic phospholipids with cholesterol have been used for the preparation of liposome.

1.1. Self-assembly and Aggregate structure: Self-assembly is a spontaneous process, constructs aggregate having various morphology from a variety of chemical building blocks. Being an aggregated form, the process of self-assembly can include verity of complexity. It can be a simple process of dimerization of two simple building blocks or as complicated as a biological membrane. Among various building blocks, amphiphiles draw the attention most over the other as its superiority to form robust assemblies.

Amphiphiles consist with hydrophilic and lipophilic part and thus has dual preference for solvent. Due to their amphiphilicity, the polar head groups interact with water or polar solvent while the lipophilic part tends to stay out of contact with water and thus try to orient in the air water interphase. In water it first dissolves as a monomer, but above certain concentration level, they form micro-structure or assemblies to avoid the unfavorable solvent hating interaction between water and lipophilic parts. This process of self-organization is spontaneous and

accompanied by increased entropy. The increased entropy begins from water-hydrocarbon interaction when amphiphiles are present as monomer. As monomer it forces water molecules in

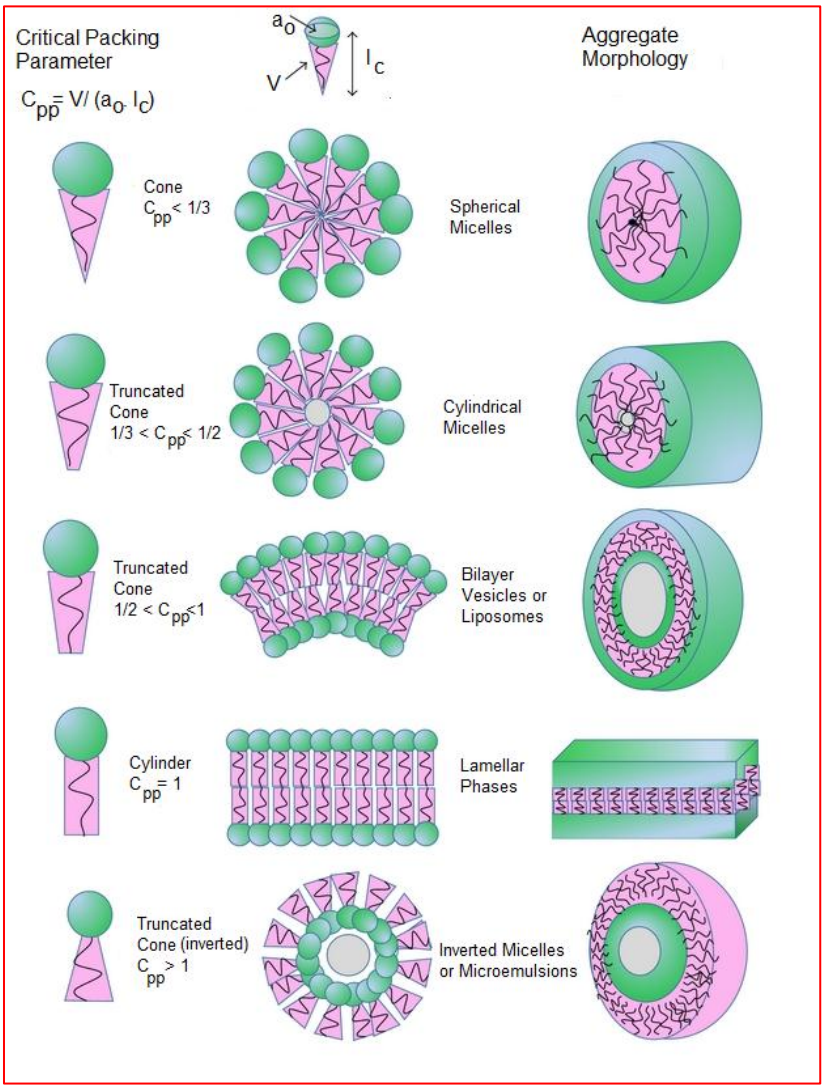


Figure 2: Aggregate structure of lipid assessed from critical packing parameter C_{pp}.

an order state around the lipophilic part. When concentration of amphiphiles is increased two possible scenarios is developed. Either they have to move at air-water interphase or to form aggregate structure. The brakeage of ordered water molecule enhances the process of entropy which leads to an overall gain of free energy that drives the amphiphiles to form aggregate structure by virtue of self-assembly. The aggregation of amphiphiles is generally driven by hydrophobic interaction, hydrogen bond, steric effect and electrostatic interaction

1.2. Critical Packing parameter and aggregates morphology:

The shape and the size, or better to say morphology of the aggregates not only depend on above mentioned forces, but also depend on the structure, molecular geometry and concentration of the amphiphiles, extent of hydration, pH and ionic strength of the of the dispersion medium. Critical packing parameter (C_{pp}) which takes into account parameters such as polar head group cross-sectional area (a_0), hydrocarbon chain length (l_c) and hydrophobic volume (V) is related as: [11,12]

$$C_{pp} = \frac{V}{a_0.l_c} \quad (1)$$

This parameter together holds the information about the geometrical structure of the aggregates. With the increasing C_{pp}, the structure of the aggregated form could be spherical (C_{pp}< 1/3), cylindrical (1/3< C_{pp}< 1/2) and lamellar (C_{pp} = 1). In case of vesicles or liposome, the range of C_{pp} is 1/2 to 1 with a inner cavity encapsulating dispersion medium. A summary of the aggregated structure predicted from critical packing parameter (C_{pp}) is represented in Fig 2. Lipids are the most common amphiphile to formulate the aggregate morphology.

2. Lipid:

The organic compounds, which are insoluble in water consisting of fats and oils, are called lipids. Chemical composition of these lipids includes carbon, hydrogen, and oxygen. Lipids belong to a diverse and large number of non-polar organic compounds having non-polar hydrocarbon chain or acyl chain attached with the polar head group. In aggregation, its head group exposed towards the polar solvent medium, the non-polar acyl chain being shielded inside. They deliver high energy and execute three vital biological functions in the body: to arrange for structure to cell membranes, to accumulation energy, and to role as signaling molecules.[13, 14]

2.1. Biological function of lipids:

Lipids play important roles in the normal function of the body:

1) Lipids works for cell membranes and organelles to serve as structural building materials.

2) Lipids greatly afford energy for the organisms than carbohydrates or proteins.

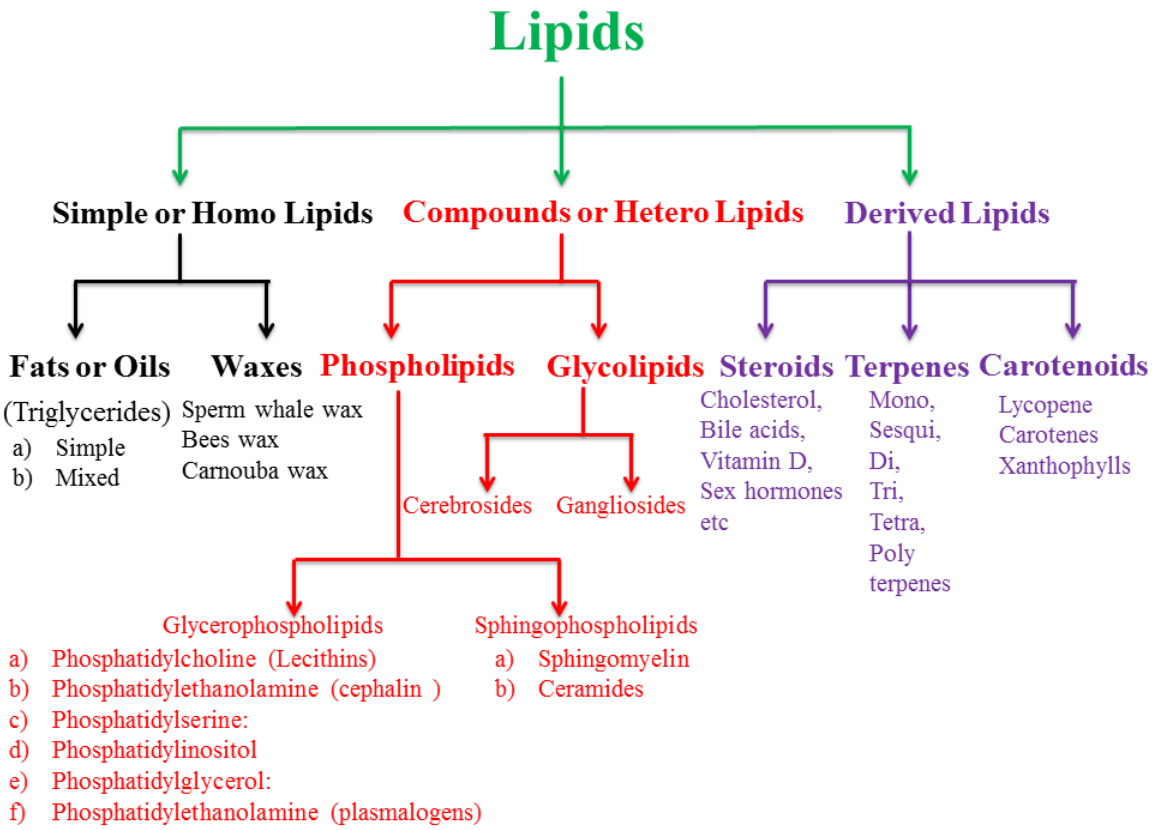
3) To serve as signaling molecules and molecular messengers in the body.

4) Lipids act as biomarkers for certain diseases and play some important role in chronic disease and genetic modification.

2.2. What are the food sources of lipids?

The lipids or fat are the macronutrients important in nutrition. They are mainly produced in dietary food and fisheries, and other foods. The delicious sources of diabetes fat include soybean, nuts and seeds, olive oil, and avocados, which contain essential fatty acids. Contrary to popular belief, lipids are an important part of our diet, and it is necessary to feed a minimum amount. However, many health problems are associated with excessive dietary fat, such as obesity, heart disease, and cancer.[15,16,17]

2.3. General classification and structure of lipids: Lipids are broadly classified into simple, complex and derived which are further subdivided into different groups. The lipid classification is shown in the chart below:[18]



2.3.1. Simple lipids: Simple lipids are esters of fatty acids with different alcohols. Even number of carbon atoms may contain saturation or unsaturation in the fatty acid chain. They are classified as triglycerides (fats and oils) and waxes. The esters of fatty acid with combination with glycerol are called triglycerides (fats and oils). Fat and oil are extensively dispersed among plants and animals. Triglycerides generally known as neutral fat, some of them are insoluble in water and non-polar in nature. The triglycerides of saturated fatty acids, which are present in solid form at room temperature, are called fats. On the other hand, when glycerides are presented in liquid-sized form at room temperature, then it is called oil. They are the unsaturated fatty acids of triglycerides. The degree of unsaturation of fatty acid residue in fat is lower than that of oil. On hydrolysis of oil and fat gives long chain monocarboxylic acid and glycerol.[19,20]

Monoglycerides:

$R_1 = C_nH_{2n}O$

$R_2 = R_3 = H$

Diglycerides:

$R_1 = R_2 = C_nH_{2n}O$

$R_3 = H$

Tryglycerides:

$R_1 = R_2 = R_3 = C_nH_{2n}O$

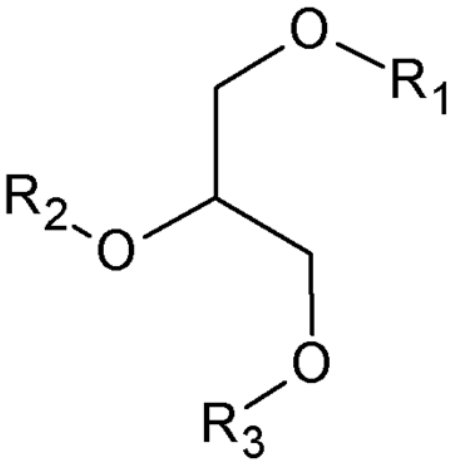


Figure 3: General structure of glycerides.

Waxes are the ester of long chain saturated or unsaturated fatty acid with long chain monohydric alcohol. Waxes are considered as the lipid which on hydrolysis produces long chain fatty acids and long chain alcohols. The fatty acid range between C_{14} and C_{36} and the alcohol rang between C_{16} and C_{36}. Because of the long hydrocarbon chain, it has high molecular mass with relatively high melting point. It is insoluble in water but soluble in organic, non-polar solvents.

Waxes→ hydrolysis→ long chain fatty acids + long chain alcohols

In the case of plankton (aquatic plants) waxes is the chief storage form of metabolic fuel. Biological waxes have different kinds of applications in the pharmaceutical, cosmetics and other industrial level.

2.3.2. Compound or complex or hetero lipids: Complex lipids are commonly found in plants, bacteria and animals. They are the major ingredients of cell membranes but are established also in circulating fluids.[21] On the basis of composition complex lipids are also categorize as: 1) Phospholipids 2) Glycolipids.

2.3.2.1. Phospholipids: The complex lipids comprising phosphoric acid is named as phospholipids. It contains nitrogen base and other elements and they are generally located in the brain and nervous tissue. They also exist in cytoplasmic membrane because of which they are elaborate in cellular activities. [22] They are classified as i) Glycerol phospholipids and ii) Sphingo phospholipids.

2.3.2.1.1. Glycerophospholipids: They are most abundant phospholipids. They comprise 2 fatty acids esterified with first and second hydroxyl group of glycerol. The third hydroxyl group of glycerol makes an ester linkage with phosphoric acid. Glycerophospholipids are more allocated into following groups shown in Figure 4.

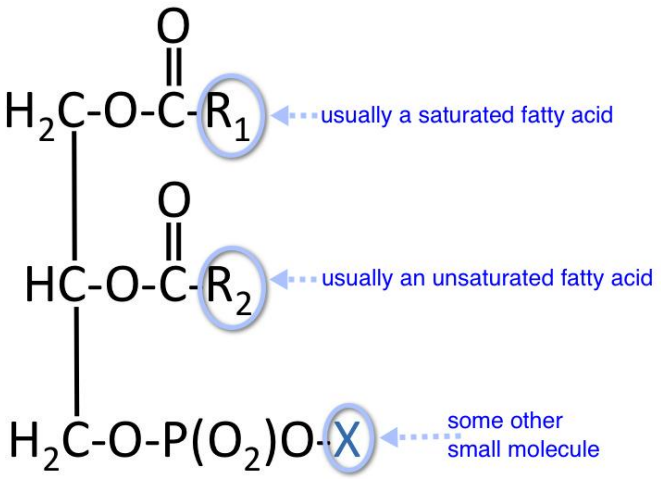


Figure 4: General structure of glycerophospholipid.

Phosphatidylcholine (Lecithins): It is extensively circulated in the body mostly in the liver. [23] In these phospholipids, collin is exist as the base which inhibits the deposition of abnormal fat in the liver. It is also the major elements of acetyl choline.

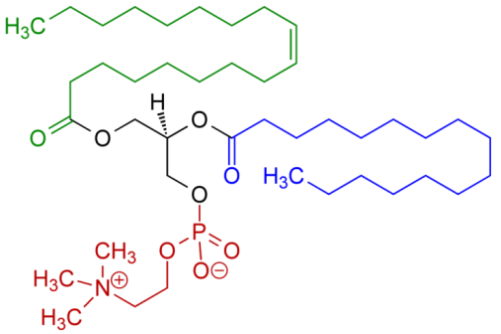


Figure 5: Structure of phosphatidylcholine (SPC).

Phosphatidylethanolamine (cephalin): They are manufactured by the addition of cytidine diphosphate-ethanolamine to diglycerides, liberating cytidine monophosphate. The base ethanolamine is present which composed with lecithin and associated with the brain. It is also exist in RBC.

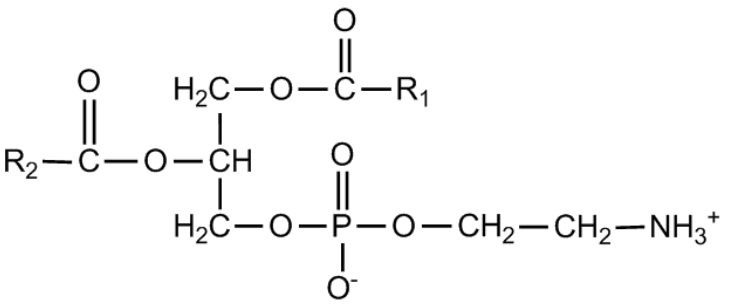


Figure 6. General structure of phosphatidylethanolamine.

Phosphatidylserine: It contains of two fatty acids connected in ester linkage to the first and second carbon of glycerol and on the other hand, serine is connected through a phosphodiester linkage to the third carbon of the glycerol. Phosphatidylserine works for blood coagulation. The base amino acid serine is present. It has similar function with lecithin.

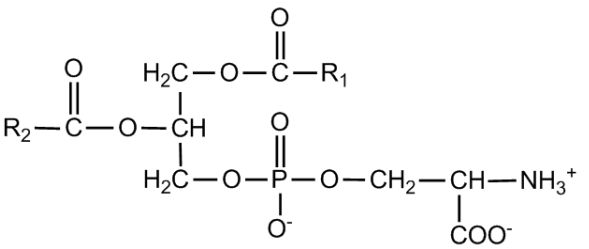


Figure 7: General structure of phosphatidylserine.

Phosphatidylinositol: It contains inositol as the base instead of nitrogen base. It is mainly found in plants and nervous tissue. Some hormones such as oxytocin, vasopressin are modified through phospholipids.

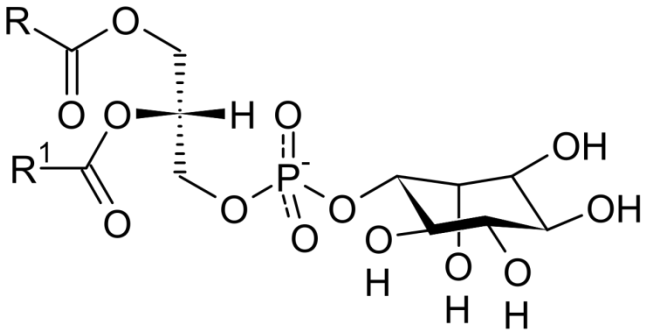


Figure 8: General structure of phosphatidylinositol.

Phosphatidylglycerol: In phosphatidylglycerol, two molecules of phosphatic acids are connected by the accumulation of glycerol to phosphate groups. The common structure of phosphatidylglycerol contains of a L-glycerol 3-phosphate backbone which are ester bonded to either saturated or unsaturated fatty acids on carbons 1 and 2. The head group substituent glycerol is bonded through a phosphomonoester. It is originated in the inner membrane of mitochondria.

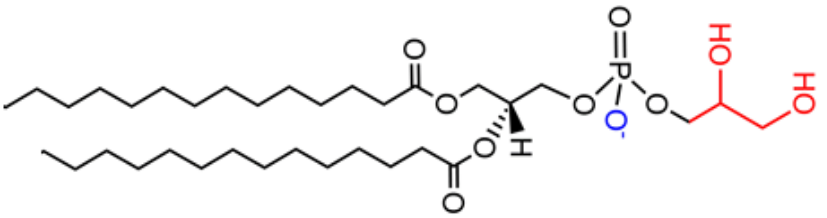


Figure 9: Structure of phosphatidylglycerol.

Phosphatidylethanolamine (plasmalogens): In these phospholipids, fatty acids are linked with carbon and glycerol by an ester linkage. Generally, unsaturated fatty acid is linked at carbon.

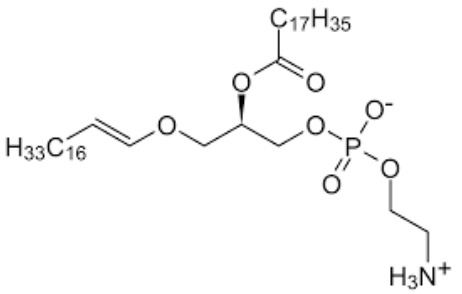


Figure 10: Structure of plasmalogens.

2.3.2.1.2. Sphingophospholipids: They are formed by the combination of long chain fatty acids with long chain amino alcohol sphingosine or one of its derivatives and the polar bond of alcohols. They are originate in the brain or nervous tissue.[24,25] On the basis of composition

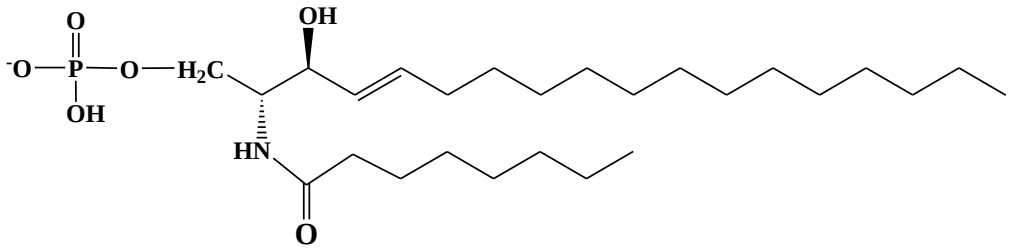


Figure 11: Structure of sphingophospholipid.

Sphingolipids are two kinds, one is sphingomyelin and another is ceramides. Sphingomyelin is the humblest and best abundant lipid which holds phosphocholine or phosphoethanolamine as the polar head region. It is founded in the animal cell as myelin sheath which covers the nerve cell. Ceramides consist of sphingosine molecule and fatty acids. It is equable of sphingosine molecule and fatty acids:

2.3.2.1.3. Functions of phospholipids: Some essential functions of phospholipids are:

1) Phospholipids in the relations with proteins make the structural constituents of a membrane and control membrane permeability.[26]
2) Phospholipids in the mitochondria are accountable for preserving the conformation of electron transport chain mechanisms and thus cellular respiration.[27, 28]
3) Phospholipids contribute in the absorption of fat in the intestine.[29, 30]
4) Phospholipids are important for the production of different proteins *i.e.* lipoproteins and thus take part in the transport of lipids.[31,32]
5) Accumulation of fat in the liver can be stopped by phospholipids; hence they are considered as the lipoprotein factor.[33, 34]
6) Phospholipids take part in the reverse cholesterol transport and thus help in the elimination of cholesterol from the body.[35, 36]
7) Cephalins are the crucial group of phospholipid contribute in the blood clotting.[37,38]
8) Phosphatidylinositol are participated in signal transmission through membrane.[39, 40]
9) Phospholipids performance as an agent for pull down surface tension. [41, 42]

2.3.2.2. Glycolipid: The lipids with combination of carbohydrates attached by a glycosidic bond or covalently bond are called glycolipids. They have no any phosphate group. It comprises C 24 fatty acid molecule like cerebric acid, lignoceric acid. It is generally two types: a) Cerebrosides b) Gangliosides. The simplest form of glycolipids is called Cerebrosides. Glucose and galactose are remains as carbohydrate residue. Generally, 2-20 units carbohydrate chain is present in cerebtosides. On the other hand, the derivative of cerebroside are called gangliosides and they contains one or more molecule of N- acetyl neuraminic (NANA). It is mostly present on grey matter of the brain and neutralized tetanus toxins.[25]

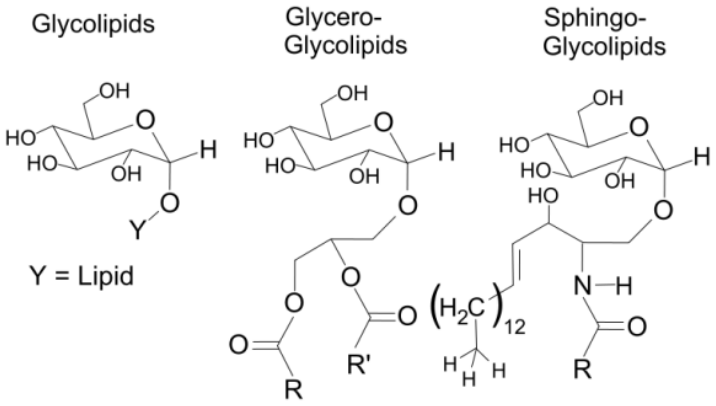


Figure 12: General structure of sphingophospholipid.

2.3.2.2.1. Function of glycolipids:

1) Glycolipids are important components of all membranes in the body.[22]
2) They are situated mainly in the outer part of the plasma membrane where they can interact with the external part of cellular environment.[43]
3) They play an important role for the cellular interaction, growth, and development.
4) They act as an antigen which decide blood group of an individual. [44, 45]
5) They function as cell surface receptors for some viruses, like cholera and diphtheria. [46]
6) It produce the structural constituent of nervous tissues.[47]

2.3.3. Derived Lipids: By the hydrolysis of simple and compound lipids derived lipids are prepared. Fatty acids, alcohols, monoglycerides, diglycerides, steroids, terpenes and carotenoids are the main components of derived lipids. This lipid contains cyclopentane rings which are the complex fat soluble molecule with four fused rings. Steroids are compounds holding a cyclic

steroid nucleus that is Cyclopentanoperhydrophenanthrene. It involves of a phenanthrene nucleus to which a cyclopentane ring is committed. There are a number of steroids in the biological system. These consist of cholesterol. vitamin D, bile acids , sex hormones etc. If the steroids enclose one or more hydroxyl group, it is famous as the sterol (means alcohol).

The best common sterol create in animal tissue is the cholesterol. The cholesterol contains of Cyclopentanoperhydrophenanthrene ring with OH group at carbon number of 3 and the double bond among carbon number 5 and 6. The state at room temperature is solid and insoluble in water but rarely in higher plants. The cell membrane of eukaryotes contains of cholesterol. It is the precursor of a large number of essential steroids comprising bile acids, sex hormones, vitamin D etc. Cholesterol is manufactured by the liver and secreted in the blood.[48, 49] It is likewise circulated in serum and plasma. The normal blood cholesterol level contains 130-250 mg/dl. But cholesterol level is studied by metabolic functions which are prejudiced by nutrition,

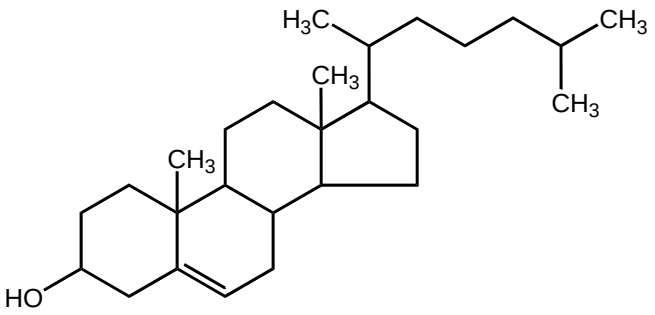


Figure 13: Structure of cholesterol.

heredity, endocrine functions, liver, kidney etc. If the cholesterol level is lower than normal value then it is called hypercholesterolemia.[50] This situation is understood in hyperthyroidism acute infection, anemia, malnutrition etc. If the cholesterol level is higher than the normal value then it is called hypercholesterolemia.[51] This condition is seen in hypothyroidism poison, less controlled diabetes mellitus, chronic hepatitis, nephrotic syndrome, obstructive Jaundice. Thus the purpose of blood cholesterol level is the significant diagnostic parameter from the medicine technology.

2.3.3.1. Function of steroids/ cholesterols:

1) Cholesterol is an important component of cell membrane that modulates the fluidity/rigidity of membrane.[52, 53]

2) Cholesterol acts as an insulating guard for the transmission of electrical impulses in the nerve systems.
3) Steroids/Cholesterol comprise the bile acids, sex hormones and vitamin D.[54]
4) Cholesterol acts as an essential role in fertility for female body. At low concentration of cholesterol in female body, it may lead to sterility. [55]
5) The cardiovascular diseases controlled by cholesterol.[56, 57]
6) Steroids preserves the serum calcium level.[58, 59]

2.3.4. Description of some others phospholipids relevant to the present study: The phospholipids which were used in this research work are given bellow:

2.3.4.1. Zwiterionic phospholipids: Generally phospahtidylcholine (PC) lipids are zwiterionic in nature. PC is derived from natural and synthetic sources. It is also known as lecithin. The two glycerol moieties attached with fatty acyl chain via ester linkage and the remaining one hydroxyl group linked with phosphate ion, which is further, connects with choline group. They are easily available in egg-yolk and soybean but hard to extract form bovine heart and spinal cord. Lecithin is chemically inert and neutral charge. Besides chemical properties, the low cost of lecithin made it as one of the major phospholipid in liposome preparation. Lecithin from vegetable oils contains one or multiple unsaturation in the acyl chain. On the other hand lecithin from animal fat produces fully saturated hydrocarbon chain. Soy phosphatidylcholine (SPC) and dipalmitoylphosphatidylcholine (DPPC) are the two important PC lipids. In case of SPC lipid, one hydrocarbon chain is unsaturated.

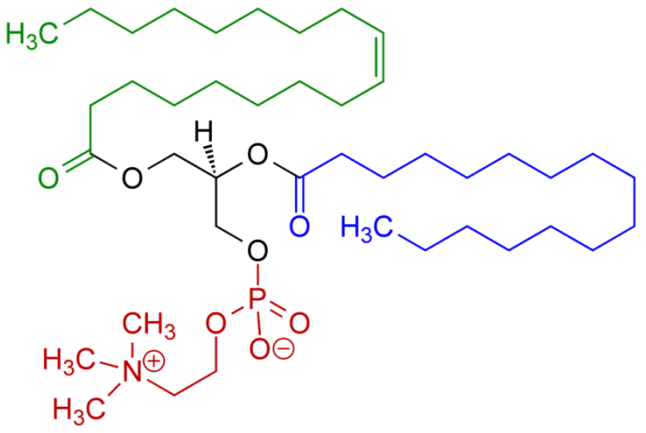
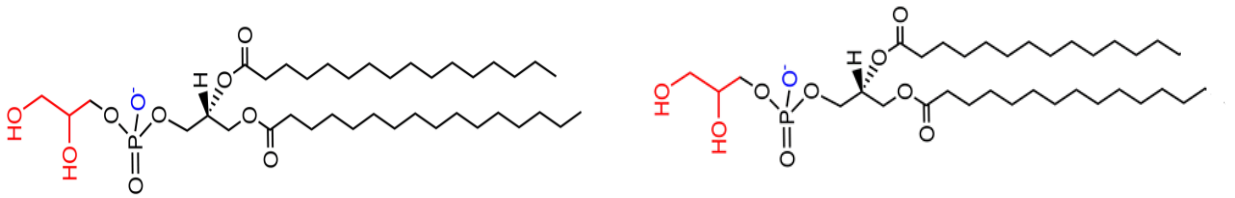


Figure 14: Structure of soy phosphatidylcholine (SPC) lipid.

2.3.4.2. Negatively charged phospholipids: Generally Phosphatidyl glycerol (PG) are anionic in nature. They found in pulmonary surfactant. It is consisting of L-glycerol 3-phosphate backbone. Two fatty acyl chains either saturated or unsaturated are connected with the ester linkage. The head group region constituted with glycerol molecule bonded with phosphomonoester.



DPPG DMPG

Figure 15: Structure of DPPG and DMPG.

Anionic lipids are freely accessible in nature and they are easily manufactured by semi-synthetically form other lipids by means of phospholipase D in the existence of glycerol. These kinds of phospholipids hold uninterrupted negative charge all over the place of body pH range. 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) and 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) are the two important anionic lipids. They are only differing by chain length.

2.3.4.3. Phosphadityl alcohol: They have the same kinds of dipalmitoyl groups, but the phosphate group is directly connected to alkyl group. Phosphadityl alcohols are generally physiologically scare.The small and anionic head group of phosphadityl alcohol has large effect on their membrane properties. They are highly portent promoters on membrane bilayer and their trans bilayer movement is three times higher than that of other naturally occurring phospholipids. 1,2-dipalmitoyl-sn-glycero-3-phospho ethanol (DPPEth) is an example of phosphadityl alcohol.

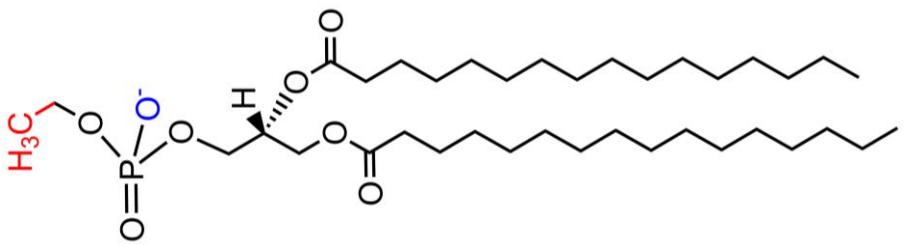
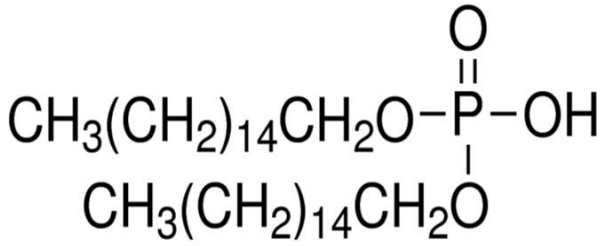
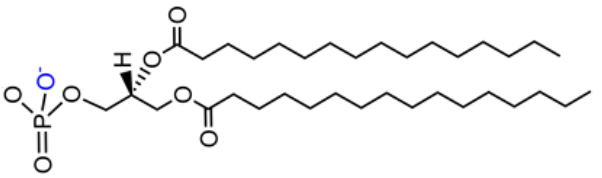


Figure 16: Structure of 1,2-dipalmitoyl-sn-glycero-3-phospho ethanol (DPPEth).

2.3.4.4. Others anionic lipids: 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPP) and dihexadecylphosphate (DHP) are the two another negatively charged phospholipids. In case of dihexadecylphosphate (DHP), the phosphate group is directly connected to the long chain hydrocarbon. On the other hand DPP has only phosphatidyl group with two palmitoyl chain length.



DHP



DPP

Figure 17: Structure of DHP and DPP phospholipids.

3. Aggregation behavior of amphiphiles: On the basis of concentration and structural complexity of the lipid (amphiphile) form aggregates like micelles, bilayer or liposomes or other form of lipids.

3.1. Micelle formation: In an aqueous medium, the polar head groups of amphipathic lipids expose the polar towards aqueous environment, while the hydrophobic tail groups decrease their contact with water and have a greater tendency to group organized called as lipid aggregates. Depending on the concentration and structural complexity of the lipid, these lipid aggregates constitute either of micelles, bilayer or liposomes. Micelles are lipid molecules that arrange themselves in a spherical form in aqueous solutions are shown in Figure 18.[60] Fatty acids form micelles generally contain a single hydrocarbon chain instead of two hydrocarbon tails. This permits them to configure into a spherical shape for lesser steric hindrance within a fatty acid. For instance, salts of fatty acids (such as sodium palmitate, a component of soap), which contain only one chain can easily form micelles.

3.1.1. Biological importance of micelles: Micelles are essential as they transport the weakly soluble monoglycerides and fatty acids to surface of enterocyte or intestinal absorptive cells (are simple columnar epithelial cells found in the small intestine) where fats are absorbed.[61] In the digestive tract, triacylglyceride is hydrolyzed through the lipase enzyme, to produce two free

fatty acids and a monoglyceride. These free fatty acids and monoglycerides along with bile salts and phopholipids create micelles.[62, 63] Also, micelles enclose fat soluble vitamins and cholesterol. Micelles continuously break down and reconstitute, feed a small group of monoglycerides and fatty acids which are present in medium.

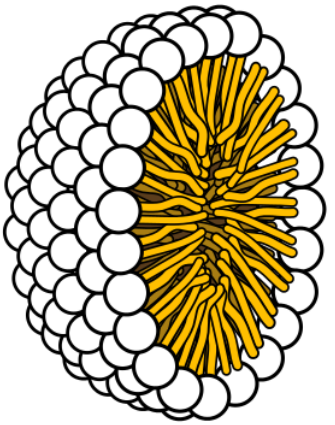


Figure 18. Schematic diagram of micelle.

3.2. Monolayer formation: Due to amphipathic nature, lipid molecules are insoluble in individually polar and apolar media. In these media, they produce monomolecular films termed as monolayers (shown in Figure 19), that decrease the surface tension. The properties of lipid

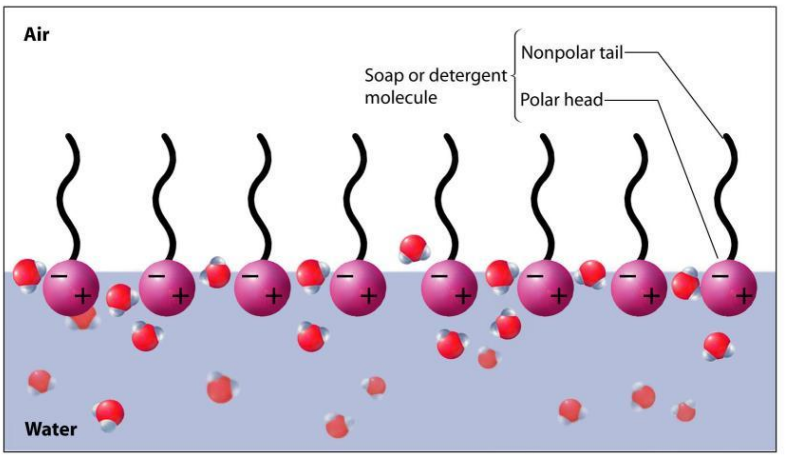


Figure 19: Structure of lipid monolayer at the air-water interface.

monolayers differ with their surface density. For example, when the density is higher, the surface tension becomes lower at the interface. At a definite high surface density further decrease of the surface tension is not likely: the monolayers turn into unbalanced at the interface and collapse.

Besides being of fundamental interest for surface science, lipid monolayer collapse is crucial for sustaining low surface tension at the gas-exchange interface in the lungs for the duration of breathing.[64, 65]

3.3. Bilayer formation: Phospholipids and glycolipids unable to generate micelles as happen in fatty acids. Because phospholipids and glycolipids comprise two hydrocarbon chains which are so large that cannot assemble them into a micelle. So the formation of lipid bilayers from phospholipids is a quick and spontaneous manner in water. A lipid bilayer is also termed as a bimolecular sheet. The hydrophobic tails of each individual plane interact with one another, constituting a hydrophobic core which acts as a permeability barrier. The interaction between hydrophilic head groups with water on both side of the bilayer is predominant. The two opposite sheets are known as leaflets, are shown in Figure 20.

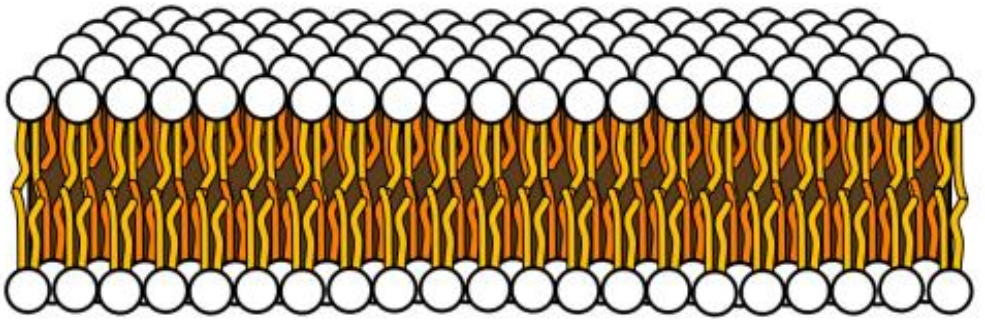


Figure 20: Structure of planar lipid bilayer.

3.3.1. Biological importance of lipid bilayers:

The development of bilayers as a replacement for of micelles by phospholipids has vital biological importance. A micelle is a smaller structure, generally less than 20 nm (200 Å) in diameter. On the contrary, a bilayer sheet can be of larger dimensions, e.g. a millimeter (106 nm or 107 Å). Phospholipids and related compounds are important membrane constituents as they easily form extensive bilayer sheets. Lipid bilayer sheet associated with proteins in biological membranes contains lipid of various structural complexities depending upon the membrane source.[66]

3.3.2. Hydrophobic interactions in lipid bilayer:

The formation of lipid bilayers is an energetically preferred and spontaneous process when the phospholipids are placed in an aqueous medium. This is called as the hydrophobic effect or hydrophobic interactions, shown in Fig 16.[67, 68] Lipid bilayers stabilized through the variety of forces which promote molecular interactions in biological systems which are followed as

1) Molecules of water are released from the hydrocarbon tails of membrane lipids as these tails become sequestered in the nonpolar core of the bilayer.[69]

2) In addition, van der Waals attractive forces among the hydrocarbon tails favor close packing of the tails.[70]

3) There are electrostatic and hydrogen bonding attractions between the polar head groups and water molecules.[71]

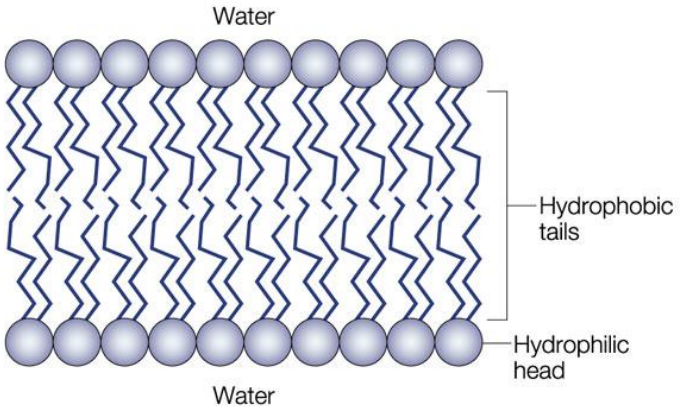


Figure 21: Schematic diagram of hydrophobic interactions in lipid bilayer.

3.3.3. Properties of lipid bilayer:

Because of hydrophobic interactions, lipid bilayer inherits unique properties.

1) Lipid bilayers have an inherent tendency to be extensive.[72]
2) Lipid bilayers tend to close on themselves so that there are no edges with exposed hydrocarbon chains and they constitute compartments.
3) Lipid bilayers are self-sealing since a hole in a bilayer is energetically unfavorable.[73],

3.4. Cubosome formation:

The liquid crystalline form nano-structured cubic states of lipids are called cubosomes. The monooleate, or other amphiphilic large molecules with the similar behaviour were used to form cubosomes. They are formed at definite temperatures where lipid bi-layer form 3-D structure with minimal surface area developing a compacted structure with bicontinuous areas of water and lipid, shown in Figure 22. Cubosomes remain in different phases of cubic structure (like P-surface, G-surface and D-surface for primitive, gyroid and diamond) arrangements correspondingly. This types of structure ultimately use as different drug delivery against verity therapeutic agent or microbial disease. [74]

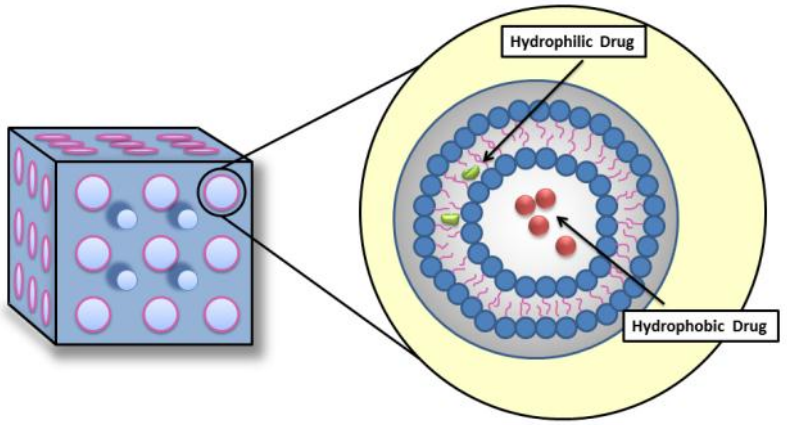


Figure 22: Schematic diagram of cubosome.

4. Further details of liposomes:

A liposome is a spherical vesicle having at least one lipid bilayer. Liposomes are artificially prepared spherical shaped vesicles consisting one or more phospholipids bilayer. Amphiphilic molecules like lipids, cholesterol etc. are the main constituting molecules in the liposome bilayer. The word liposome comes from Greek words: "*lipo*" means fat and "*soma*" means body.[75] Liposomes were first describe in the year of 1961 by British haematologist Alec D. Bangham . Today, liposome become one of the important tools in different scientific discipline, including mathematics, theoretical physics, chemistry, colloid science, biochemistry and in various clinical trial. The representative image of liposome is given below in Figure 23.

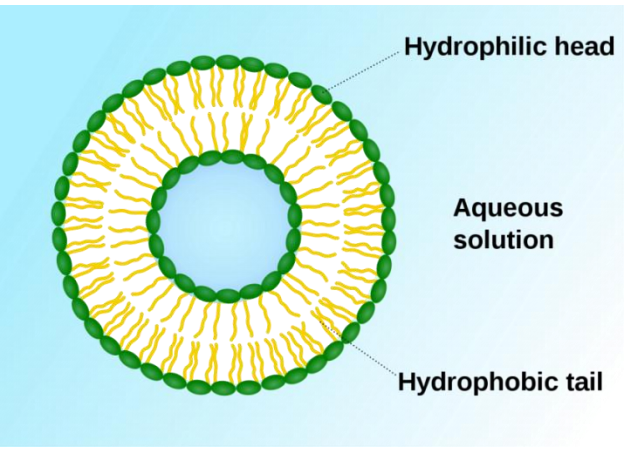


Figure 23: Schematic structure of liposome.

Due to their unique hydrophilic - lipophilic environment, it can host hydrophilic drug into the polar head group region or into the interior whereas lipophilic drug could be trapped inside the hydrocarbon region, *i.e*, acyl chain region.[76, 77] Thus artificially prepared liposome become one of the important agents for improving the delivery of the large number of drugs: enzyme vaccines, genetic material, drugs against microbial agents, anticancer drug, antifungal drugs, peptide hormones etc. [78,79, 80,81]

In many cases with many drugs, the direct oral administration may always not leads to the better result. Most of the drugs are not good enough to tolerate the resistance of enzymes in our mouth and stomach. Bile salt, intestinal flora that produce in our body, alkaline solution, digestive juices and free radicals in human body etc are smart enough to reduce the efficacy of the drug and may promote the side effect. Here liposome made its attention as a protecting vehicle for the drug and transfer it to the desire cell or tissue with controlled release. [82, 83] It also draws attraction because of its unique inert behaviour towards the entrapped drug (hydrophilic and lipophilic), ease of biodegradability, non-toxicity and improved bio-distribution of the drug with fewer side effects.[84, 85] The life time of the drug encapsulated inside of liposome also get increase as it shields the drug from different pH in human body system.

Liposomes act as drug carrier for management of nutrients and pharmaceutical purpose. By disrupting the biological membranes through sonication process liposomes are prepared. [86, 87, 88] The main composition of liposomes are phospholipids, mainly the phosphatidylcholine, besides other lipids, like egg phosphatidyl ethanolamine also used for preparation of liposome as they

easily form lipid bilayer structure. These are some different kinds of liposomes: multilamellar liposomes/vesicles (MLV), small unilamellar liposome/vesicles (SUV), the large unilamellar liposomes/vesicles (LUV), and the cochleate liposomes/vesicles. In case of MLV a number of lipid bilayers present and on the other hand for SUV have one lipid bilayer. A fewer anticipated form of liposomes is multivesicular liposomes in which one vesicle surrounds one or more smaller vesicles. Liposomes and lysosomes are two different things.

4.1. Liposome formulations: Liposomes are made by the hydration of thin lipid films or lipid cakes and stacks of liquid crystalline bilayers turn into fluid and swell. The hydrated lipid sheets separate during agitation and self-close to produce large multilamellar vesicles (LMV) which inhibits the interaction between water and hydrocarbon core of the bilayer at the boundaries. When these elements have made, the size of these particles have reduced by sonic energy (sonication) or another mechanical energy (extrusion).

The main goal in liposome formulation method is to prepare drug loaded liposome bilayer or vesicles with acute accuracy. Narrow size distribution and long term stability of the dispersion are also the primary concern in choosing the method for liposome preparation. Generally all the methods involve the lipid film hydration followed by sizing of the particle and finally removing the non-encapsulated drug. There are two kinds of methods involve in liposome preparation: Passive loading mechanical dispersion method and active loading methods.

In passive loading mechanical dispersion method the drug either in aqueous phase or organic phase is encapsulated by adding it before or during liposome preparation. One would get high drug encapsulation efficiency by using this method. On the other hand in active loading method, a diffusion gradient of the ions or drugs around the inner and outer phase of the aqueous layer is generated. The diffusion of the drugs thus gets loaded into the liposome. The most common used methods for the preparation of the liposome are: thin-film hydration method, injection methods, sonication, membrane extrusion, micro emulsification, reverse phase evaporation method and calcium induced fusion method.

Properties of lipid formulations can vary depending on the composition (cationic, anionic, neutral lipid species), however, the same preparation method can be used for all lipid vesicles

regardless of composition. The different technique for preparation of liposomes are given bellow:

4.1.1. Thin film rehydration: It is one of the most simple and common method for the preparation of liposomes. The desire amounts of lipidic components were taken in round bottom flask chloroform, dichloromethane, ethanol and chloroform-methanol mixture (2:1 v/v; 9:1 v/v; 3:1 v/v). A thin lipidic layer was formed by the evaporation of organic solvent under the

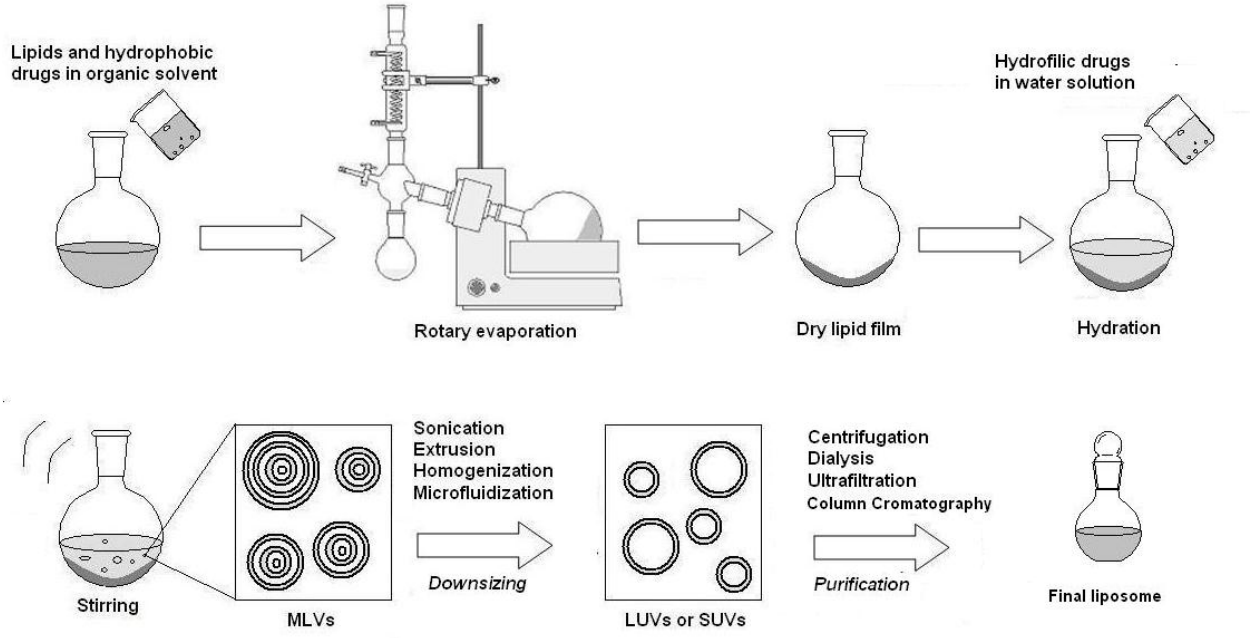


Figure 24: Thin film rehydration method for preparation of liposome.

influence of vacuum. The traces/minute quantity of organic solvent was further removed by the stream of nitrogen at temperature 4 ºC. Distilled water, phosphate buffer solution with varying pH and normal saline buffer solution could be used for the hydration of the thin film. It was hydrated at temperature 60-70 ºC for 1h. Total lipid hydration of the liposomal dispersion was done by allowing the lipid film to hydrate overnight. All kind of lipidic mixture can be used in this method for the preparation of the liposome. One of the important drawbacks of this method is the irregularity of the particle size which leads to the formation of heterogeneous system.

4.1.2. Ether injection: Lipids are dissolved in ether or ether/methanol combination solution. Then it is gradually mixed into an aqueous solution of encapsulated resources at 55-65 ºC with decrease pressure. Under the vacuum, the evaporation or elimination of the organic layer leads to

the liposome preparation. Reports suggest the drawbacks for this method are the exposure of the encapsulated material to the organic solvent and high temperature. Irregular particle size distribution also leads to the heteroginaty.[90]

4.1.3. Ethanol Injection Method: A lipid solution of ethanol is quickly injected to a large amount of buffer or distilled water. Instantly the liposomes are made in the mixture. The drawbacks of this method are the heterogeneity in liposome size. Liposomes are very dilute and the exclusion of the ethanol is very tough as it produce azeotrope with water. Formation of the azeotrope restricted this kind of liposomes to apply in biological system. [91, 92, 93]

4.1.4. Ultrasonic homogenization: This method includes the transformation of size of the liposomes. MLV prepared in thin film rehydration technique, is sonicated by bath or probe sonicator to prepare homogeneous dispersion of small unilamellar vesicles. The difficulties associated with this method are: low encapsulation of the drug due to lesser volume of each particle, the overheating could possibly degrade the liposomal dispersion. The tip of the probe sonicator tends to release Ti particle that may contaminate the dispersion. [88, 90]

4.1.5. Reverse phase evaporation: This method involves the formation of water-in-oil emulsion by means of brief sonication of a two phase system. The two phase system containing a lipidic solution of organic solvent (diethyl ether, isopropyl ether or mixture of isopropyl ether-cholroform) and aqueous phase. Phosphate buffer solution or citric-Na_2HPO_4 buffer is added to the aqueous phase to improve the efficiency of the liposomal dispersion. The final liposomal gel was formed by the evaporation of the organic solvent by rotary evaporator under constant vacuum. The residual solvent was removed by continued rotary evaporation under reduced pressure. The principle advantage of this method is high encapsulation efficiency. However a disadvantage is also there. Possible reaming of the solvent in the formulation may restrict its application. [87, 94, 95]

Due to the curve like structure, it enclosed some part of the solvent into their interior. The size of the vesicles starts from 20 nm up to several micrometers depending upon the pattern of aggregations between the amphiphiles. However liposome's properties could be changed by altering or tuning the charge of the head group, acyl chainlength, addition of other amhiphiles etc. The properties of liposome could be altered by altering the type of PL, head group charge, size, hydrocarbon chain saturation-unsaturation. Liposome of various types has been formulated

so far.[96, 97] Apart from the precursor, different ways of liposome preparation also leads to create different type of liposomes.

4.2. Classification of liposome: Liposome can be classified into two categories: on the basis of composition and hydrodynamic size.

4.2.1. On the basis of composition: Liposomes are the aggregative product of natural and/or synthetic lipids along with 30% cholesterol. Other hydrophilic polymer conjugate lipids could also play the role in formation of liposomes. Thus liposome with different surrogates generate liposomes with different properties. The major focus on liposomes relate to its capability as a drug delivery agent. Being a drug delivery agent its efficacy towards the target cells and effect on normal cell, tissues is primary concern. Depending upon the terms of composition and mechanism of drug delivery, it could be classified into five different types. (i) Conventional liposome (ii) pH-sensitive liposome (iii) Cationic liposomes (iv) Immunoliposomes and (v) Long-cerculating liposomes (LCL)

4.2.2. On the basis of hydrodynamic size: The size of the liposome can range from 50 to 1000 nm. The size of the liposome is vital parameter to determine its circulation life time. Bilayer controls the incorporation of the drug; which is a direct size dependent phenomenon.[98] Depending on the size, liposome is divided into three categories: (i) small unilamellar vesicles (SUV) (ii) large unilamellar vesicles (LUV) and (iii) multilamellar vesicles (MLV). For SUV, the size is less than 50 nm, whereas for LUV it's higher than 50 nm. MUV consisted of more than one bilayers, size ranged from 500 to 1000 nm.

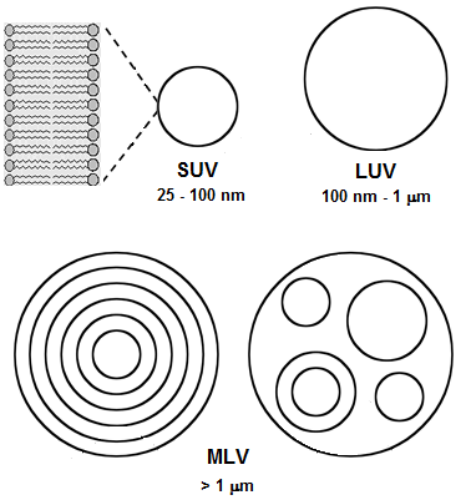


Figure 25. Representative images of different liposomes.

4.3. Advantages and disadvantages of liposome:

Advantages	Disadvantages
Liposomes increase the stability and therapeutic index of drug (actinomycin-D)	Solubility of liposomes is very low
Liposomes enhance the stability via encapsulation	Liposomes show short half-life
Liposomes are non-toxic and flexible in nature. They are biocompatible, biodegradable, and non-immunogenic for widespread.	Sometimes liposome containing phospholipid go through oxidation and hydrolysis-like reaction
When drugs/molecules/toxic agents (amphotericin B, Taxol) are encapsulated by liposomes, it reduces the toxicity.	Leakage and fusion of encapsulated drug/molecules
Liposomes support to reduce the exposure of sensitive tissues to toxic drugs.	Liposome manufacture cost is high
Liposomes show site avoidance effect.	Fewer stables
Elasticity to couple with site-specific ligands to attain active targeting	

4.4. Applications of liposome:

Liposomes are used as pharmacology and medicine technology which can be distributed into therapeutic and diagnostic applications of liposomes comprising drugs carrier or usage as a model or any reagent in the undeveloped studies of cell membrane. Unluckily various drugs have a very constricted therapeutic window, significance that the therapeutic application is not considerably lower than the toxic one. In a number of cases the toxicity can be decreased or the efficacy improved by the use of a suitable drug carrier which modifies the temporal and spatial scattering of the drug, i.e. its pharmacokinetics and biodistribution. Various applications of liposomes are specified bellow.

1. Liposomes are used in chelation therapy for treatment of heavy metal poisoning.
2. Liposomes performance as protein carriers in immunology.
3. They used as sustained or controlled delivery.
4. Liposomes used as diagnostic imaging of tumours.
5. They show intracellular drug delivery properties.
6. They used as site-avoidance delivery.
7. Enzyme replacement
8. Liposomes are used to study the membrane property.
9. They are used as oral drug delivery systems.
10. Liposomes are used as gene therapeutic agent.
11. Liposomes are used to prepare various cosmetics in our recent days.

4.5. Loading of Drugs by Liposomes: There are two types of drug loading capacity were seen for liposome.

4.5.1. Encapsulation of Hydrophilic Drugs: Encapsulation of hydrophilic drugs effects in hydration of lipids hydrophilic drugs mixture. By this method, drugs can move in the liposome core and other materials persist in exterior part of the liposome. Stayed materials will eliminate drug entrapment in liposome. In order to clean these two parts (drugs and remained outside materials), gel filtration column chromatography and dialysis are used. In addition, dehydration and rehydration method may be applied for high encapsulation of the DNA and proteins.

4.5.2. Encapsulation of Hydrophobic Drugs: The phospholipid bilayer of liposomes is a region of hydrophobic drug encapsulation. By entrapment of this type of drugs (such as curcumin), movement of drug will be reduced towards the exterior aqueous and interior parts of liposomes. These drugs are encapsulated through solubilizing of drug in the organic solvent and phospholipids. Region of drug entrapement in liposome is the hydrophobic part of liposome.

After that, it is promising to use laser light for activation of drug due to the treatment of wet macular degeneration.

4.6. Advantage of liposome drug delivery systems:

1) Liposomes are suitable for delivery of hydrophobic, hydrophilic and amphipatic drugs and agents.
2) They are chemically and physically well characterized entities
3) Liposomes are bio compatible.
4) Liposomes are used as carrier for suitable for controlled release drug delivery.
5) They are suitable to give localized action in particular tissues.
6) Liposomes are suitable to administer via various routes.
7) They increase the efficiency and therapeutic index.
8) Liposomes improve the pharmacokinetics properties.
9) They can be made into verity of drug.
10) They show lowest antigenicity properties.

4.7. Disadvantages of liposome drug delivery systems:

1) Their rapid clearance from circulation due to uptake
2) By the reticuloendothlial system (RES), primarily in the order
3) Leakage of encaptulation drug delivery during storage
4) Batch to batch variation
5) Once administered, cannot removed
6) Difficult in large scale manufacture and sterilization
7) Physical and chemical stability

8) Very production cost

9) Possibility of dumping due to faulty administration.

5. **Dendrimer:**

Dendrimers are synthetically prepared hyper branched macromolecules which contain high number of active termini that describe their properties and functions.[8-9] The term dendrimers arises from Greek words "dendron" meaning "tree" and "meros" meaning "branches". Because of perfect branching; dendrimers have the maximum number of terminal functionalities of any polymeric material at a given molecular weight and are perfectly mono dispersed (shown in Figure 26). Dendrimers compared to the corresponding linear polymers possess architectural advantages in terms of drug delivery: (i) reproducible pharmacokinetic behavior (due to mono disperse nature);[10] ii) globular shape provides superior biological and rheological properties;[11-12] and iii) controlled multivalency can attach several molecules (drugs, imaging agents, cell-penetrating peptides, targeting groups, and solubilizing moieties etc.

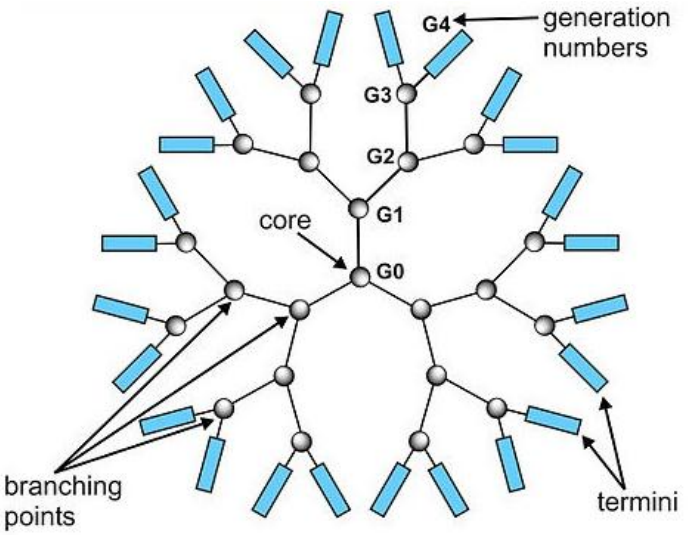


Figure 26. Schematic diagram of dendrimer.

Dendrimers are polymeric, hyper branched three dimensional tree-like architecture widely used in the field of nanotechnology.[99] These synthesised macromolecules can be fabricated by controlling its molecular weight, degree of branching, globular shape and surface functionalities and the size measured as reported ranging from 2.5 nm to 10 nm.[99-100] It exhibits homogeneous

symmetrical monodisperse structure with a typical core inside, involving an inner shell and outer shell structure. Its solubility in the solvent is highly dependent on the nature of the surface groups. Hydrophilic surface groups of dendrimers are soluble in polar solvent like water whereas hydrophobic surface group are soluble in non-polar solvent. Interaction can be proceeds either with the groups inside called "endorecepters" or the groups on the periphery well-known as "exorecepters" liable on the type of host-guest. Dendrimer structure can be categorized into three parts. (i) a multifunctional core moiety connecting the dendrons, (ii) radially linked repeating layer of branches connected with the core and (iii) a terminal surface groups as represented in the figure. Dendrimers have grown a wide-ranging of applications in supra molecular chemistry, mostly in host-guest reactions and self-assembly methods. Dendrimers are described by special features that make them auspicious aspirants for a lot of applications.[101] The role of dendritic molecules for anticancer therapies and diagnostic imaging is outstanding. The benefits of these well-defined materials make them the latest class of macromolecular nanoscale delivery procedures. Dendritic macromolecules have a tendency to linearly rise in diameter and espouse a more globular shape with enhancing dendrimer generation. Hence, dendrimers act as model delivery vehicle for clear study of the properties of dendrimer size, charge, and composition on biologically related properties.[102], [103]

5.1. Structure and chemistry of dendrimer: The structure of dendrimer molecules generates with a central atom or group of atoms termed as the core. From this central groups or atoms, the subdivisions of other atoms entitled 'dendrons' generates via various chemical reactions. There continues to be a argument about the proper structure of dendrimers, Especially whether they are fully extended with maximum density at the surface or whether the end-groups fold back into a densely packed interior different chemical events taking place at the dendrimer surface. [101] The unique building block structure of dendrimers gives a special prospects for host-guest type chemistry and is mainly well prepared to comprise in multivalent interactions. At the same time, one of the first recommended applications of dendrimers was as carrier like compounds, wherein small substances are bound inside the core side of the dendrimer

5.3. Physical properties of different generation of dendrimer.

Generation	Molecular weight	Diameter	Surface group
0	517	15	4
1	1,430	22	8
2	3,256	29	16
3	6,909	36	32
4	14,215	45	64
5	28,826	54	128
6	58,048	67	256

5.4. Synthesis of dendrimer: Dendrimers are not only the material from molecular chemistry but also polymer chemistry. As they prepared by the feature of their step-by-step well-ordered synthesis thus they tell to the molecular chemistry substance. On the other hand as dendrimers have the repetitive structure made from monomers so they are class of polymeric substances. They are in three types macromolecular architectural classes like linear, cross-linked, and branched. Dendrimer have definite size and molecular weight. Dendrimers are usually synthesized using two different methods like divergent and convergent. In the altered methods, dendrimer produces outward from a multifunctional core molecule. After reaction between core molecules and monomer molecule which is enclosed by one reactive and two dormant groups, form the first-generation dendrimer. After that again the new molecule is activated for reactions with more monomers. Dendrimer can be producing by changing functionality in each of this portion to categorize properties such as solubility, thermal stability, and addition of compounds for meticulous application. Synthetic procedure can also correctly manage the size and number of branches on the dendrimer. There are two different methods of dendrimer synthesis, divergent synthesis and convergent synthesis. [101]

5.4.1. Divergent Method: The dendrimer is manufacturing to from a multifunctional voids, which prepared by the subsequent of reactions, commonly a Michael addition type of reaction.

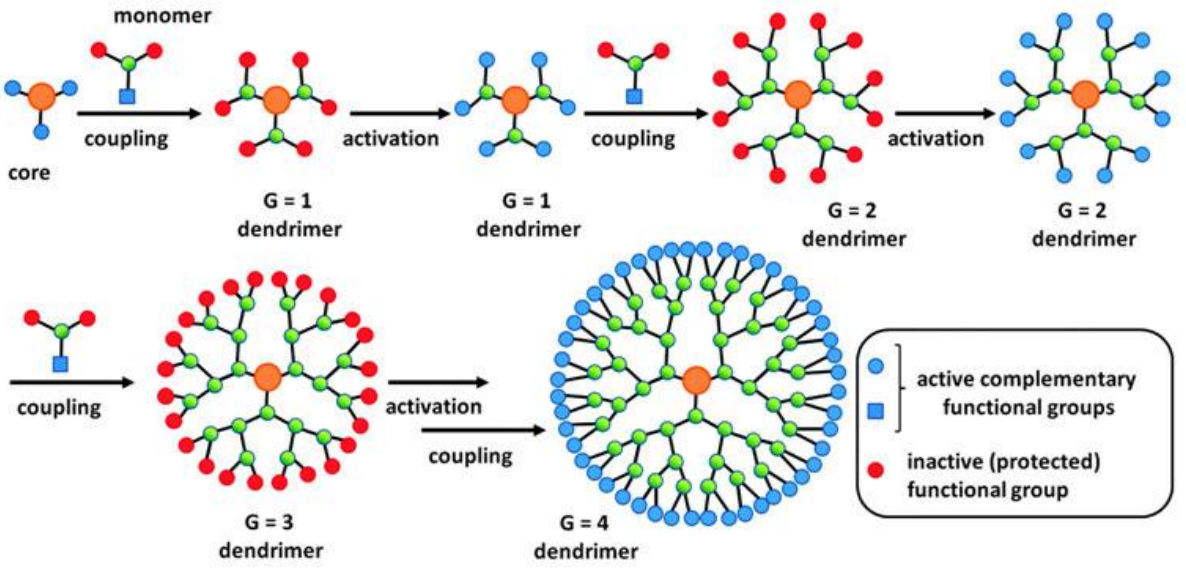


Figure 27. Divergent growth method for dendrimer synthesis. ***J. Chem.*, 2014, 38, 2168-2203**

For a single step of the reaction must be strong-minded to full completion of the reaction to inhibit mistakes in the dendrimer, that can grounds trailing generations (few branches are smaller than the others). These impurities can pileup the functionality and structure of symmetry of the dendrimer, but are terrifically hard to cleanse out because the virtual size variation between pure and impure dendrimers are very small. The main disadvantage of this method is that the incomplete development and the side reactions lead to defect of dendrimers. To decrease these side reactions and imperfections needs to recommend to usage a higher amount of reagents. The divergent growth reaction of dendrimer is shown bellow. [104]

5.4.2. Convergent Method: Dendrimers are constructed from starting point of small molecules which finished up at the surface of the sphere. The reactions precede deepest building internal and are suddenly attached to a core side. This types of method creates it very easier to remove the impurities, so that the final dendrimers are the type of mono-disperse. Therefore, the dendrimers completed by this way are not so high as those made by other method (divergent methods). Because by this method it is crowding due to stearic property of the core is restrictive 10. The convergent growth reaction are shown show in figure 28. Main problem of convergent method is that we cannot manufacture a large molecule for drug loading due to crowding and stearic effect. [105]

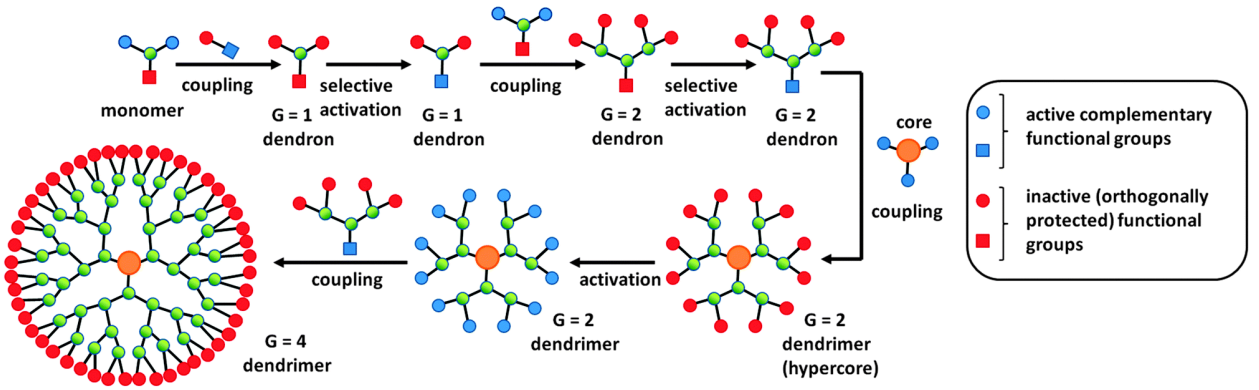


Figure 28: Convergent growth method for dendrimer synthesis. *New J. Chem.*, 2014, 38, 2168-2203

5.5. Classification of dendrimer:

Classifications of dendrimer sort out through the basis of dendrimer generation. Generation depend on the number of repeated branching cycles that are accomplished throughout its synthesis. For example when a dendrimer is prepared by convergent synthesis by performing the branching reactions onto the core molecule three times, then it is called 3rd generation of dendrimer. For each consecutive generation affects in a dendrimer approximately twice the molecular weight of the former generation. Higher generation of dendrimers having higher number of end group can later be used to modify the dendrimer for a particular application.

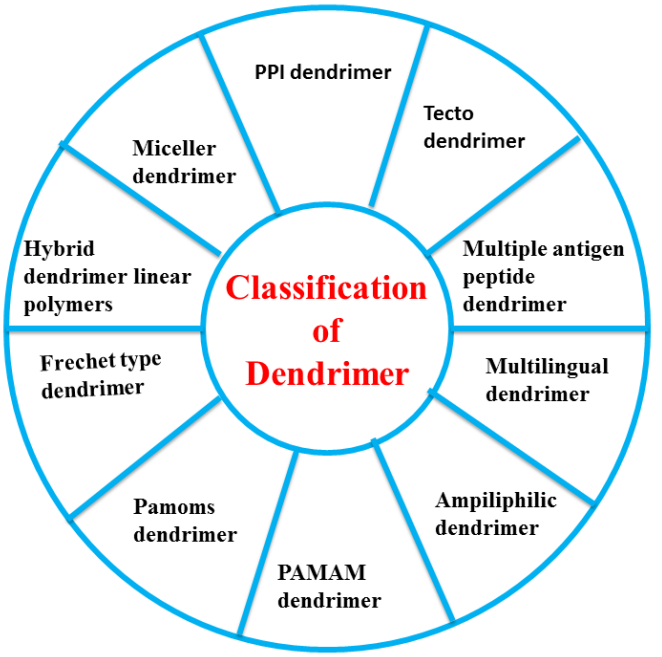


Figure 29: Classification of dendrimer.

5.6. PAMAM dendrimer: Poly (amidoamine), or PAMAM dendrimers are the fourth class of synthetic polymers, characterized as highly branched, globular, monodispersed (single molecular weight distribution) and reactive molecule which is prepared of repetitively branched subunits of amide and amine functionality. Since the first successful synthesis of poly(amidoamine) (PAMAM) dendrimer in the mid-1980s by Tomalia's group, dendrimers have received much attention, in particular, the biomedical applications of dendrimers in various medical and pharmaceutical field including drug delivery, gene transfection, nanoscale drug and diagnostic tools have regarded to be highly promising and have drawn eminent interest and studies in these few areas. PAMAM dendrimers, sometimes referred to by the trade name Starburst and represent the most well-characterized dendrimer. Similar to other dendrimers, they have a sphere-like structure having the internal molecular construction of tree-like branching, with each outward 'layer'. This branched architecture differentiates PAMAMs and other dendrimers from other normal polymers, as it permits for low polydispersity and a high level of structural control for the duration of synthesis, and gives increase to a huge number of surface

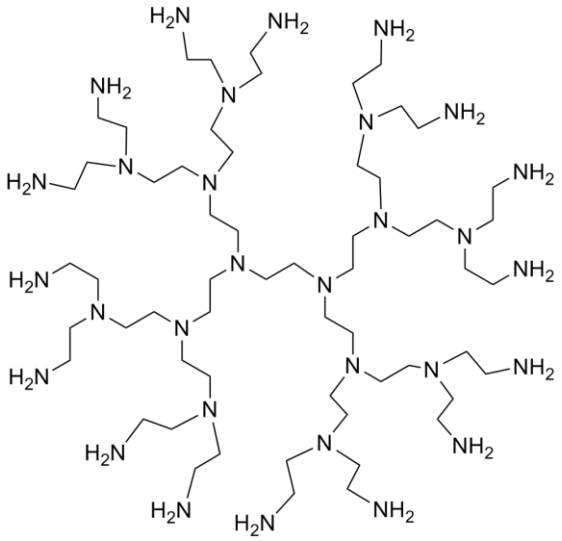


Figure 30: Molecular structure of PAMAM dendrimer.

sites comparative to the total molecular volume. Furthermore, PAMAM dendrimers display higher biocompatibility than other dendrimers, possibly due to the organization of surface amines and interior amide bonds; these bonding themes are greatly important for biological chemistry as PAMAM dendrimers shows similar properties with globular proteins. Comparatively ease/little cost of production of PAMAM dendrimers (mainly comparative to

similarly-sized biological molecules like proteins and antibodies), with their biocompatibility, functional property, structural control and, have ready PAMAMs viable candidates for use in drug improvement, biochemistry, and nanotechnology. [106]

5.7. Application of dendrimer: Now a day's dendrimers have numerous medicinal and practical applications.

5.7.1. Dendrimers in biomedical field: Dendrimers have improvement in different biomedical uses. They are equivalent to protein, enzymes, and viruses, and are simply functionalized. Dendrimers can either be close to the outside edge or can be encapsulated in their internal cavities. Present drug uses a variety of this substance as potential blood substitutes, e.g., polyamidoamine dendrimers.[106]

5.7.2. Anticancer drugs: Possibly the best auspicious potential of dendrimers is in their probability to execute controlled and definite drug delivery, which concerns the area of nanomedicine. One of the best important problems that are established toward recent medicine is to recover pharmacokinetic assets of drugs for cancer. Drugs are conjugated with these polymers which are described by half-life, higher stability, water solubility, reduced immunogenicity, and antigenicity. Distinctive pathophysiological behaviors of tumors like extensive angiogenesis causing in hypervascularization, the enlarged penetrability of tumor vasculature, and restricted lymphatic drainage allow impassive targeting, and as a consequence, selective addition of macromolecules in tumor tissue.[107], [108],

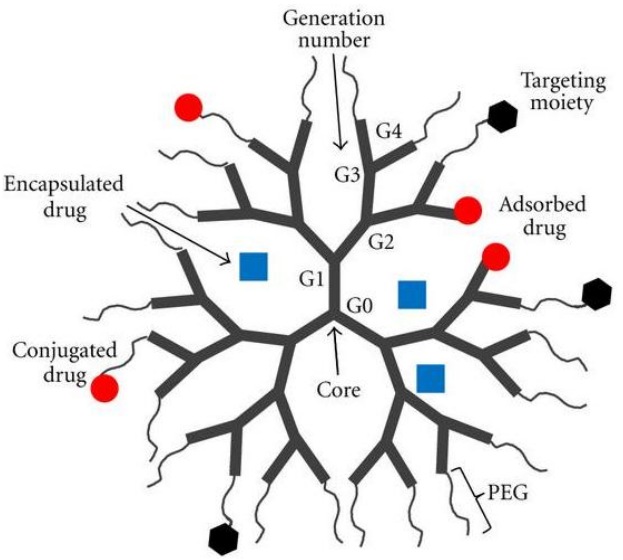


Figure 31. Schematic diagram of dendrimer as drug delivery.

5.7.3. Dendrimers in drug delivery: In 1982, Maciejewski planned, fat first, the use of these highper branched polymeric molecules as molecular containers. Host-guest properties of dendritic polymers are currently under scientific investigation and have gained crucial position in the field of supramolecular chemistry. Host-guest chemistry is based on the reaction of binding of a substrate molecule (guest) to a receptor molecule (host) shown in Figure 31. Transdermal drug delivery Clinical have used the NSAIDs, which is limited due to antagonistic reactions such as GI side special effects and renal side special effects when specified orally. Transdermal drug delivery incapacitates these corrupt effects and also conserves therapeutic blood level for a long time. Transdermal delivery undergoes lowly rates of transcutaneous delivery because of barrier function for the skin. Dendrimers have originate variety of applications in transdermal drug delivery schemes. Commonly, in bioactive drugs which contains hydrophobic parts in their structure and low soluble in water, dendrimers are a good carrier in the field of different delivery system. [109], [108], [108]

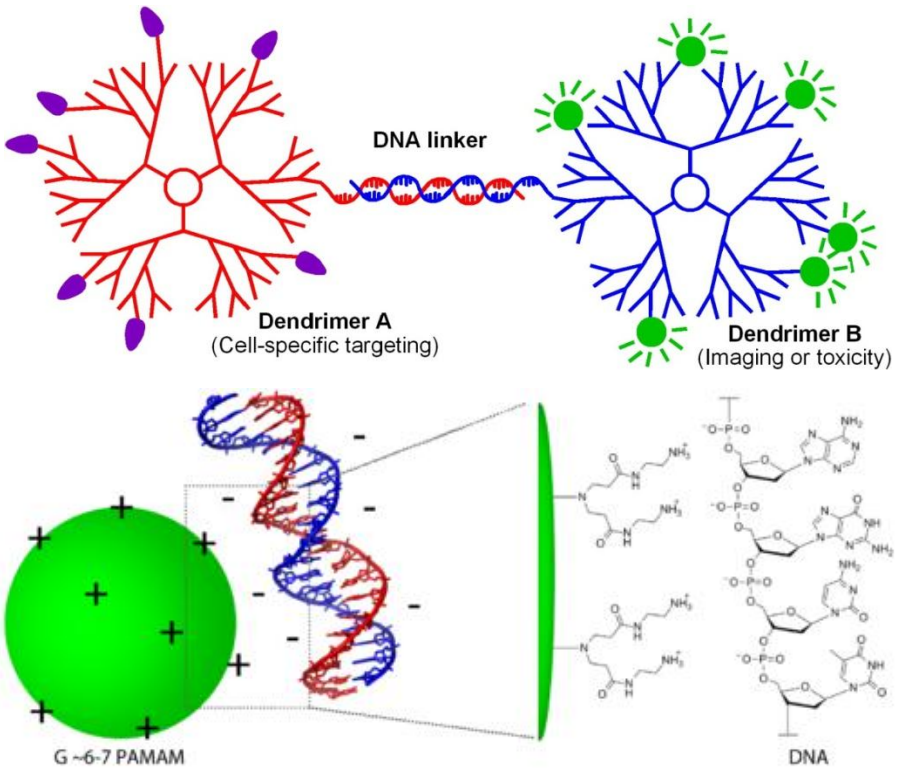


Figure 32. Schematic diagram of dendrime as gene delivery.

5.7.4. Gene delivery: The primary potential that the mixture of thoughtful the molecular paths of infection and the whole human genome arrangement would produce nontoxic and more effective medicines and change the way we pleasure patients has not been satisfied to date. Conversely, there is tiny hesitation that genetic therapies will create a important support to our therapeutic armamentarium when some of the key contests, such as definite and well-organized delivery, have been resolved. The capability to deliver parts of DNA to the requisite parts of a cell comprises many contests. Present investigation is being completed to discovery ways to custom dendrimers to transportation genes into cells without harmful or disengaging the DNA. To conserve the activity of DNA during dehydration, the dendrimer/ DNA complexes were captured in a water soluble polymer and then dropped on or sandwiched in well-designed polymer films with a fast degradation speed to facilitate gene transfection. PAMAM dendrimer/DNA complexes were used to capture functional biodegradable macromolecule polymer films for substrate-mediated gene delivery. Investigation has shown that the fast-degrading functional polymer has abundant potential for restricted transfection. [110], [111], [112]

5.7.5. Dendrimers as magnetic resonance imaging contrast agents:

Dendrimer-based metal chelates act as magnetic resonance imaging contrast agents. Dendrimers are extremely appropriate and used as image contrast media because of their properties. [113]

5.7.6. Dendritic sensors:

In a single molecule, dendrimers have large numbers of functional groups on their surface side. This makes them outstanding for applications where covalent linking or close proximity of a large number of classes is significant. Balzani and coworkers inspected the fluorescence of a 4^{th} generation of poly (propylene amine) dendrimer containing 32 dansyl units at the boundary. As this dendrimer contains 30 number of aliphatic amine units in the structure, it is appropriate for metal ions which are able to coordinate. It has been seen that when a Co2+ ion is amalgamated into the dendrimer, the heavy fluorescence for dansyl units is quenched. Little concentrations of Co2+ ions ($4.6 \times 10{-}7$ M) can notice by a dendrimer concentration of $4.6 \times 10{-}6$ M. The many fluorescent groups on the surface help to intensify the sensitivity of the dendrimer as a sensor. [114, 115]

5.7.7. Dendrimers used for enhancing solubility:

PAMAM dendrimers are probable to have potential uses in increasing solubility for drug delivery schemes. Dendrimers have hydrophilic surfaces, which are accountable for its unimolecular micelle like properties. Dendrimer-based transporters offer the chance to increase the oral bioavailability of difficult drugs. Thus, dendrimer nano transporters suggest the potential to increase the bioavailability of drugs that are slightly soluble and/or substrates for efflux transporters.[116], [117], [118]

5.7.8. Photodynamic therapy:

Photodynamic therapy (PDT) trusts on the initiation of a photosensitizing mediator with visible or near-infrared (NIR) light. By excitation, a extremely energetic state is made which, by the reaction with oxygen, gives a extremely sensitive singlet oxygen accomplished of making necrosis and apoptosis in tumor cells. Delivery of PDT agents has been examined within the last few years in order to progress upon tumor choosiness, holding, and pharmacokinetics. [119], [120],

6. Other drug delivery systems:

Colloidal drug carrier systems such as micelle, vesicles, liquid-crystalline system as well as nanoparticle dispersion etc with diameter 10 to 400 nm show promising agent as drug carrier. With the aid of advance technologies, researchers are developing drugs with high and promising activity. Most of the drug mainly consists with DNA and/ or protein and are characterized on the basis of its bioactivity; liberate their bioactive material right time with right concentration and limited toxicity. When developing the formulation of the drug carrier, the aim is to get an optimized system that has well encapsulation efficacies, long shelf life and low toxicity with controlled release of the drug. So carrier such as colloidal medium, nanoparticle gets special attraction due to the biocompatibility, biodegradability and biomimiking phenomenon. General description about different drug carrier system is discussed below.

6.1. Microemulsions and nanoemulsions: Microemulsions is composed of water, oil, surfactant and co-surfactant[121] are transparent and optically isotropic single –phase liquid solution. The system is bicontinuous with low viscosity and is thermodynamically stable. The solubilisation of both hydrophilic and lipophilic drug in microemulsions could be achieved. The

limited use of microemulsions for dermal and oral application is a significant drawback. Nanoemulsions come into the picture in 1950's to the need of parenteral nutrition. It has been introduced into the market as various pharmaceutical products, such as: disoprivan, Etomidat lipuro, diazepam lipure, steasolidand lipotalon. Nanoemulsions are the two component heterogeneous system where one liquid is dispersed as droplets in the other one. As a drug delivery agent, it reduces the local and systematic side effect. However it has some limitation when lipophilic drug is concerned as the presence of oil solubilizes the drug.

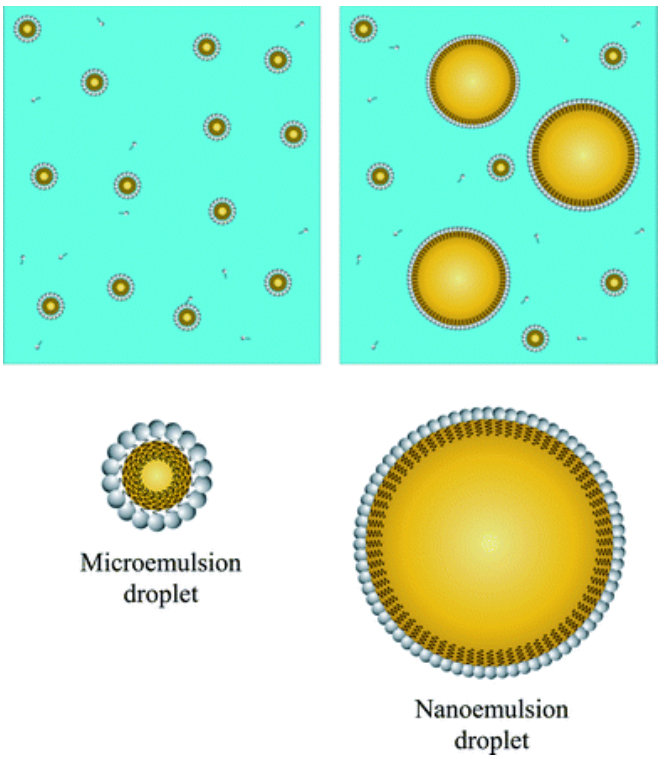


Figure 33. Scheme representative of microemulsion and nanoemulsion.

6.2. Hydrogels. Hydrogels are hydrophilic three dimensional polymeric networks composed of homopolymers and co-polymers and can absorb large quantity of biological fluid or water. [122] Due to the chemical or physical crosslinks between the polymers, they are insoluble in water. Hydrogels are on the forefront of control drug delivery as it shows thermodynamic stability in water and can modulates the drug release with respect to temperature, ionic strength, pH, electric field etc. This versatility of hydrogel makes it as an superior agent for the specific drug delivery.

6.3. Nanoparticles. It includes nanocapsuls and nanospheres having size 10 – 200 nm and is crystalline or amorphous. They have the capabilities to encapsulate or absorb the drug and act as a protective barrier. In the recent years it draws researcher's attention because of the controlled release of the drug and targeting drug delivery. It has the ability to deliver genes, proteins and peptide through the peroral route. [123-124]

6.4. Solid lipid nanoparticles. Solid lipid nanoparticles (SLN) are composed with solid lipid core matrix that can solubilise lipophilic molecules. It is spherical shape and has the diameter 10 – 1000 nm. Lipids such as triglycerides, diglycerides, monoglycerides, faty acids, wasex, and steroids are used to prepare SLN. The lipid core is stabilized by adding surfactants based on the charge and molecular weight. SLN is used in variousapplications such as parental, pulmonary, ocular deliver of drugs. [125]

7. Importance of monolayer study:

The mutual miscibility and stability of the binary monolayers of zwitterionic phospholipids with negatively charged phospholipids at the air-buffer interface and the interaction of positively charged dendrimer with the stable monolayers have been studied in detail using surface pressure area isotherms. The surface pressure-area isotherms indicated that the binary monolayers of zwitterionic lipid and negatively charged lipid at the air-water interface were miscible and more stable than the monolayers of the two individual components. Based on the regular solution theory, the miscibility and stability of the two components in the monolayer were analyzed in terms of compression modulus and excess Gibbs free energy. Our main object is to recognize which binary composition is considered to be liposome formulation. Also the kinetic behavior of liposome disintegration to monolayer is carried out by monolayer study.

8. Dendrimer-liposome interaction:

Since the first successful synthesis of poly (amidoamine) (PAMAM) dendrimer in the mid-1980s by Tomalia's group, dendrimers have received much attention, in particular, the biomedical applications of dendrimers in various medical and pharmaceutical field including drug delivery, gene transfection, nanoscale drug and diagnostic tools have regarded to be highly promising and have drawn eminent interest and studies in these few areas . A few numbers of these dendrimer molecules are already now available on the market (e.g. Polyfect® and SuperFect® as in vitro

transfection agent) or have proceeded to clinical trial as dendrimer-based pharmaceutical (e.g. Viva Gel TM as microbicide against HIV or other sex transmitted diseases) , and the numerous successful studies of dendrimers in various other applications including Boron neutron capture therapy for cancer treatment have allowed dendrimer to replace other traditional polymers as the revolutionary agent in the biomedical field. They include development as: inherently active drugs (e.g., as antiviral and antimicrobial agents and modulators of angiogenesis); drug-carriers for targeting and controlled release (particularly as anticancer agents); non-viral vectors to promote oligonucleotide and gene delivery. Dendrimers are also being studied as medical imaging agents, as components of tissue engineering scaffolds and as adjuvants for vaccine delivery.

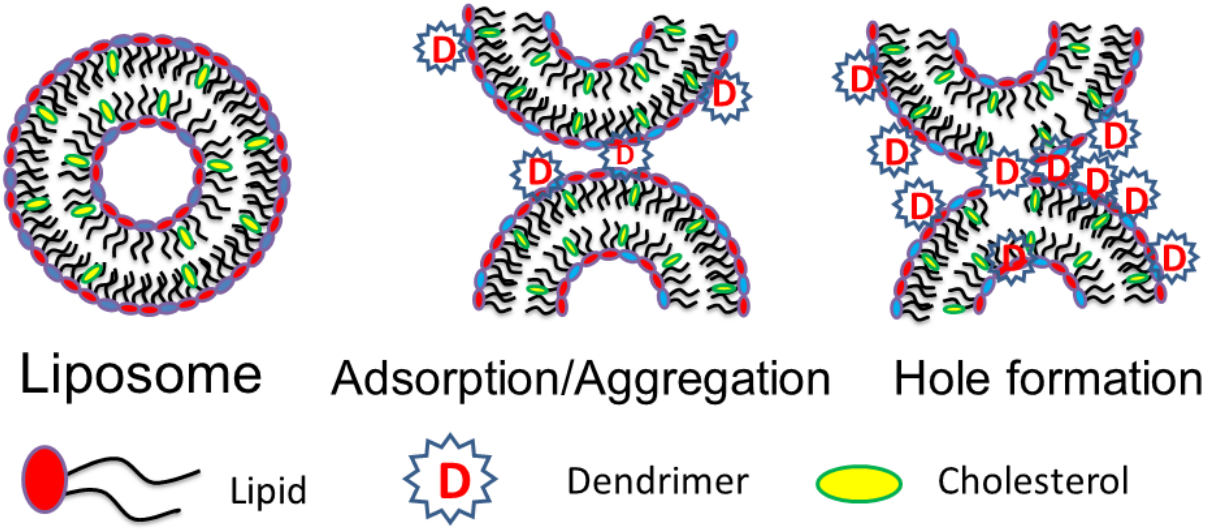


Figure 34. Schematic diagram for the formation of dendrimer-liposome aggregates

The field of biomedical dendrimers is yet not completely studies, but the eruption of attention in dendrimers and dendronised polymers as fundamentally active therapeutic mediators, as vectors for engaged delivery of drugs, peptides and oligonucleotides, and as permeability enhancers capable to stimulate oral and transdermal drug delivery creates it timely to analysis current understanding concerning the toxicology of these dendrimer chemistries (at present under improvement for biomedical applications). Apart from their antiviral properties, dendrimers have also been demonstrated to have antibacterial properties. In general, the mechanism of action of antibacterial dendrimer is based on the electrostatic interaction between the ammonium groups

on the dendrimers with the anionic charged membrane of bacteria, which in turn caused their membrane to lose integrity and thus lysis.

The physical properties of liposomes are highly dependent on the type of phospholipids constituted (i.e. negative charged DPPG or neutral charged DPPC phospholipids). intermolecular interactions as well as the conditions of the environment which surround it. Nevertheless, due to their easy generability and readily modifiable nature in terms of its lipid contents and structure (*i.e.*, unilamellar and multilamellar), liposomes have received particular attention as the simplified version of cell membrane and have been commonly employed in the investigation of drug-lipid bilayer interaction. In this proposed program, liposomes will be employed in order to study the interaction between dendrimer and lipid bilayers.

Adsorption of dendrimers on liposomes and the formation of dendrimer-liposome aggregates are the most common aspect in the interaction between dendrimers and liposomes. It is believed that the dendrimers could act as glue for the vesicles. Most of the recent studies have focused on the interactions of positively charged dendrimers with cell membranes, since greater interaction potency is expected between these dendrimers with the negatively charged cell membrane compared to other neutral or negatively charged dendrimers. However, it has also been shown that besides the surface charge of the dendrimer molecules, there are many other physicochemical properties of dendrimers which influence the interaction between dendrimers and lipid bilayers. These properties of dendrimers include the dendrimer type, generation, surface charge and also solution conditions (e.g. pH and salt) as well as the composition of membrane lipids.

Studies on the change of the zeta-potential of liposomes are assumed to provide better understanding of the nature of dendrimer-liposome interaction. It is known that the zeta-potential of liposomes would change as a result of the adsorption of charged dendrimers, while remain constant if dendrimers are internalized from the interaction with the liposomes.

The current barrier to the application of dendrimers as various therapeutic agents as mentioned above, however, is the lack of established knowledge on biocompatibility and toxicity of dendrimers in biological systems. The kinetics and mechanisms of dendrimer-lipid bilayer interaction have received considerable attention as cells contain many layers of membranes

which dendrimers need to recognize, interact and internalized before it could reach its intracellular targets. As discussed, the knowledge on this area has been expanding over the past few years, but there are still many questions left to be answered before dendrimers could be safely employed as a novel therapeutic agent.

As part of the effort to provide a better understanding of the mechanism of dendrimer interactions with lipid bilayers, this research is aimed to investigate the mechanism involved in the interaction between different generation and types of PAMAM dendrimers and liposomes (i.e. electrostatic and hydrophobic interaction), and also to provide an understanding on how the various physicochemical properties of dendrimers and liposomes could affect the dendrimer-liposome interaction. Specifically, in this research, different types of phospholipids including 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC), dihexadecyl phosphate (DHP), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPP), 1,2-dipalmitoyl-sn-glycero-3-phospho ethanol (DPPEth), L-α-phosphatidylcholine (soy Lecithin) and 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (Sodium Salt) (DPPG) and cholesterol (CHOL) will be employed to synthesize a range of liposomes of varying chemical composition, structural and physical properties. The information obtained from their interaction, including adsorption isotherm, packing bilayer and palisade layer, changes in surface charge as a result of adsorption process and aggregate formation (changes in particle size) are believed to be useful as they would provide a basis for future studies which investigate the interaction between dendrimers and cell membranes, which structurally and chemically more complex than simple lipid bilayer (*i.e.* liposomes).

Also, as dendrimers with different physical and chemical properties would be employed as therapeutic agent in future according to the need of the specific therapeutic purpose, the studies are also assumed to investigate the influence of dendrimer types on its interaction with liposomes. Three different types of PAMAM dendrimers that contain a similar surface group (amine), but different generations will be employed. Besides some polymer anchored dendrimers and dendrimers with different end functional groups will also be explored. The influences of the dendrimer type and generation on the dendrimer-liposome interaction will be studied in terms of variation in the adsorption isotherm, changes in zeta potential as well as particle size. The information obtained would be useful as they would provide an idea in future for the rational

design of dendrimers which are biocompatible and are optimized for therapeutic application. In another attempt, efforts will be put to study the kinetic and mechanistic aspects of dendrimer-liposome interaction. To meet up the final goal of the proposed work, dendrimers of different kinds and generations will be employed to perform cell (bacterial/ cell lines) growth studies. This will eventually be helpful in obtaining a proper denderimer based formulation for therapeutical uses.

9. Characterization of monolayer and bilayer:

Preparation of samples with high purity is the most important criteria. Once the sample was prepared and purified one could measure the particle size, surface charge (or zeta potential) and polydispersity of the medium by using DLS studies. Monolayer studies another important one to get knowledge about the mutual misicibility among the bilayer components. Electron microscopy (TEM and FF-TEM) and AFM studies also helpful determining the bilayer thickness. The effect of temperature on vesicles bilayer could be scrutinized. Apart from this inclusion of drug in its subsequent effect on the bilayer could also be viewed on such studies. Vesicles hosted drug also be scrutinized by measuring their entrapment efficiency, release kinetics and cytotoxicity effect. In case of vesicles/dendrimers interaction, apart from the above mentioned studies, one could perform vesicles disintegration kinetics and Isothermal carometric (ITC) measurement.

9.1. Dynamic light scattering (DLS) technique:

Liposomes are known as colloidal particles render surface charge and have hydrodynamic size in nano dimension. Being in dispersion state, it involves in Brownian motions. DLS technique is useful in determining the hydrodynamic size, zeta potential as well as polydispersity of the medium. It is worthy to mention that the hydrodynamic diameter is the hypothetical diameter of the hydrated particles that diffuse through the medium. In practice the colloidal particles are non-spherical, solvated and tumbling. Hence based on diffusion properties of particles, DLS calculates the apparent dynamic hydrated size of the particles; known as hydrodynamic size. Figure 18 represent particle size measurement curve.

A He- Ne leaser light with an emission wavelength 628 nm at an angle 90º collide with particles involved in the process of diffusions. It measures the translational diffusion coefficient (D) by collecting the light scattered by the diffused particles. Based on Stokes-Einstein's equation.

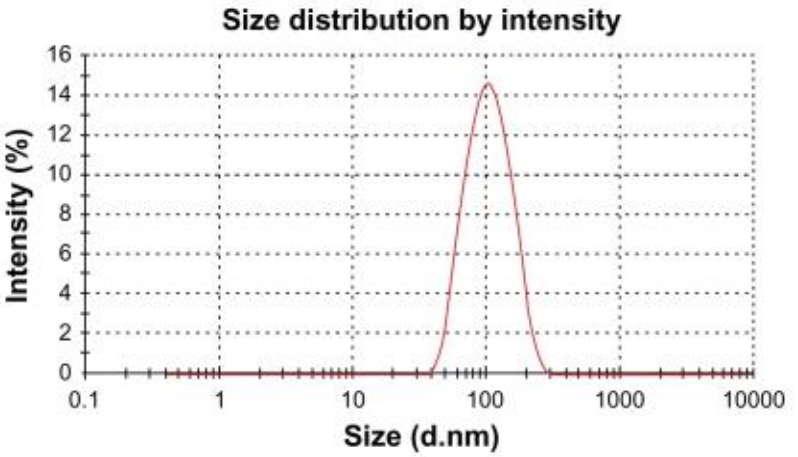


Figure 35. Hydrodyanmic size distribution as function of intensities.

$$d_h = \frac{kT}{3\pi\eta D} \quad (1)$$

where, k, T and η indicate the Boltzmann constant, temperature and viscosity of water respectively.

Hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z.P.) measured by Dynamic Light Scattering (DLS) technique. DLS measures the autocorrelation function $g^1(\tau)$ that is correlated with the size distribution of the particles by the given equation:

$$g^1(\tau) = \int_0^\infty m_\Gamma^2 P(q,\Gamma) G(\Gamma) e^{-\Gamma\tau} d\Gamma \quad (2)$$

where, $G(\Gamma)$ is the normalized number distribution function for the decay constant Γ, m_Γ is the particle mass, $P(q,\Gamma)$ is the particle scattering factor, the decay constant $\Gamma = q^2 D_T$, the scattering vector $q = \left(4\pi n/\lambda\right)\sin(\theta/2)$, θ is the angle between the incident and scattered beam, n is the refractive index of the medium and D_T is the translational diffusional coefficient. D_T is related to the hydrodynamic radius (R_h) of the particles by the given equation[126]:

$$D_T = \frac{k_B T}{6\pi\eta R_h} \quad (3)$$

where, k_B is the Boltzmann constant, T is the temperature of the dispersion medium and η is the viscosity of the medium. The zeta potential was measured using folded capillary cells. The PDI and zeta potential measurements were carried out at the temperature of 25 °C; however the size

of the liposome systems was measured in the temperature range between 25 to 50 °C. DLS studies were undertaken for three different samples of liposome formulations. The DLS results of each sample were the average of four consecutive measurements.

ZP is one of the crucial parameter that maintains the stability of the vesicles. It prevents particle collision wing to the repulsion and hence it could be understood, higher the ZP, higher is the stability of the particles. Substitution of other substances in vesicle bilayer may often change the value of ZP. Thus measurement of ZP could be useful in determining the role of other components in the bilayer. Like any charge particles, vesicles can also involve in of electrophoresis under the influence of an applied electric field. The speed of the particles depends on the size, ZP, viscosity and dielectric of the dispersion medium. Figure 18 describes a representative image of ZP of an vesicle medium.

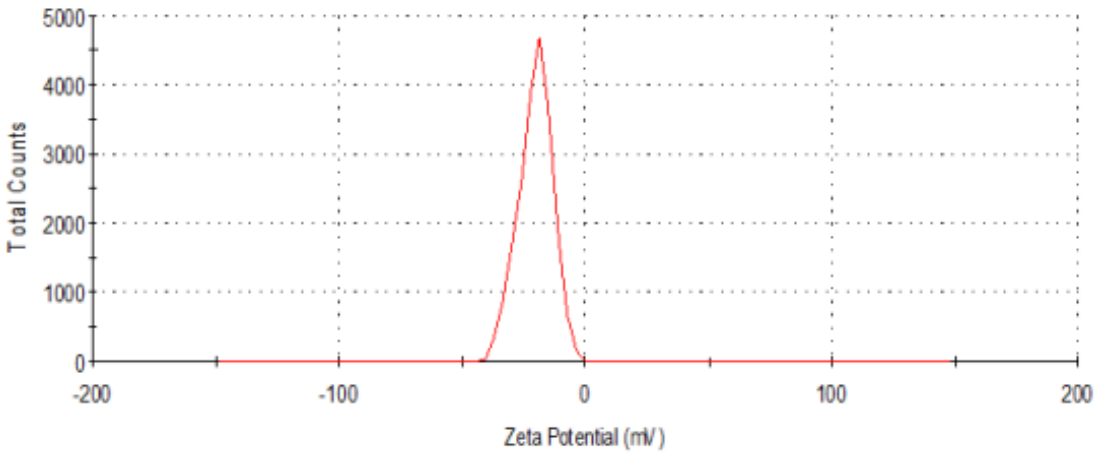


Figure 36. Representative image of ZP of a vesicular dispersion.

9.2. Turbidity measurements: Interaction of dendrimers with different liposomes was studied by measuring the % transmittance (%T) at 420 nm. It was studied by measuring the absorbance by U.V. spectrophotometer with varying concentration of ligand at a particular wavelength. At this wavelength, the turbidity of a solution is assumed to be proportional to (100-%T). [127]

9.3. TEM and FF-TEM technique: DLS study delivers the information about apparent dynamic size of the particles but the morphology of liposome can not be seen by DLS technique.

However, the details size and morphology were studied by TEM and FF-TEM technique. [128] Morphology of the sample was studied by the transmission electron microscopy by taken a drop of dilute dispersion solution was placed on Formver™ carbon-coated 200 mesh copper grid and dried under air. It was then analyzed to obtain TEM images using TEM instrument (like Hitachi H-600 transmission electron microscope (Japan) operating at 80 kV). In case of FF-TEM studies, a drop of the sample was placed onto the sample holders and frozen in liquid propane. FR-7000A (Hitachi High Technologies Ltd., Japan) was used at -150 °C for the freeze fracturing. Samples were then replicated by evaporation using platinum carbon. The replica was placed on 300- mesh copper grid, dried, and examined in a transmission electron microscope (H-7650, Hitachi High Technologies Ltd., Japan) with an accelerating voltage of 120 kV.

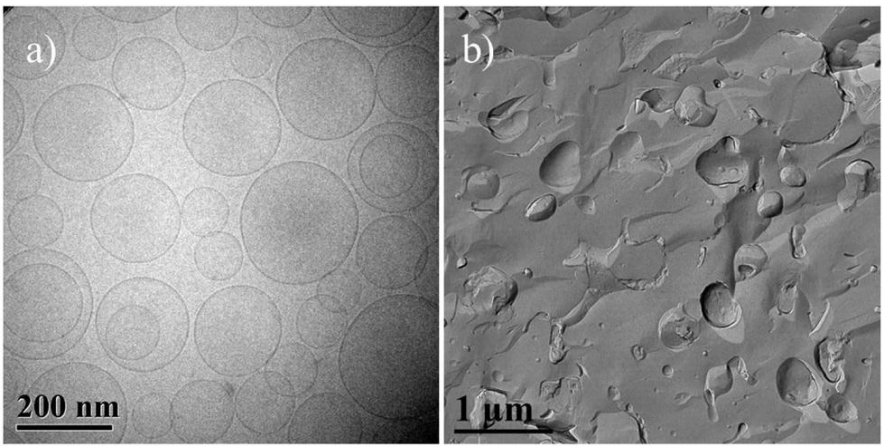


Figure 37. TEM and FF-TEM images of liposomes.

9.4. Atomic Force Microscopy (AFM) technique: A supported bilayer could be generated by way of vesicle fusion on mica/glass/gold substrate. Vesicles have the tendency to get fused over glassy substrates. Thus if a vesicular solution is placed over glass or freshly cleaved mica, it will fuse to form bilayers. These solid supported bylayers could also be considered as model membranes. Feature of the bilayers, generated onto freshly cleaved mica, will be visualized by way of atomic force microscopic (AFM) measurements. Bilyaer disruption can then be studied by way of addition of dendrimers. Bilayer images are grabbed at different resolutions. Then dendrimer solutions of desired concentration is used to rinse the bilayer. After certain time, it is again scanned to visualize the effect of dendrimers. [129, 130, 131]

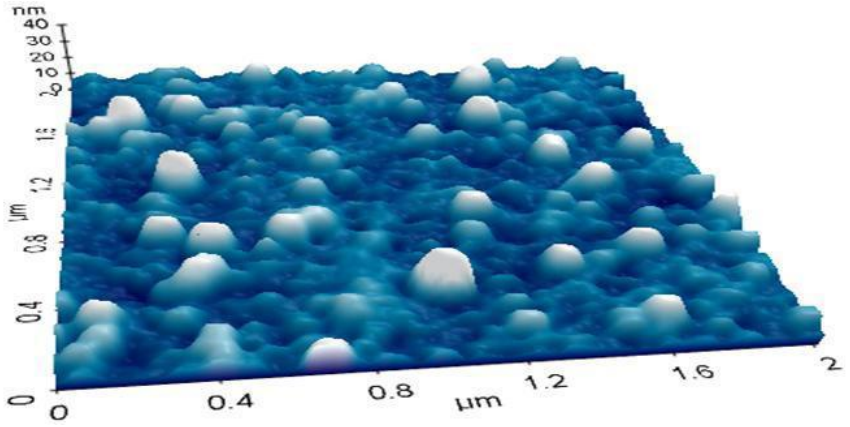


Figure 38. AFM image of liposome.

9.5. Fluorescence Spectroscopy: It is known that the dendrimers have the capability to rupture cell membranes. Therefore, if a dendrimer is added to a liposomal solution, where some fluorescent dye (like curcumin, 7-hydroxy coumarine, *etc.*) is entrapped, the dye will start leaking out of the liposome. Such leakage of fluorescent dyes will be monitored through fluorescence spectroscopic measurements. Extent of leakage would then be correlated with the membrane rupture. Steady state fluorescence spectra, as well as fluorescence anisotropy and fluorescence life time studies were carried out by a bench-top spectrofluorimeter (Quantummaster-40, Photon Technology International Inc., NJ, USA). Curcumin, used as the probe, was excited at 415 nm. Fluorescence anisotropy value was determined using equation mentioned below:

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2G.I_{VH}} \tag{4}$$

where, I_{VV} corresponds to the intensity obtained when the excitation and the emission polarizers are oriented vertically. I_{VH} is the intensity obtained for vertical excitation polarizer and horizontal emission polarizer. G is the grating correction factor which is the ratio of sensitivities of the instrument for vertically and horizontally polarized light. Thus:

$$G = \frac{I_{HV}}{I_{HH}} \tag{5}$$

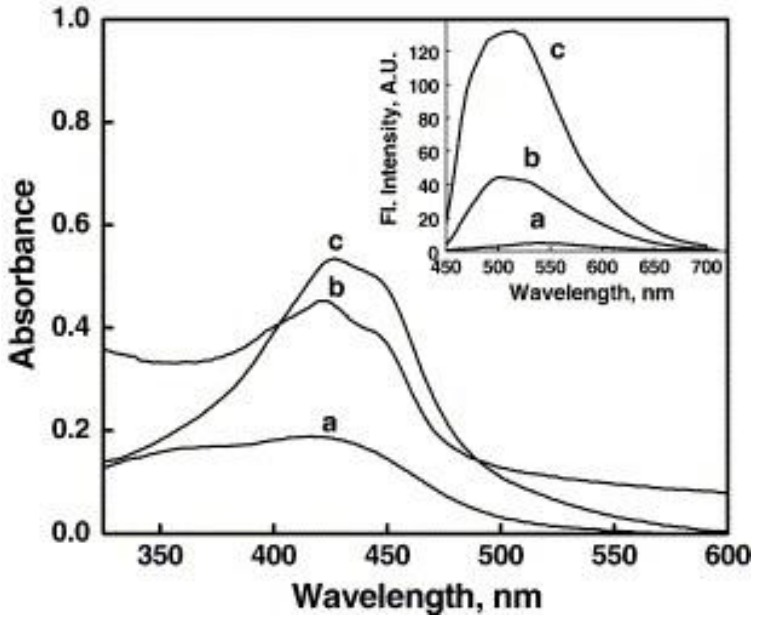


Figure 39. Absorbance and fluorescence spectra of curcumin.
https://doi.org/10.1016/j.bbagen.2006.06.012

Fluorescence lifetime measurements for fluorescent dye were recorded with the same fluorimeter where a nano LED (Photon Technology International Inc.) of different wavelength was used to excite the dye. Fluorescence decays were obtained by Strobe technique. [132]

9.6. Differential Scanning Calorimetry (DSC). DSC is useful techniques that can detect the hydrocarbon chain melting process also known as phase transition. The temperature at which chain melts, known as chain melting temperature (T_m). By measuring T_m one could also get other thermodynamic parameters like enthalpy change, change in heat capacity, crystalinity of hydrocarbon chain etc. In this experiment, the vesicle bilayer were heating or cooling with desire temperature range. In chain melting process, the orientation of hydrocarbon chains get tilted leading the creation of endothermic event as reflected in the Figure 2. Hence bilayer strength and other thermodyanmical entities are heavily depend on bilayer composition and the process of chain melting.

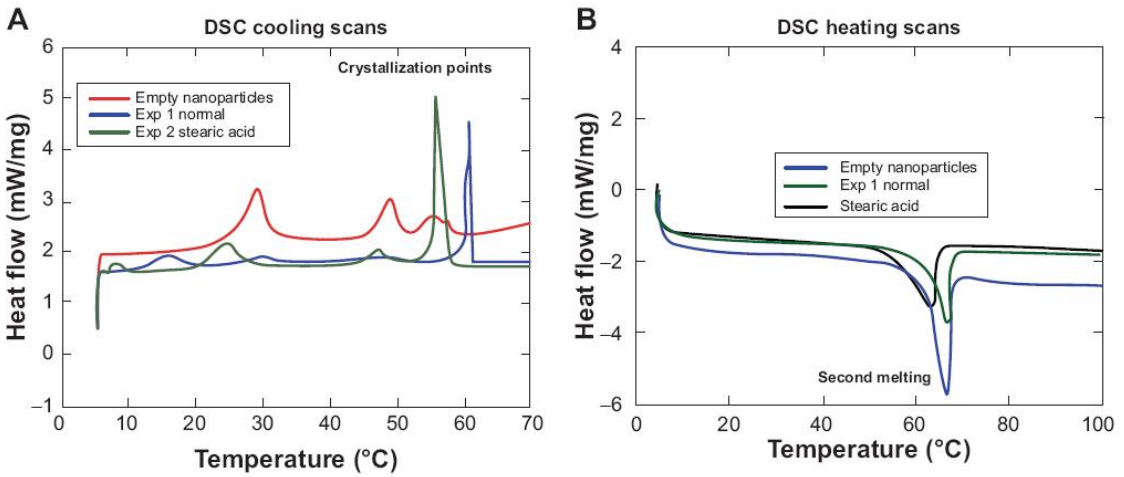


Figure 2 (**A**) DSC cooling scans; (**B**) DSC heating scans.
Notes: Empty nanoparticles refers to solid lipid nanoparticles that do not contain drug; Exp 1 normal refers to primaquine-loaded solid lipid; Exp 2 refers to stearic acid alone, which was used as the matrix in forming the solid lipid nanoparticles.
Abbreviations: DSC, differential scanning calorimetry; Exp 1, experiment number 1; Exp 2, experiment number 2.

Figure 40. DSC thermogram of SLN. https://doi.org/10.2147/IJN.S62630

9.7. Isothermal Titration Calorimetry (ITC). ITC measurements were performed in a VP-ITC titration calorimeter (MicroCal, Northampton, MA). Before loading, the solutions were thoroughly degassed. The preformed samples were kept in a sample cell, and a syringe of aliquot volume sample was filled by the ligand in the same buffer. The sequential titrations were performed to ensure full occupancy of the binding sites by loading and titrating with the same ligand without removing the samples from the cell until the titration signal was essentially constant. The respective heat of dilution was subtracted from the corresponding binding experiments prior to curve fitting. Typically three replicate titration experiments were performed.

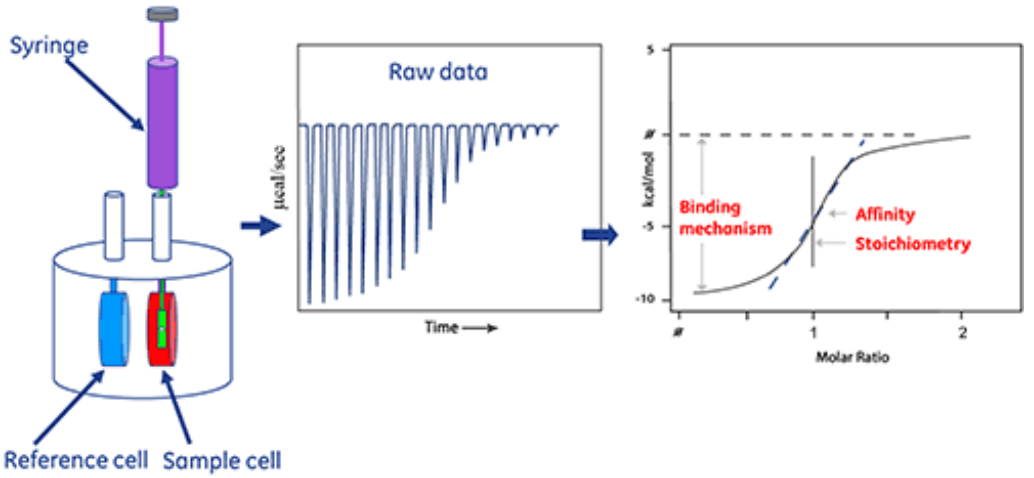


Figure 41. Schematic diagram of ITC technique.

9.8. Entrapment efficiency (EE) and release kinetics: It is known that liposome can accommodate both hydrophilic and lipophilic drug. Hence Drug loading capacity of vesicles need to be determined. Once drug loaded liposome dispersion was prepared, it filled into the cellulose dialysis bag (MWCO 12 KDa) and dialyzed in PBS or water with constant stirring (50 rpm) under sink conditions for about 20 min. The un-entrapped drug was removed from the formulations and the absorption spectra of collected samples were recorded through UV-VIS spectrophometer. The EE was calculated by considering the following equation: [133, 134] where,

$$\text{E.E. (\%)} = \frac{T-C}{T} \times 100 \qquad (6)$$

T is the total amount of drug presents both in the sediment and supernatant, C is the amount of drug identified only in the supernatant. T and C values were determined colorimetrically.

9.9. Drug release Kinetics. Liposomes are marked to be specific when they liberate the drug in target cell with sustained release. Hence drug incorporated vesicles often undergo such kinetics measurement. Dialysis method is generally used where the drug loaded samples were placed in dialysis bag and immersed in the same medium or solvent that was used in liposome preparation. [135, 136, 137] UV-VIS spectra of the sample were recorded with time which reveals the release pattern of the drug. Figure 40 put on a view of such release kinetics of drug loaded liposomes.

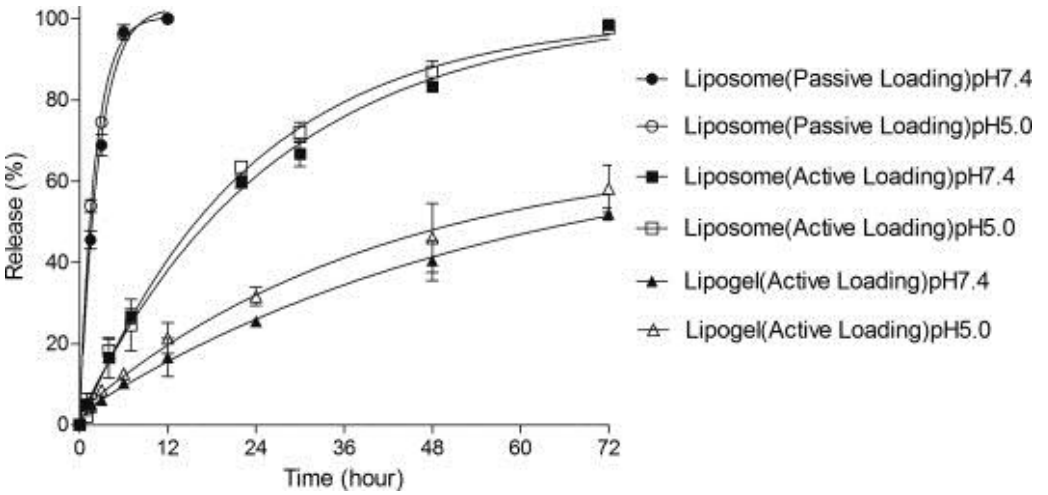


Figure 42: Representative image of % of drug release with time form liposome bilayer.

9.10. Antibacterial activity: Antibacterial activity of drug loaded systems was assessed by the conventional cup-plate method. Nutrient broth and agar were used as the culture growing and solidifying agent respectively. In the incubated plates, bacterial suspension (0.1 mL of 5×10^5 CFU mL^{-1}) was homogeneously spread over the agar surface and then grooves of equal diameter (0.8 cm) were made. The plates were incubated at 37°C for 24 h.

9.11. Studies on the behavior of monomolecular films: Surface pressure (π) – area isotherm (A): Bilayer could be viewed as superimposition of two monolayer's. Hence through investigation of monolayer would shed light on hydrocarbon chin miscibility, bilayer compressibility, free energy of chain mixing which helps to understand the role of hydrocarbon wall in bilayer.[138] Excess area of mixed monomolecular film where more than one amphiphiles are present are often guide to understand the associative or repulsive type interaction between the hydrocarbon chains of the amphiphiles. Π-A Isotherm is measured in a A Langmuir–Blodgett trough (LB trough that is used to compress monolayer of amphiphiles molecules on the surface of a given subphase (usually water) and measures surface phenomena (viz. surface pressure, surface potential etc.) due to this compression. Water must be purified to remove impurities. Impurities as small as 1ppm can radically change the behaviour of a monolayer. Representative image of Π-A isotherm is given in Figure 42.

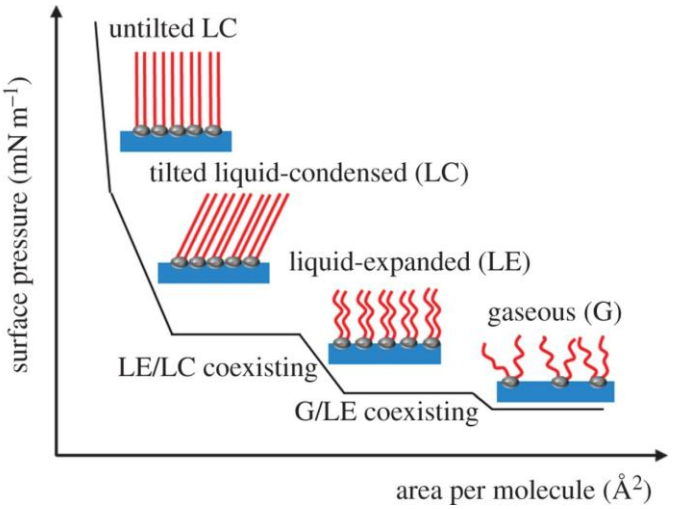


Figure 43: Representative surface pressure – area isotherm of lipid.

After careful addition of lipidic materials to the sub-phase the trough was closed by a glass box to avoid the entrance of dust. 30 min after addition the compression starts with a speed 5 mm per

sec. Due to the compression, surface pressure increases as the lipidic molecules come closer. As reflected from the figure, the isotherm consist of four differ parts and each describe the state of the monolayer. In gaseous state, the molecules are apart from each other whereas in liquid expanded and in liquid condense phase, they orient themselves in a same fashion. Finally in the solid state, most of the molecules are very close to each other that beyond this no further addition of molecule would not be possible and will break the pattern. Thus characterization of bilayer would not be successful unless one performed monolayer study. [139, 140]

The physical properties of liposomes are highly dependent on the type of phospholipids constituted, intermolecular interactions as well as the conditions of the environment which surround it. Nevertheless, due to their easy generability and readily modifiable nature in terms of its lipid contents and structure (i.e. unilamellar and multilamellar), liposomes have received particular attention as the simplified version of cell membrane and have been commonly employed in the investigation of drug-lipid bilayer interaction. In this proposed program, liposomes will be employed in order to study the interaction between dendrimer and lipid bilayers.

Adsorption of dendrimers on liposomes and the formation of dendrimer-liposome aggregates are the most common aspect in the interaction between dendrimers and liposomes. It is believed that the dendrimers could act as glue for the vesicles. Most of the recent studies have focused on the interactions of positively charged dendrimers with cell membranes, since greater interaction potency is expected between these dendrimers with the negatively charged cell membrane compared to other neutral or negatively charged dendrimers. However, it has also been shown that besides the surface charge of the dendrimer molecules, there are many other physicochemical properties of dendrimers which influence the interaction between dendrimers and lipid bilayers. These properties of dendrimers include the dendrimer type, generation, surface charge and also solution conditions as well as the composition of membrane lipids.

Studies on the change of the zeta-potential of liposomes are assumed to provide better understanding of the nature of dendrimer-liposome interaction. It is known that the zeta-potential of liposomes would change as a result of the adsorption of charged dendrimers, while remain constant if dendrimers are internalized from the interaction with the liposomes.

The current barrier to the application of dendrimers as various therapeutic agents as mentioned above, however, is the lack of established knowledge on biocompatibility and toxicity of dendrimers in biological systems. The kinetics and mechanisms of dendrimer-lipid bilayer interaction have received considerable attention as cells contain many layers of membranes which dendrimers need to recognize, interact and internalized before it could reach its intracellular targets. As discussed, the knowledge on this area has been expanding over the past

few years, but there are still many questions left to be answered before dendrimers could be safely employed as a novel therapeutic agent.

As part of the effort to provide a better understanding of the mechanism of dendrimer interactions with lipid bilayers, this research is aimed to investigate the mechanism involved in the interaction between different generation and types of PAMAM dendrimers and liposomes (i.e. electrostatic and hydrophobic interaction), and also to provide an understanding on how the various physicochemical properties of dendrimers and liposomes could affect the dendrimer-liposome interaction. Specifically, in this research, different types of phospholipids including 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC), soyphosphatidylcholine (SPC), 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (Sodium Salt) (DPPG), dihexadecyl phosphate (DHP), 1, 2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphate (DPP) and 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanol (DPPEth) and cholesterol (CHOL) will be employed to synthesize a range of liposomes of varying chemical composition, structural and physical properties. The information obtained from their interaction, including adsorption isotherm, packing density and affinity, changes in surface charge as a result of adsorption process and aggregate formation (changes in particle size) are believed to be useful as they would provide a basis for future studies which investigate the interaction between dendrimers and cell membranes, which structurally and chemically more complex than simple lipid bilayer (i.e. liposomes).

Also, as dendrimers with different physical and chemical properties would be employed as therapeutic agent in future according to the need of the specific therapeutic purpose, the studies are also assumed to investigate the influence of dendrimer types on its interaction with liposomes. Different types of PAMAM dendrimers that contain a similar surface group (amine), but different generations will be employed. The influences of the dendrimer type and generation on the dendrimer-liposome interaction will be studied in terms of variation in the adsorption isotherm, changes in zeta potential as well as particle size and other physiochemical parameters. The information obtained would be useful as they would provide an idea in future for the rational design of dendrimers which are biocompatible and are optimized for therapeutic application. In another attempt, efforts will be put to study the kinetic and mechanistic aspects of dendrimer-liposome interaction. To meet up the final goal of the proposed work, dendrimers of different kinds and generations will be employed to perform cell (bacterial/ cell lines) growth studies. This

will eventually be helpful in obtaining a proper denderimer based formulation for therapeutical uses.

In 1st chapter we have studied the different physicochemical properties of liposomes with respect to lipid composition, pH, time and temperature. The liposome were prepared by conventional thin film hydration technique by using soy phosphatidylcholine (SPC), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) and a 7:3 (M/M) mixture of DPPC+DPPG along with 30 mole% cholesterol in each combination. Hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z.P.) of liposome were determined with respect to time, temperature and pH by DLS. Except SPC, hydrodynamic diameter of other liposomes did not vary significantly up to 100 days indicating its substantial stability. The size of liposomes depended on lipid composition, pH and temperature. On the other hand the zeta potential was found to be independent of the pH of the medium, although it varied with liposome type. Lower polydispersity index (PDI) values (0.15 to 0.4) of all the liposomes indicate that liposomes are monodisperse nature in the entire pH range. Liposomes were thermally reversible in the temperature range 25 to 50°C. All the liposomes exhibited size constriction at ~40°C, both during the heating and cooling cycles, which correspond to the chain melting temperature of the phospholipid bilayer. But PDI values did not change appreciably with temperature. Spherical morphology was observed from electron microscopy for all the liposomes. The size of the liposomes, as obtained by the TEM measurements was found to be comparable with the DLS data. Bilayer thickness of the liposomes was found to be ~5.6 nm, comparable with the literature values. Hydration on the surface of liposome could also be noticed. DSC studies on the liposomes were performed with an aim to understand the bilayer chain melting process. The presence of higher amount of cholesterol (30 mole%) resulted in the broadening of DSC peaks. The main phase transition temperature of the liposomes was in the range of 38 to 50°C for the different studied pH. Influence of pH on the transition temperature (also known as the temperature of the maximum heat flow, T_m) was more significant among the negatively charged liposomes. The phase transition temperature increased with decreasing pH. To understand the physicochemistry of membrane property, curcumin were used as probe. Incorporation of curcumin into liposomes caused fluorescence enhancement with significant blue shift in λ_{em} values indicating its transfer to the less polar region of liposome. Curcumin, predominantly in its neutral form at lower pH, binds strongly to liposomes through hydrophobic

interactions; thus the fluorescence intensity of curcumin is enhanced in liposomes at lower pH. However, at higher pH, curcumin decomposes from its conjugated diene; leading to its poor adherence to the liposomes. The membrane viscosity of liposomes was studied by anisotropy measurement. Anisotropy value followed the order: SPC > DPPC+DPPG > DPPG, irrespective of the pH of the medium. The movement of curcumin in SPC liposomes is hindered to a greater extent compared to other systems which explains the highest anisotropy value of curcumin for SPC liposome. Lowest 'r' value for DPPG could be due to the similarity in the charge of the probe and the lipid. Anisotropy value for all the systems (except DPPC) increased with pH. In conclusion, membrane micro viscosity unveiled highest value for SPC, whereby the rigidity of membranes increased with increasing pH. Entrapment efficiency of liposomes with reference to curcumin followed the order: DPPC > DPPC+DPPG > DPPG > SPC. *In vitro* release kinetics of curcumin were studied to compare the four different liposomal systems and also to define the nature of the release processes. Curcumin release from the liposomes at pH 7.4 followed the order: SPC> DPPG> DPPC+DPPG> DPPC. Sustained release of curcumin was noticed for all the liposomes. The antibacterial efficacy of the liposomes, loaded with and without curcumin, was assessed on gram negative (*Klebsiella pneumoniae* and *Pseudomonas putida*) and gram positive bacteria (*Bacillus amyloliquefaciens* and *Bacillus subtilis*). No antibacterial activity was recorded with the liposomes in the absence of curcumin. Curcumin loaded liposomes exhibited substantial antibacterial activity on the gram positive bacteria *Bacillus amyloliquefaciens*. The comprehensive studies could provide a deeper understanding of the effect of formulation variables on the physicochemical stability of the liposomes.

In second chapter the interaction of different generation poly(amidoamine) (PAMAM) dendrimers and with the combinations of liposomes were studied. Second, fourth and sixth (2G, 4G, and 6G) generation PAMAM dendrimers were used, which are cationic under normal conditions. Liposomes comprised of soy lecithin + cholesterol (SLC+CHOL) (negative surface charge), DPPC+CHOL (positive surface charge), DPPG+CHOL (negative) and a biologically simulated mixture of DPPC + DPPG (7:3) + CHOL (negative) were used as model bilayers. Silica was used as a negatively charged hard sphere model to make a comparative study. The interaction between dendrimer-liposome systems can be assessed by turbidity measurements. There is a threshold concentration after which the absorbance increases, reaches a maximum and decreases again. Similar observation was found in case of size analysis by DLS measurements.

Initial size increment and attainment of maxima is probably due to aggregation/association of liposomes, assisted by dendrimers. Dendrimers being oppositely charged, compared to the surface charge of liposome, get easily bound/attached to the liposome surface. The extent of interaction between dendrimer and liposome depends on dendrimer generation and follow the order 6G>4G>2G. Interaction between negatively charged surface (liposome bilayer) and the positively charged dendrimer were further explored by zeta potential measurements. It has been found that a negative value of zeta potential gets decreased to the positive side upon addition of dendrimers. At a certain concentration zeta potential of the mixture attains zero value, which suggests the charge neutralization. Further increase in zeta potential suggests that it is possible for the non-charged liposomal particles to further interact and aggregate with the PAMAM. After a certain concentration limit, the zeta potential attains a platue, indicating the saturation of liposomes. Interaction after the charge neutralization is probably driven through hydrogen bond and/or hydrophobic interaction. Presence of secondary and tertiary amino groups might induce such interaction. Also bilayer disruption, induced by dendrimers, can also be visualized by AFM studies. Bilayer structures of vesicles, fused on freshly cleaved mica surface, were supported by height analysis. When dendrimer added, get adsorbed onto the bilayer, preferably near the bilayer edges, while at higher dendrimer concentration, the bilayer structure gets completely disrupted. At higher concentration of dendrimers, possibly dendrimers forms some aggregates with the liposomes. Further details about dendrimer-liposome interaction were studied in 3rd chapter.

In 3rd chapter, interaction of liposomes carrying net negative charges with cationic polyamidoamine dendrimers (PAMAM) of different generations were investigated by combined size, zeta potential, turbidity, electron microscopy, atomic force microscopy, fluorescence spectroscopy and calorimetric studies. Firstly, the stability of the liposomes comprising 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) + dihexadecyl phosphate, DPPC+1, 2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, DPPC+1, 2-dipalmitoyl-*sn*-glycero-3-phosphate and DPPC+1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanol were checked through their size and zeta potential with the variation of time. Hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z. P.) of four different liposomes (DHP+DPPC), (DMPG+DPPC), (DPP+DPPC) and (DPPEth+DPPC) were measured at pH 7.4 as a function of time by dynamic light scattering. The size remained almost constant up to 60 days for all the systems, indicating their substantial

stability. The lower PDI values designate that all the liposomes are fairly monodispersed. Size of the liposome depends on the lateral packing of lipid molecules within the membrane bilayer and is mainly determined by the van der Waals interactions between the hydrocarbon chains. The PDI values follow the sequence DPPEth+DPPC> DMPG+DPPC> DPP+DPPC> DHP+DPPC. The zeta potential for different liposome follows the sequence DHP+DPPC > DPPEth+DPPC > DPP+DPPC > DMPG+DPPC. The interaction efficiency between dendrimers and liposomes can be assessed by the turbidity measurements. Turbidity values of the dendrimer-liposome complexes pass through maxima likewise the size after a threshold dendrimer concentration. Initial size or turbidity increments and the attainment of maxima are due to the formation of liposome aggregates assisted by dendrimers. The dendrimers being oppositely charged, get adsorbed on to the liposome surface. Decrease in size and turbidity upon further addition of dendrimers are due to the formation of water soluble dendrimer-liposome aggregates. The ability in imparting turbidity or size enhancement depends on the dendrimer generation. PAMAM dendrimers follow the order 5G>4G>3G while considering the size and turbidity variation. The interactions between a negatively charged surface and the positively charged dendrimers were further explored by zeta potential measurements. Similar effects were studied in 2nd chapter. Spherical morphology with smooth surfaces were observed for all the liposomes; the sizes were comparable to the DLS data. To double check the morphological informing FF-TEM studies were performed. It was observed that with increasing concentration of PAMAM, liposomes surfaces lost their homogeneity with the ultimate formation of aggregated heterogeneous entities. These results further support the proposition of the formation of dendrimer-liposome complexes are discussed in the 2nd chapter. State of polarity of the liposome surface and the packing of bilayer are the two important parameters while considering the dendrimer-liposome interaction processes. Solvatochromic dye 7-hydroxycoumarine (7-HC) was used as the molecular probe to understand the state of polarity as well as the rigidity/fluidity of the palisade layer of the liposome and the effect of dendrimers. Additionally, the hydrophobic probe 6-diphenyl-1,3,5-hexatriene (DPH) were used to understand about the bilayer packing of dendrimer-liposome complexes. Fluorescence anisotropy value of 7-HC loaded liposomes was lower than DPH loaded liposomes in absence of dendrimer. These fluorescent probe monitors interactions between the external and internal regions of the membrane with dendrimers. Increase in the fluorescence anisotropy was due to the adsorption of dendrimer liposome surface; further

addition of dendrimer leads to mild decrease in the fluorescence anisotropy probably due to the formation of holes in the bilayer, reflecting the fact that some dendrimers probably move through the liposome bilayer. Time-resolved fluorescence decay studies of 7-HC were carried out to further understand the emission decay parameters. Initially, with the progressive addition of dendrimer, fluorescence life time increased, and then attained constancy. Rotational diffusion of 7-HC decreased with increasing dendrimer concentration due to the formation of stable dendrimer-liposome complex that lead to overall increase in the viscosity of the medium as also revealed from the anisotropy studies. DSC studies were carried out in order to understand the thermal behavior of dendrimer-liposome interaction process. With increasing dendrimer concentration, the pre-transition temperature of lipids declined and the main transition peak became shorter and wider the high concentrations of dendrimers in DPPEth+DPPC lipid bilayer, that led to the abolishion of the transition peak indicating the perturbation of lipid bilayer. A decrease in the pre-transition peak of liposomes suggests that dendrimers can interact with this liposome surface, whereas alteration of the main transition peak suggests that dendrimers can lead to the generation of holes in the bilayer. With increasing dendrimer concentration, the membrane fluidity increases and the endothermic heat change decreases that lead to the lowering of the enthalpy changes of the chain melting processes. Binding constant is another important parameter to quantify the extent of the interaction processes between the dendrimer and liposome. The binding tendency of dendrimer on liposomes follows the order DMPG+DPPC> DPP+DPPC~ DPPEth+DPPC > DHP+DPPC which were found from ITC and colorimetric technique. With increasing dendrimer generation the binding constant values decreases. Due to presence of larger number of polymeric amino groups, higher generation of dendrimers interact effortlessly than that of lower generation of dendrimer. The understanding of the mechanism of dendrimer-liposome interactions is important as the cytotoxicity issue is vital for acceptance and development of dendrimer-liposome aggregates as pharmaceutical agents. The cytotoxicity results obtained from MTT assay clearly demonstrate that the all the liposome formulations and dendrimer-liposome aggregates were completely non-toxic towards normal human blood lymphocyte as well as in human RBCs suggesting the potential of such aggregates as drug delivery systems against microbial diseases.

In the last chapter, it contains two parts. One is the mutual miscibility and stability of the mixed monolayers of zwitterionic phospholipid, dipalmitoylphosphatidylcholine (DPPC) with

negatively charged phospholipids (dihexadecyl phosphate (DHP), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPP) and 1,2-dipalmitoyl-sn-glycero-3-phospho ethanol (DPPEth) and another part is the interaction between the positively charged dendrimer with the monolayers which has been studied in detail using surface pressure-area isotherms. Thermodynamic analysis indicates miscibility of the binary mixtures when spread at the air/buffer interface with synergistic interaction between the components. The surface pressure-area isotherms of the binary monolayers of DPPC and negatively charged lipids at the air-water interface showed maximum deviation for DPPC : anionic lipid at 7:3 M/M ratio mixed monolayer was more stable than the monolayers individual components. Based on the regular solution theory, the miscibility and stability of the two components in the monolayer were analyzed in terms of compression modulus (C_s^{-1}) and excess Gibbs free energy ($\triangle G^0$) and these physiochemical parameters dependent on phospholipids composition. Stable liposomes were formulated by the binary mixture in 7:3 molar ratio of DPPC with negatively charged phospholipids. Subsequently adsorbed monolayers were generated through vesicles disruption technique. Effect of polyamidoamine (cationic) dendrimers on the adsorption kinetics at the vesicles was followed. Bylayer disintegration and subsequent interfacial adsorption of lipids were followed up through the surface pressure. Time analysis bilayer disintegration kinetics was governed by the lipid head groups, chain length as well as the dendrimer generation and concentration.

CHAPTER I

CHAPTER I

The influence of lipid composition, pH and temperature on the physicochemical properties of liposomes with curcumin as model drug.

Abstract:

Physicochemical properties of large unilamellar vesicles (LUVs) were assessed with respect to lipid composition, pH, time and temperature by monitoring their size, zeta potential, drug payload as well as thermal behavior. Conventional thin film hydration technique was employed in preparing liposomes with soy phosphatidylcholine (SPC), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) and a 7:3 (M/M) mixture of DPPC+DPPG along with 30 mole% cholesterol in each combination. While the size of liposomes depended on lipid composition, pH and temperature, the zeta potential was found to be independent of the pH of the medium, albeit it varied with liposome type. Spherical morphology and the existence of bilayer were established from electron microscopy. The phase transition temperature increased with decreasing pH. Membrane micro viscosity unveiled highest value for SPC, whereby the rigidity of membranes increased with increasing pH. Entrapment efficiency of liposomes with reference to curcumin followed the order: DPPC > DPPC+DPPG > DPPG > SPC. Sustained release of curcumin was noticed for all the liposomes. Curcumin loaded liposomes exhibited substantial antibacterial activity on the gram positive bacteria *Bacillus amyloliquefaciens*. The comprehensive studies could provide a deeper understanding of the effect of formulation variables on the physicochemical stability of the liposomes.

1. Introduction

Among the diverse drug delivery systems, liposomes are advantageous with a multitude of application potentials.[1] Liposomes are synthetically prepared spherical, self-enclosed microscopic entities with membrane bilayer like structures.[2] They are formed with the exposure of the lipid head groups to an excess aqueous environment.[3] Use of such systems for different mode of administration is advantageous because of their biocompatibility and biodegradability. Drug incorporation into liposomes can increase its uptake to target cells, thus minimizing its degradation.[4] Liposomes can act as an effective carrier for both hydrophilic and hydrophobic drugs owing to its amphiphilic character (hydrophobic fatty acyl chains and polar head groups).[5]

Vigorous ongoing research in the field of liposomes has resulted in the emergence of novel liposomes apart from the conventional one, *viz.*, long circulating, ligand targeted, triggered release, pH and temperature sensitive systems, to mention a few.[6-7]

Phospholipids are the structural components of liposomes as well as one of the major components of mammalian cell membranes. Cholesterol is also an important component of liposome that modulates the fluidity/rigidity of membrane.[8] In a recent study by Magarker *et al.* [9] it has been reported that with increasing amount of cholesterol, zeta potential of liposome decreases owing to the reduction of sodium ion binding. While soy phosphatidylcholine (SPC) is a mixture of phospholipids with different fatty acids mimicking the natural membrane, dipalmitoylphosphatidylcholine (DPPC) is a pure lipid which can act as model biomembrane. SPC is fluid at physiological temperature due to one unsaturated fatty acyl chain. On the contrary, DPPC with both the acyl chains being saturated is solid upto 41^{o}C.[10] Dipalmitoylphosphatidylglycerol (DPPG) is negatively charged for its uncharged glycerol head group[11)]. DPPC-DPPG mixture is the major component of lung surfactant and also such a combination is necessary for the formation of lipid-protein conjugates, *viz.*, tubular myelin located in the alveoli of the lungs.[12] Thus, physicochemical investigations on liposome with such biomimetic combinations are considered to be worth investigating.

pH and temperature can significantly affect the stability of a liposome.[8] It has been reported by Zhang *et al.*[13] that pH and temperature can dictate the extent of hydrolysis of phospholipids and thus the shelf-life of liposome. Likewise, Sulkowski *et al.*[8] asserted that the fluidity of the membrane increases with pH and temperature; protonation of phospholipid occurs at lower pH resulting in favorable hydrogen bonding among them, resulting in an increase in the phase transition temperature.[12]At enhanced temperature, associative interaction between phospholipid molecules become less probable, thus causing the fluidization of membranes.[8] Began *et al.*[14] similarly observed that an increase in temperature of the liposomal system decreased the stability of drug-liposome aggregates. With rising temperature, hydrocarbon chain packing of the bilayer gets randomized, thereby reducing the rigidity of the membrane due to the formation of the pore like defects and bilayer discs. This ultimately reduces the stability of a molecular probe or a drug incorporated in the liposome[15)].

Curcumin, a natural polyphenol, isolated from the rhizome of *Curcuma longa Linn*, exerts a number of pharmacological activities like anti-inflammatory,[16] anti-oxidant,[17] anti-bacterial,[18] anti-HIV[19] and anticancer activities[20] *etc*. However, its poor aqueous solubility retards its effective use as therapeutic agent.[21] Additionally, its rapid metabolism and elimination contributes to its poor bioavailability.[22] It is susceptible to hydrolysis and its photolytic degradation in alkaline medium results in the formation of other phenolic compounds such as vanillin, *4*-dioxo-*5*-hexanol, trans-*6*-(*4*'-hydroxy-*3*-methoxyphenyl)-*2*,*4*-dioxo-*5*-hexanol, feruloyl methane and ferulic acid[9)]. Thus suitable carriers are warranted for curcumin ensuring site specific delivery with improved bioavailability. Liposomes are considered as promising vehicle for curcumin to overcome such issues.[23-24]

Synthetic phospholipid based vesicles, analogous to the mammalian cell membranes, are recognized for their capability to act as potential drug delivery systems.[5] These systems have been reported to promote the biological activities of curcumin such as improved drug level in cells,[24] increased antioxidant,[25] anti-angiogenic activity,[26] anticancer activity,[27] *etc*. The physicochemical properties of liposomes, *viz.*, size, surface charge, membrane viscosity, phase transition temperature have obvious implications on the stability and bioactivity of loaded therapeutic agents,[28] In view of the above mentioned facets, the studies relating to the influence of parameters like membrane's surface charge and composition, pH and temperature of dispersion medium, which are responsible for manipulating physicochemical behavior of vesicles are warranted. However, reports on the systematic studies concerning the effects of surface charge variation on physicochemistry of liposomes at different pH and temperature are not so common in the literature. Protonation/deprotonation phenomena and variation in viscosity of biomembrane mimetic systems at different pH have also not yet been systematically investigated. Thus, the present study aims to investigate the effect of pH, composition and temperature on the physicochemical behavior of four different biomimetic membranes. Liposomes using SPC, DPPC, DPPG and DPPC+DPPG (7:3 M/M) were formulated in combination with 30 mole% cholesterol. Size, polydispersity index and zeta potential were monitored by dynamic light scattering (DLS) technique. Morphology of the bilayer dispersions was assessed by transmission electron microscopy (TEM). Differential scanning calorimetric (DSC) studies were carried out to determine the phase transition temperature of the bilayer. Membrane polarity and microviscosity of the palisade layer were evaluated by fluorescence

spectroscopy using curcumin as a probe. The entrapment efficiency of different liposomes for curcumin and *in vitro* release kinetics studies were conducted to assess the therapeutic effectiveness of curcumin loaded liposomes. Antibacterial activity of the four systems in combination with curcumin was executed for the confirmation of the same property of curcumin. It is believed that the combined aforementioned studies can provide information on the optimization of the formulation parameters (pH and temperature) in order to prepare stable curcumin loaded drug delivery systems.

2. Materials and methods

2.1 Materials

Soy phosphatidylcholine [(2R)-2,3-di(tetradecanoyloxy)propyl]-2-(trimethylazaniumyl)ethyl phosphate, (SPC)] was purchased from Calbiochem, Germany. 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DPPG), (3*β*)-cholest-5-en-3-ol (cholesterol), curcumin [1,7-*bis*(4-hydroxy-3-methoxyphenyl)-1,6-hepatadiene-3,5-dione] were the products from Sigma-Aldrich Chemicals Pvt. Ltd. (USA). All the chemicals were ≥99% pure. AR grade 99% pure disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), sodium chloride (NaCl), HPLC grade chloroform and methanol were the products of Merck Specialities Pvt. Ltd, India. The nonionic surfactant polyoxyethylenesorbitanmonooleate (Tween 80) with a stated purity of ≥ 99.0% was purchased from Sisco Research Laboratory, India. Double distilled water with specific conductance 2-4 μS (at 25^oC) was used in preparing the solutions. All the chemicals were stated to be ≥99% pure and were used as received.

2.2 Methods

2.2.1 Preparation of liposomes

Liposomes were prepared by the conventional thin film hydration technique.[29] The quantitative amount of phospholipid and cholesterol were dissolved in chloroform and methanol (3:1, v/v) in a round bottom flask. The solvent was evaporated on a rotary evaporator at 40°C. The resultant thin film was further maintained under vacuum for 6 h. It was then hydrated with 10 mM phosphate buffer saline (PBS) for 1 h at 70ºC (temperature above chain melting temperature of all lipids). Ionic strength was maintained at 150 mM using sodium chloride. Systems were then

frozen at -20°C and thawed followed by sonication at 45°C by ultrasonic cleaner (ultrasonic power: 150W). The freeze-thaw-sonication process was repeated for four cycles in order to obtain large unilamellar vesicles. Liposomes were prepared separately using SPC, DPPC, DPPG and DPPC+DPPG along with 30 mole% cholesterol at different pH (6.0, 7.0, 7.4 and 8.0). In case of curcumin loaded liposomes, curcumin was mixed along with the lipids in the chloroform: methanol mixture prior to the generation of thin film. Dispersions were filtered through 0.45 µm cellulose nitrate membrane filter prior to size and zeta potential measurements.

2.2.2 Instrumentation

Hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z.P.) of the liposomes were measured by Dynamic Light Scattering (DLS) technique using Zetasizer Nano ZS90 ZEN3690 (Malvern Instruments Ltd, U. K). He-Neon laser with an emission wavelength at 632.8 nm was used and the scattered data were recorded at 90° angle. DLS measures the autocorrelation function $g^1(\tau)$ that is correlated with the size distribution of the particles by the given equation[30]:

$$g^1(\tau) = \int_0^\infty m_\Gamma^2 P(q,\Gamma) G(\Gamma) e^{-\Gamma\tau} d\Gamma \quad (1)$$

where, $G(\Gamma)$ is the normalized number distribution function for the decay constant Γ, m_Γ is the particle mass, $P(q,\Gamma)$ is the particle scattering factor, the decay constant $\Gamma = q^2 D_T$, the scattering vector $q = \left({}^{4\pi n}/_{\lambda}\right)\sin({}^{\theta}/_{2})$, θ is the angle between the incident and scattered beam, n is the refractive index of the medium and D_T is the translational diffusional coefficient. D_T is related to the hydrodynamic radius (R_h) of the particles by the given equation:[30]

$$D_T = \frac{k_B T}{6\pi\eta R_h} \quad (2)$$

where, k_B is the Boltzmann constant, T is the temperature of the dispersion medium and η is the viscosity of the medium. The zeta potential was measured using folded capillary cells. The PDI and zeta potential measurements were carried out at the temperature of 25 °C; however the size of the liposome systems was measured in the temperature range between 25 to 50 °C. DLS studies were undertaken for three different samples of liposome formulations. The DLS results of each sample were the average of four consecutive measurements.

Morphology of the liposomes was studied by transmission electron microscopic studies. A drop of dilute (10^{-4} M) dispersion was placed on Formver™ carbon-coated 200 mesh copper grid and dried under air.[31] It was then analyzed to obtain TEM images using Hitachi H-600 transmission electron microscope (Japan) operating at 80 kV. In order to provide better contrast, uranyl acetate staining agent was used.

Differential Scanning Calorimetry (DSC) studies were carried out to determine the chain melting temperature of liposome bilayer[32] by a differential scanning calorimeter (DSC 1, STAR^e system, Mettler Toledo, Switzerland) with a scan rate of 2°C /min. The temperature range was set in the range of 15 to 65°C for SPC, DPPC, DPPG and DPPC+DPPG liposome. SPC, with an unsaturated double bond in one of its fatty acyl chains, was additionally scanned in the temperature range -25 to 25°C. 40 µL Al pan, sealed after sample insertion, was used with buffer as a reference. Results on the third scan were reported and used for further analyses.

Steady state fluorescence spectra, as well as fluorescence anisotropy studies were carried out by a bench-top spectrofluorimeter (Quantummaster-40, Photon Technology International Inc., NJ, USA). Curcumin, used as the probe, was excited at 415 nm. Fluorescence anisotropy value was determined using equation mentioned below:[33-34]

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2G.I_{VH}} \tag{3}$$

where, I_{VV} corresponds to the intensity obtained when the excitation and the emission polarizers are oriented vertically. I_{VH} is the intensity obtained for vertical excitation polarizer and horizontal emission polarizer. G is the grating correction factor which is the ratio of sensitivities of the instrument for vertically and horizontally polarized light. Thus:

$$G = \frac{I_{HV}}{I_{HH}} \tag{4}$$

2.2.3 Entrapment efficiency (EE) and release kinetics studies

Amount of curcumin entrapped in liposome was estimated by the method of centrifugation[35)]. Curcumin loaded liposome was centrifuged at 14000 rpm for 1 h in Eppendorf™ tube whereby the drug entrapped liposome got sedimented.[15] The sediment was analyzed colorimetrically by dissolving it in appropriate volume of methanol to quantify the encapsulated curcumin. %EE for curcumin was calculated as:[36]

$$\%EE = \frac{W_{entrapped\ curcumin}}{W_{total\ curcumin}} \times 100\ \% \quad (5)$$

where, W term represents the corresponding weight of curcumin (entrapped or total).

In vitro release kinetics of curcumin from liposome was investigated by using the dialysis bag method:[37] A dialysis bag (12 kDa molecular weight cut-off, Sigma-Aldrich, USA) was used. While the donor medium comprised 5 mL curcumin loaded liposome or aqueous curcumin solution (control), the receptor compartment contained 20 mL PBS buffer (pH 7.4) with 0.5% Tween-80 and 20% ethanol (v/v) as sink at 37°C:[36] At definite time intervals, 3 mL receptor medium was withdrawn with concomitant addition of the same volume of fresh medium and analysed colorimetrically.

2.2.4 Antibacterial activity studies

Antibacterial activity of curcumin loaded liposomes was assessed by the conventional cup-plate method.[38] Nutrient broth and agar were used as the culture growing and solidifying agent respectively. In the incubated plates, bacterial suspension (0.1 mL of 5×10^5 CFU mL^{-1}) was homogeneously spread over the agar surface and then grooves of equal diameter (0.8 cm) were made. The plates were incubated at 37°C for 24 h.

All the experiments, unless otherwise stated, were carried out at a controlled ambient temperature.

3. Results and discussion

3.1 DLS studies

Hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z.P.) of liposome were determined with respect to time, temperature and pH by DLS method. Figure 1 shows size-time correlation for liposomes at different pH. Most of the cases size of the liposomes passed through minima which was due to the structural reorganization of the lipidic components.[39] Asymmetric unimodal size distribution, representative of the existence of uniform particle size, was established from the nice autocorrelation curves of the DLS studies (as shown in Figure 2, supplementary section). The auto correlation curves were found to be independent of the pH and lipid composition, indicating its stability with respect to the pH and lipid composition. Except SPC, hydrodynamic diameter of other liposomes did not vary significantly up to 100 days

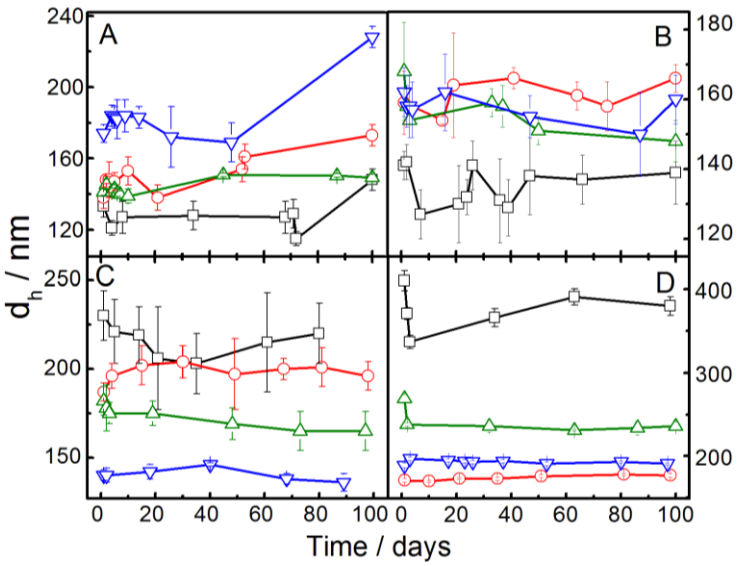


Figure 1. Variation in the hydrodynamic diameter (d_h) of liposomes with time at different pH. Systems: A, SPC; B, DPPC; C, DPPC+DPPG and D, DPPG. pH □, 6.0; ○, 7.0; △, 7.4; ▽, 8.0. 30 mole% cholesterol was used in each case. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

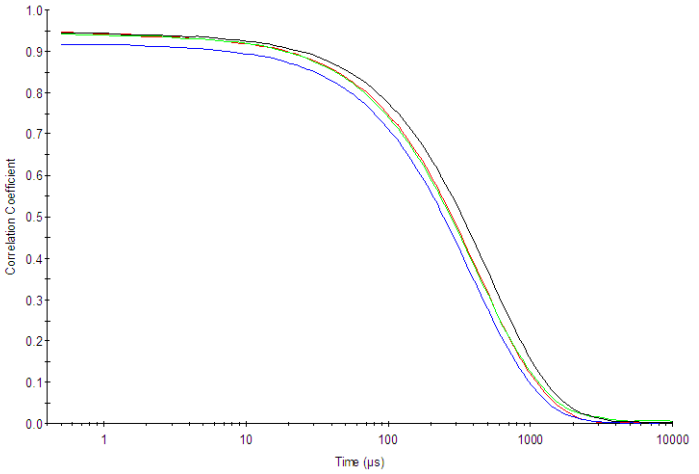


Figure 2 Auto corelation curve for the all liposome at pH 7.4. Lipid conc. 10^{-4}M. 30 mole% cholesterol was used in each case. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C. (1, DPPG; 2, SPC; 3, DPPC+Chol and 4, DPPC+DPPG). 30 mole% cholesterol was used in each case. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

indicating its substantial stability. For SPC, prominent size enhancement after 50 days, especially at pH 8.0, indicates aggregation. In case of SPC and DPPC, the size of the liposomes generally increased with increasing pH of the medium. However, the opposite trend was observed for the

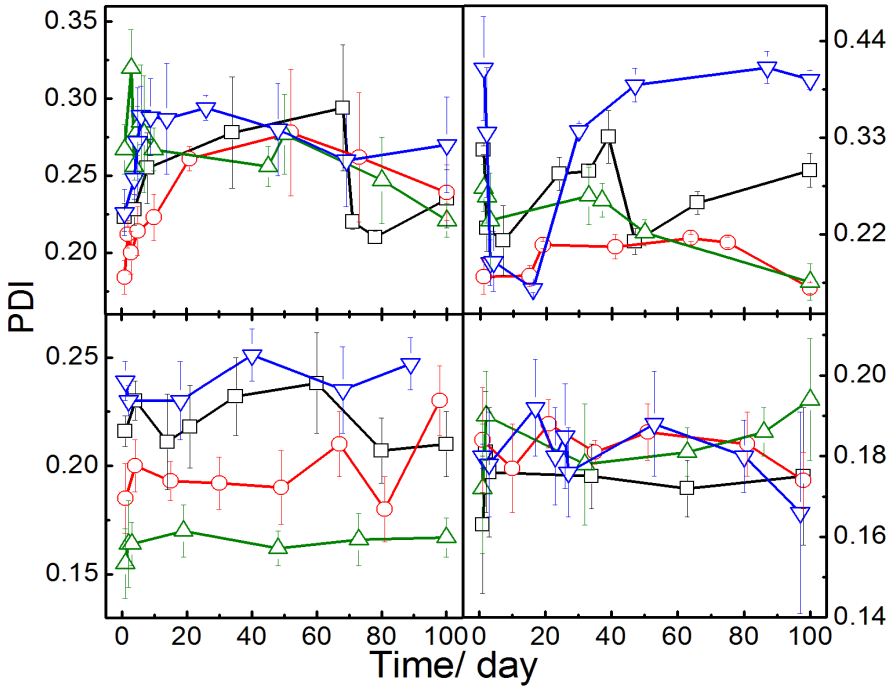


Figure 3. Variation in the polydispersity index (PDI) of liposomes with time at different pH (□, 6.0; ○, 7.0; Δ, 7.4 and ▽, 8.0). Liposomes: (A= soy phosphatidylcholine (SPC), B=DPPC, C=DPPC+DPPG and D= DPPG). 30 mole% cholesterol was used in each case. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

negatively charged liposomes (DPPG, DPPC+DPPG). Results indicate the existence of inter-vesicle repulsions in case of SPC and DPPC in acidic pH. At lower pH, the phosphate group of the lipids, being protonated, result in hydrogen bonding between the neighbouring phospholipid molecules.[8] On the other hand, electrostatic repulsion between protonated choline groups is also possible. However, fewer phosphatidylcholine molecules become protonated for its zwitterionic nature at pH 6.0. Thus the electrostatic repulsion dominates over the hydrogen bonding.[12] which corresponds to the smaller sizes recorded for SPC and DPPC in acidic pH. In case of DPPG, extent of protonation is higher than phosphocholine. Thus, the hydrogen bonding between the neighboring phospholipid molecules is likely to be more probable. Subsequently, the extent of

hydration of the phospholipids would decrease (due to the reduced hydrophilicity of the head groups) resulting in further instability. DPPC+DPPG liposome exhibited largest size in the acidicpH. This was due to the dominance of hydrogen bonding over the repulsive forces between phospholipids in presence of DPPG. However, in the neutral and alkaline pH, the repulsive Figure 4 describes the effect of temperature on liposome size. Liposomes were thermally reversible in the temperature range 25 to 50°C (Figure 5, supplementary section for DPPC+DPPG as representative). All the liposomes exhibited size constriction at ~40°C, both during the heating and cooling cycles, which correspond to the chain melting temperature of the phospholipid bilayer (to be explained later during the DSC studies). Phospholipid bilayers undergo a change from highly ordered gel phase to fluid phase[40] resulting in size constriction.

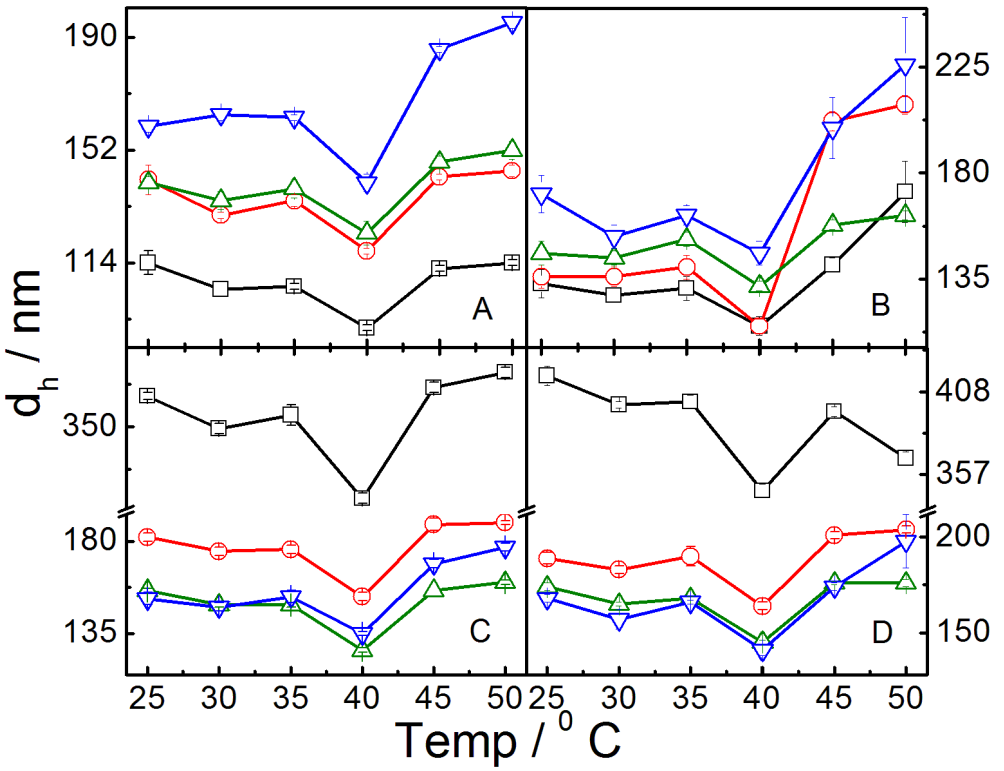


Figure 4. Effect of temperature on the hydrodynamic diameter (d_h) of liposomes at different pH. Systems: A, SPC; B, DPPC; C, DPPC+DPPG and D, DPPG. pH □, 6.0; ○, 7.0; △, 7.4; ▽, 8.0. 30 mole% cholesterol was used in each case with the lipids. Liposomes were prepared in 10 mM PBS +0.15 M NaCl.Temperature 25°C.

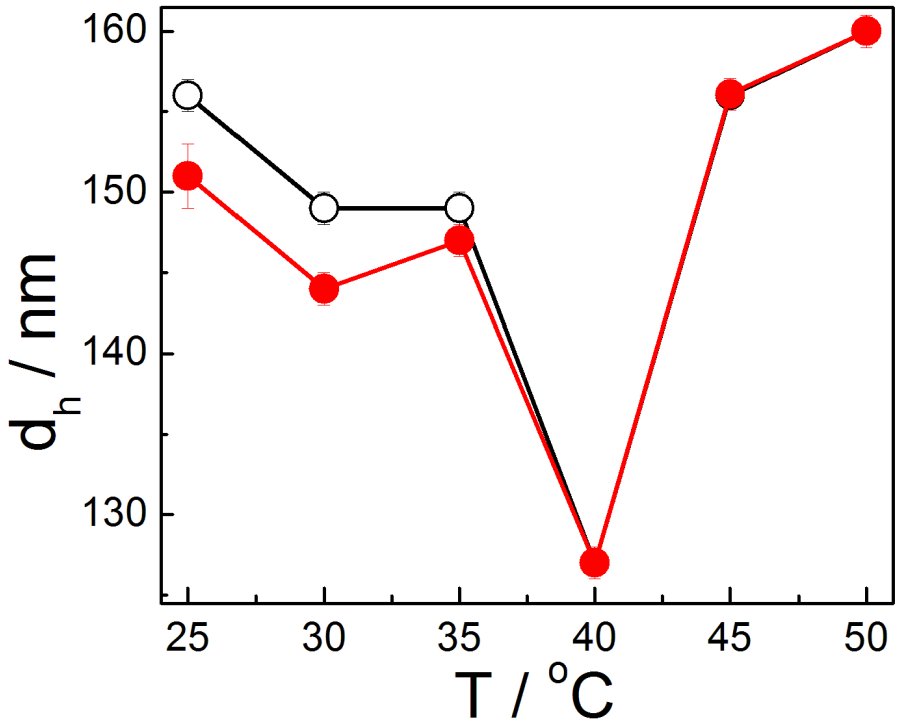


Figure 5. Reversibility in the variation of hydrodynamic diameter (d_h) of DPPC+DPPG liposome during the heating and cooling process. Open symbol (○) represents heating and closed symbol (●) represents the cooling curve. 30 mole% cholesterol was used in each case. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

Above the transition temperature, size increment, mostly irreversible, was simply due to the increase in the volume of liposomes as common for any dispersion.[41] Because of similarities in the hydrocarbon chains[11] except SPC, dependence of size variation on temperature, was almost similar for all the liposomes. PDI values did not change appreciably with temperature shown in Figure 7. Independence of PDI with respect to temperature indicates the absence of aggregation or de-aggregation phenomena during the heating and cooling processes.

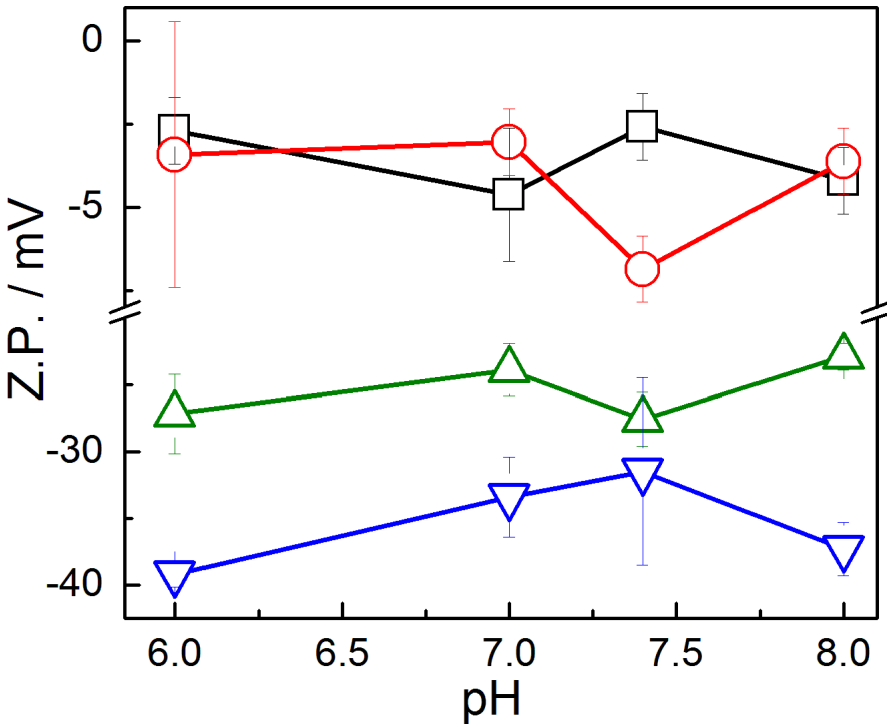


Figure 6. Variation in the zeta potential of different liposomes with pH. Systems: □, SPC; ○, DPPC; △, DPPC+DPPG and ▽, DPPG. 30 mole% cholesterol was used in each case with the lipids. Liposomes were prepared in 10 mM PBS +0.15 M NaCl. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

Zeta potential of liposomes, a consequence of the formation of electrical double layer, was also investigated (Figure 6). Mildly negative zeta potential was experienced by SPC and DPPC while the magnitude of negative zeta potential was higher for the other two systems[42)]. Magnitude of negative zeta potential for DPPC+DPPG liposome lied in between the values for DPPG and SPC/DPPC liposomes. Negatively charged liposomes are expected to exhibit enhanced stability compared to the zwitterionic liposomes.[43] Independence of zeta potential on the pH of the medium indicates that varying pH cannot significantly alter the electrostatic interaction between the phospholipid molecules.[44]

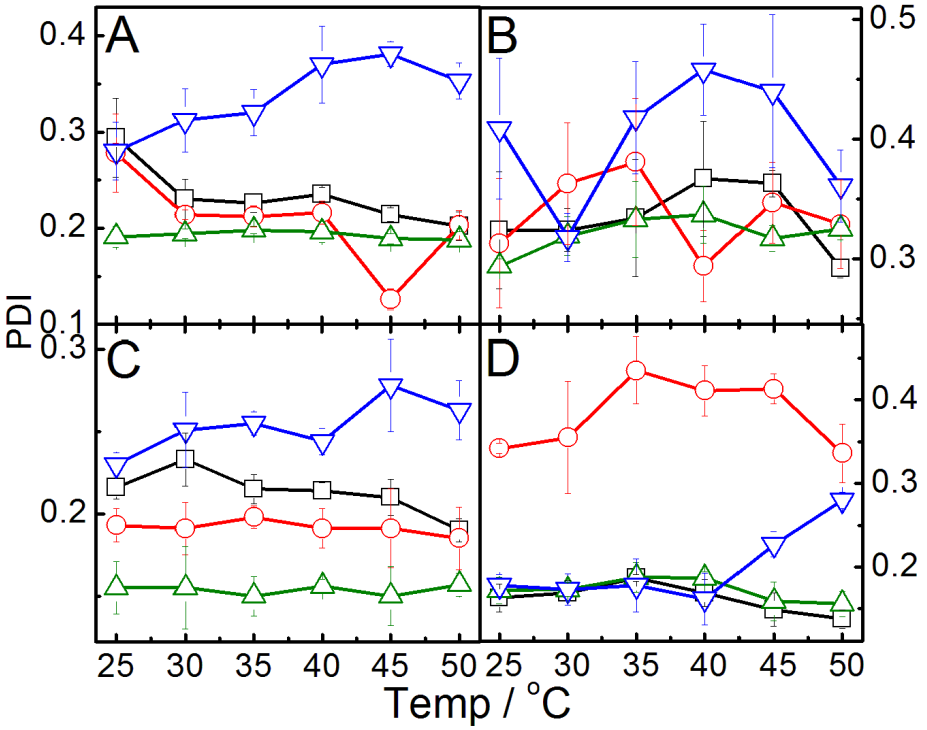


Figure 7. Variation in the polydispersity index (PDI) of liposomes with temperatures at different pH (□, 6.0; ○,7.0; Δ,7.4 and ▽, 8.0) with respect to temperature. Liposomes: (A= SPC, B=DPPC, C=DPPC+DPPG and D= DPPG). 30 mole% cholesterol was used in each case. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

3.2 Morphological studies

Representative micrographs of SPC and DPPC+DPPG liposomes are shown in Figure 8. Spherical morphology was observed for all the liposomes. The size of the liposomes, as obtained by the TEM measurements was found to be comparable with the DLS data. The existence of the bilayer could also be noticed for some of the liposomes (panel A). Bilayer thickness of the liposomes was found to be ~5.6 nm, comparable with the literature values.[45] Hydration on the surface of liposome could also be noticed (panel B). In order to have a better view of the liposome as well as the bilayer structure, further studies, like cryo-TEM or FF-TEM could be performed[25)] which are considered as one of the future perspectives.

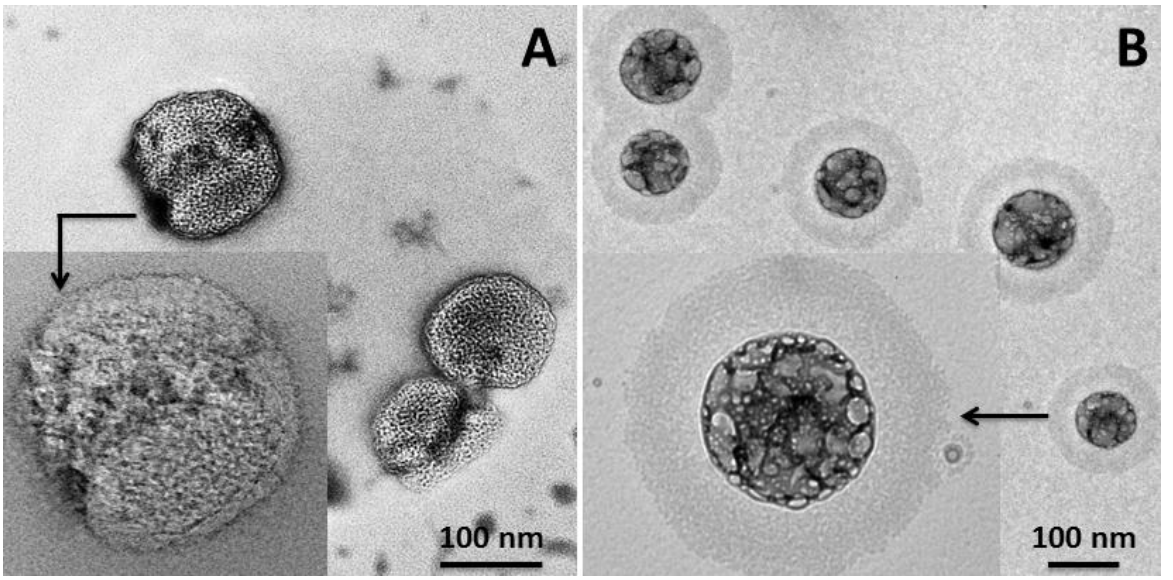


Figure 8. Representative TEM image of A, SPC and B, DPPC+DPPG liposome. 30 mole% cholesterol was used in each case with the lipids. Liposomes were prepared in 10 mM PBS +0.15 M NaCl. Numerical values are in the unit of nm.

3.3 DSC studies

DSC studies on the liposomes were performed with an aim to understand the bilayer chain melting process. DSC thermograms for SPC and DPPC+DPPG liposomes at different pH have been shown in Figigure 9A. Variation of chain melting temperature with respect to composition and pH for all the system were showed in inset of the Figure 9A. Thermal transitions with the maximum heat flow were relatively broader compared to the literature values. The presence of higher amount of cholesterol (30 mole%) resulted in the broadening of peaks[46)]. The main phase transition temperature of the liposomes was in the range of 38 to 50°C for the different studied pH (as shown in panel A). Influence of pH on the transition temperature (also known as the temperature of the maximum heat flow, T_m) was more significant among the negatively charged liposomes. However, no appreciable change in T_m was recorded for SPC and DPPC (Fig. 9A, inset). Protonation of phosphate groups of the phospholipids occur at acidic pH to a higher degree especially for the negatively charged liposomes (DPPG) that explains their increased T_m values in acidic environment (pH 6.0).[8, 12, 47] However, in case of zwitterionic lipids (SPC and DPPC), lesser extent of protonation, due to their zwitterionic nature, is responsible for the insignificant variation in T_m in the acidic pH. The presence of 30 mole% cholesterol also has

significant role in modulating the fluidity/rigidity of the lipid membranes, herein the rigidifying and fluidizing effect of cholesterol is expected for unsaturated SPC and saturated DPPC membranes respectively[8]. SPC showed additional peaks with three types of transitions (panel B, Figure 9). The first endotherm appeared in the temperature range -21 to -18°C (marked as "a", panel B). The second peak appeared in the temperature range 1 to 5°C (marked as "c"). The exothermic one, "b" appeared in the temperature range 0 to 4°C. The exact T_m values were dependent on the pH of the medium. The first endotherm indicates the melting of the unsaturated hydrocarbon chain of SPC [36, 39, 48-49]. The exothermic peak is the Kraft temperature representing the onset of the hydration of phospholipid head groups. The second endothermic peak is due to the melting of water[50-51]. The appearance of cooling peak was not found for all these systems.

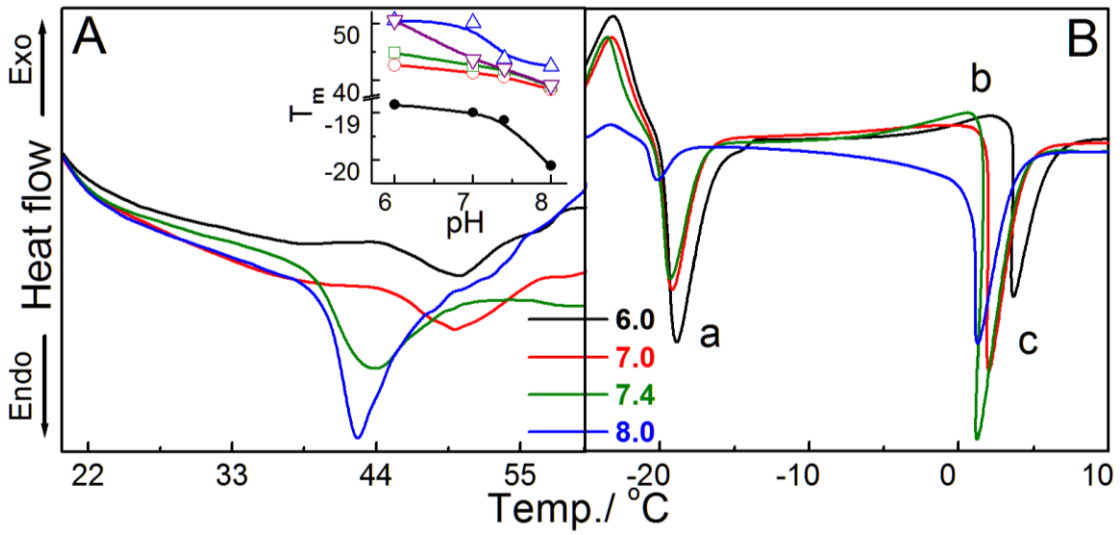


Figure 9. DSC thermogram of DPPC + DPPG + cholesterol (panel-A) and SPC + cholesterol (panel-B) liposomes at different pH (as indicated inside the figure). Inset: Chain melting temperature dependence on pH. Systems:●, SPC-a; ○, SPC-main; □, DPPC ; Δ, DPPC+DPPG and ▽, DPPG. 30 mole% cholesterol was used in each case. Scan rate 2 °C min^{-1}. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

3.4 Steady state fluorescence spectroscopic studies

Curcumin can act as an ideal molecular probe to understand the physicochemistry of membrane because of the dependence of its fluorescence on the polarity of the surrounding environment.[52-53] As the stability of curcumin depends on the pH of the medium[9, 54], its fluorescence spectra

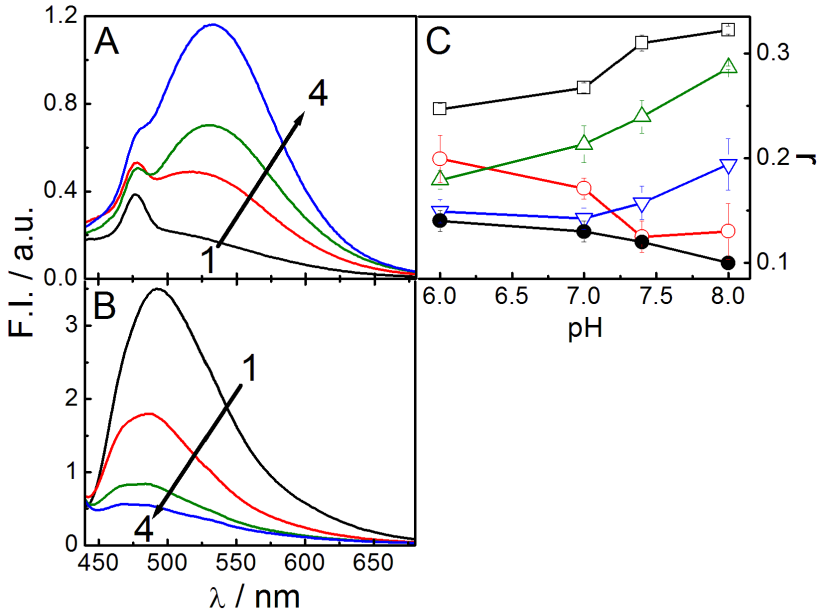


Figure 10. Fluorescence spectra of 15 µM curcumin in buffer (panel A), in DPPC+DPPG liposome (panel B) (1, pH 6.0; 2, pH 7.0; 3, pH 7.4 and pH 8.0). Panel C describes the variation in fluorescence anisotropy of curcumin with the pH of the medium. Systems: ●, buffer alone; ○, DPPC; ▽, DPPG ; Δ, DPPC+DPPG and □, SPC. Lipid conc. 500 µM. 30 mole% cholesterol was used in each case. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

was also recorded at different pH (Figure 10 A). When excited at 415 nm (λ_{ex}), curcumin exhibits weak fluorescence in water with the maximum (λ_{em}) appearing at ~534 nm. Fluorescence intensity increased with pH with a progressive shift in the λ_{em} value because of the deprotonation of curcumin hydroxyl groups in alkaline media (owing to its higher pK_a values, 8.38, 9.88 and 10.51).[53] Besides, the appearance of small shoulder at ~477 nm indicates the chemical degradation of curcumin to smaller molecules[9)]. Incorporation of curcumin into liposomes caused fluorescence enhancement with significant blue shift in λ_{em} values indicating its transfer to the less polar region of liposome[15, 24, 55-56] *i.e.* curcumin in liposomes experiences nonpolar environment. With increasing pH, fluorescence intensity decreased (Figure 10 B) for DPPC+DPPG. Other systems exhibited almost similar behavior (Figure 11). Curcumin, predominantly in its neutral form at lower pH, binds strongly to liposomes through hydrophobic interactions; thus the fluorescence intensity of curcumin is enhanced in liposomes at lower pH.

However, at higher pH, curcumin decomposes from its conjugated diene; leading to its poor adherence to the liposomes.[54]

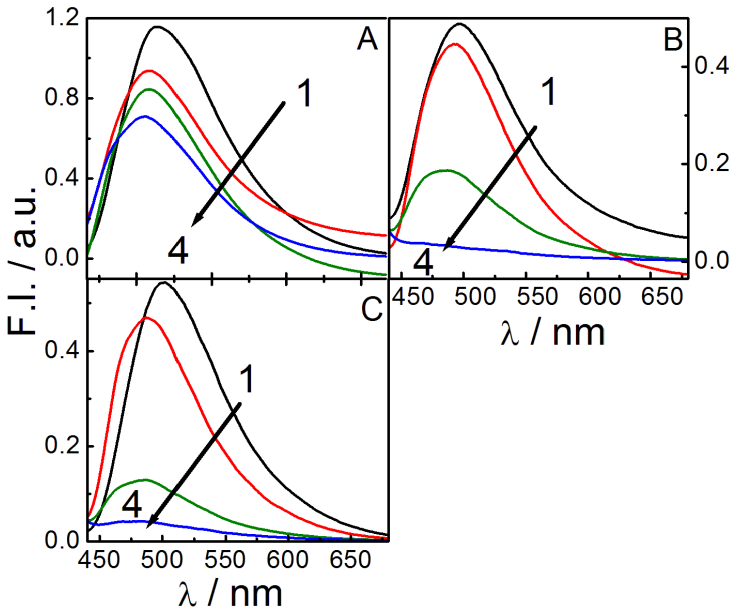


Figure 11. Fluorescence spectra of 15 µM curcumin in liposome systems: SPC (panel A), DPPC (panel B) and DPPG (panel C). (1, pH 6.0; 2, pH 7.0; 3, pH 7.4 and 4, pH 8.0). λ_{ex}= 415 nm. 30 mole% cholesterol was used in each case. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25 °C.

3.5 Fluorescence anisotropy studies

Fluorescence anisotropy has a direct relationship with the micro viscosity of a medium[11, 57)]. Variations in fluorescence anisotropy (r) of curcumin, loaded in liposomes, with pH have been shown in panel C of Figure 10. Increase in 'r' value of curcumin, when incorporated in the liposome, is due to the restricted motion, thereby confirming its entrapment.[15, 58] Anisotropy value followed the order: SPC > DPPC+DPPG > DPPG, irrespective of the pH of the medium. DPPC behaved differently than the other systems. SPC, being fluid in nature at room temperature, is more flexible and permeable[59] than the saturated lipids. On account of this fact, the insertion ability of the curcumin molecules into the bilayer core of the SPC liposomes is enhanced compared to other systems. Besides, the polarity of head groups of SPC is higher than other lipids due to the presence of unsaturation in one of its hydrocarbon chains.[60] These dual combined effects result in better adherence of curcumin into the hydrocarbon chains as well as

onto the palisade layer of the SPC liposome respectively[15)]. Thus, the movement of curcumin in SPC liposomes is hindered to a greater extent compared to other systems which explains the highest anisotropy value of curcumin for SPC liposome. Lowest 'r' value for DPPG could be due to the similarity in the charge of the probe and the lipid[61)]. Anisotropy value for all the systems (except DPPC) increased with pH, which could be rationalized in terms of increased ionization of curcumin, thereby resulting in the availability of fewer neutral curcumin molecules at higher pH.[62] It has been demonstrated by Niu *et al.*[15] that the curcumin molecule (in the neutral form) at high concentration causes disorderness in the packing of lipid bilayer. As minimal numbers of neutral curcumin molecules are present at higher pH, disruption of bilayer packing is less probable in such condition compared to the same at lower pH. Thus, at elevated pH, restriction in rotation of curcumin is enhanced which elucidates the inclination tendency of 'r' value of curcumin with increasing pH. The typical declining trend of 'r' value in DPPC liposome with increasing pH could be ascribed to the rigid/gel state of DPPC liposomes[59)]. The ability of curcumin molecules to penetrate deeper into the bilayer region of DPPC membrane is less pronounced compared to the other systems.[59] Thus, the quantitative amount of curcumin molecules adhering to the DPPC bilayer is relatively lesser and the concentration of curcumin in DPPC membrane is presumably not sufficiently high to cause disordering of lipid bilayer packing, as evidenced with other systems. With increasing pH, the concentration of neutral curcumin molecules bound to the DPPC bilayer is decreased owing to the ionization tendency of the probe. Thus, the freedom of curcumin movement in the DPPC liposome is enhanced, thereby resulting in the decrease of anisotropy value with the rise of pH.

3.6 Curcumin entrapment and in vitro release kinetics studies

Entrapment efficiency (EE) of liposomes for curcumin is shown in Figure 12A at different pH. EE values of liposomes, irrespective of the pH of the medium, followed the order: DPPC > (DPPC+DPPG) > DPPG > SPC. SPC is fluid at the physiological temperature owing to the presence of unsaturation in acyl chains.[59] It is therefore expected that the withholding capacity of the SPC liposome for curcumin will be lesser in contrast to other liposome systems. The lowest entrapment efficiency of curcumin in SPC systems can thus be explained on the basis of above mentioned rationale. DPPC exhibits opposite (rigid) behavior due to saturated hydrocarbon chains. Hydrophobic interaction between curcumin aromatic moiety and the acyl chain of lipids

is more favorable in DPPC; thus, the liposome can entrap larger number of curcumin molecules. DPPG, being negatively charged, exerts repulsive interaction with curcumin for which its EE is less. As expected, EE of DPPC+DPPG showed intermediate behavior between DPPC and DPPG. With increasing pH, EE of all the liposomes decreased due to deprotonation of curcumin[62]. This subsequently resulted in the entrapment of fewer curcumin molecules.

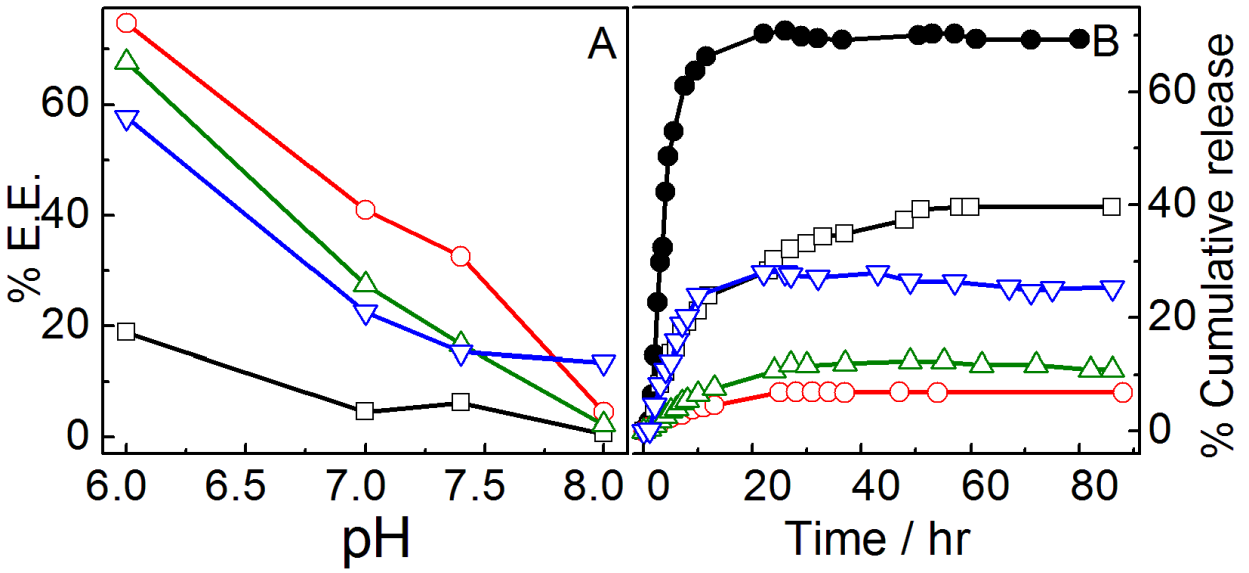


Figure 12. Panel A: Effect of pH on curcumin entrapment efficiency (%EE) of liposomes. Panel B: Dependence of curcumin release profile on the type of liposome at pH 7.4. Systems: ●, control (buffer alone); ○, DPPC; Δ, DPPC+DPPG; ▽, DPPG and □, SPC. 30 mole% cholesterol was used in each case. Curcumin conc. 15 µM; Lipid conc. 500 µM. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

In vitro release kinetics of curcumin were studied to compare the four different liposomal systems and also to define the nature of the release processes. Figure 12B compares the cumulative percentage release of curcumin from the liposomes for 80 h. Diffusion of curcumin in aqueous medium across the dialysis bag was also studied as control. Curcumin release from the liposomes at pH 7.4 followed the order: SPC> DPPG> DPPC+DPPG> DPPC. Faster diffusion of curcumin without liposome compared to that embedded in liposome proves the sustained release of entrapped curcumin. It is therefore proposed that liposomes can act as an effective drug delivery system, ensuring sustained and prolonged release of curcumin.[36, 63] Initially, burst release was recorded for curcumin in all the formulations which was due to the

release of the curcumin adsorbed on the liposome surface as well as the free curcumin molecules. However, the slower and sustained release was due to the entrapped curcumin inside the liposomes. Faster release for SPC is due to its reduced tendency to withhold curcumin. Due to the fluidic nature of SPC, curcumin can elute out more rapidly from SPC systems.[64] DPPC liposomes can withhold a larger number of curcumin molecules resulting in the slowest release rate. DPPG showed the initial burst release (~25%) similar to SPC but the attainment of constancy was different; while it was at 20 h for DPPG, the same appeared at 60 h for SPC. This was due to easier detachment of curcumin from negatively charged DPPG liposome due to similarity in charges.[63] Similar to the entrapment efficiency studies, DPPC+DPPG liposome showed an intermediate release profile. In an attempt to understand the role of membrane microviscosity on the release pattern of drug, herein curcumin, the release data of drug were correlated with its anisotropy value in different systems at pH 7.4. Unlike the general assumption, the results indicated that the release rate of drug increased with rise in the microviscosity (anisotropy value) of the medium. Although the movement of curcumin in SPC liposomes was restricted to the greater extent (highest 'r' value) compared to other systems, the drug release rate was higher for such systems. Likewise, curcumin in DPPC liposome with minimum hindrance (lowest 'r' value) showed slowest release rate. The corresponding drug release behavior is due to the fluidic and gel state of SPC and DPPC liposome respectively. The resultant experimental evidence suggests that the drug release rate was predominantly monitored by lipid composition/nature rather than the binding affinity between curcumin and lipid bilayer. Data of the drug release profile were fitted to different models, *viz.*, pseudo first order, Higuchi, Korsemeyer-Peppas, Hixson-Crowell using DD Solver 1.0, an Add-In program, and the equations of the respective models are presented below[65]:

Higuchi model: $F = k_H . t^{0.5}$ (6)

Korsemeyer–Peppas model: $F = k_k\, t^n$ (7)

Hixson-Crowell model: $F = 100.[1 - (1 - k_{HC}\,.\,t)^3]$ (8)

Pseudo first order model: $F = 100.(1 - e^{-k_1 . t})$ (9)

where, F is the percentage of the drug released, k_H, k_k, k_{HC} and k_1 are the release rate constants of Higuchi, Korsemeyer-Peppas, Hixson-Crowell and pseudo first order model respectively, t represents the time lag of the dissolution process, and n is the release exponent obtained from

Korsemeyer-Peppas model. The selection of the above mentioned kinetic models on drug release was based on the fact that such models are the most widely studied models for the drug release through sustained/controlled drug delivery systems.[66] The release kinetics parameters for all the models are summarized in (Table 1). Release kinetics of curcumin in all the formulations were found to be best fitted to Korsemeyer-Peppas model as indicated from its highest regression values (r^2). In this model, the data obtained from drug release studies are plotted as logarithm of cumulative percentage drug release versus log time. The release rate constant (k_{KP}) was found to be higher for SPC followed by DPPG, DPPC+DPPG and DPPC systems respectively. Korsemeyer-Peppas model is the most preferred one to define the release mechanism of drug through delivery systems which is characterized by the release exponent (n) value determined from the same model. The 'n' value less and greater than 0.5 is indicative of Fickian and non-Fickian diffusion respectively. In all the investigated systems, 'n' value was found to be less than 0.5 revealing Fickian diffusion as predominant drug release mechanism.

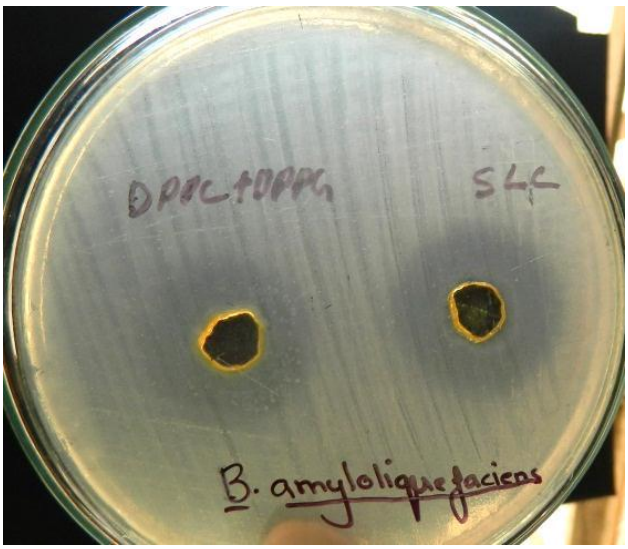


Figure 13. Effect of curcumin loaded liposome on the growth of *Bacillus amyloliquefaciens*. 30 mole% cholesterol was used in each case. Liposomes were prepared in 10 mM PBS + 0.15 M NaCl. Temperature 25°C.

Table 1 Release kinetics parameters of the respective models for curcumin loaded liposome.

Liposomes	Pseudo first order		Higuchi		Korsemeyer-Peppas			Hixson-Crowell	
	r^2	k_1/hr^{-1}	r^2	$k_H/hr^{-0.5}$	r^2	k_K/hr^{-n}	n	r^2	$k_{HC}/mg^{1/3}hr^{-1}$
SPC	0.9170	0.011	0.9627	5.442	0.9726	9.581	0.412	0.9035	0.003
DPPC	0.7891	0.001	0.9162	1.054	0.9364	1.613	0.381	0.7851	0.001
DPPC+DPPG	0.8078	0.002	0.9055	1.586	0.9250	2.504	0.380	0.8035	0.001
DPPG	0.6982	0.006	0.8081	3.832	0.8766	7.484	0.402	0.6866	0.002

*r^2= regression coefficient, k_1, k_H, k_K & k_{HC}= release rate constant of respective models, n=diffusional exponent.

3.7 Antibacterial activity

Curcumin loaded nanoparticulate systems are reported to exhibit substantial antibacterial activity. Recently, an enhancement in the *in vitro* antibacterial activity of curcumin loaded vesicle system against *Propionibacterium acnes* in the skin was observed by Liu *et al.*[67]The antibacterial efficacy of the liposomes, loaded with and without curcumin, was assessed on gram negative (*Klebsiella pneumoniae* and *Pseudomonas putida*) and gram positive bacteria (*Bacillus amyloliquefaciens* and *Bacillus subtilis*). No antibacterial activity was recorded with the liposomes in the absence of curcumin. All the liposomes loaded with curcumin exhibited substantial antibacterial activity against gram positive bacteria; however, effects on the gram negative bacteria were insignificant. Basniwal *et al.*[23] also demonstrated the pronounced antimicrobial activity of aqueous solution of nanocurcumin compared to curcumin solution and the activity was more prominent against gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) than gram negative bacteria. The obtained distinct zones of inhibition (24 h of incubation) for *Bacillus amyloliquefaciens* by curcumin loaded liposomes (SPC and DPPC+DPPG) have been presented in Figure 13. Percentage of inhibition zone, calculated for all the systems, was found to be independent of composition as there was no significant difference between the different systems. Nearly 74% growth inhibition were recorded in all the cases.

4. Conclusions

Physicochemical properties of different liposomes, *viz.*, size, PDI, surface charge, thermal behavior, and membrane micro viscosity as well as their encapsulation efficiency and release behavior of curcumin were pronouncedly influenced by liposome composition along with pH and temperature of the medium. All the studied liposomes (SPC, DPPC, DPPG and DPPC+DPPG) were found to be stable in terms of size, PDI and zeta potential values. Size of liposomes did not vary significantly with temperature in terms of composition, albeit the minimum size was evidenced at ~40°C corresponding to phase transition temperature of the saturated phospholipids. All the systems turned out to be more rigid with increasing acidity of the medium as revealed from DSC results; this was due to increased hydrogen bonding among phospholipid molecules. Maximum anisotropy value of curcumin for SPC liposome reflected its higher binding affinity compared to the other systems. Increase in membrane microviscosity of the vesicles (except DPPC) with the rise in pH of the medium could be rationalized on the basis

of the ionizing tendency of curcumin in basic environment and its ability (in neutral form) to disrupt bilayer packing at high concentration. Entrapment efficiency, however decreased with increasing pH of the medium due to the acidic nature of drug. Entrapment efficiency and release kinetics studies revealed opposite phenomenon with respect to liposome composition. SPC exhibited fastest drug release and lowest entrapment efficiency owing to its fluid nature. Curcumin loaded liposomes exhibited pronounced antibacterial activity against the Gram positive bacteria *Bacillus amyloliquefaciens*. Experimental evidences led to conclude that the optimization of the lipid composition and formulation conditions like pH is necessary to prepare liposomes with enhanced stability and efficient drug carrier property. *In vivo* studies of the curcumin loaded liposomes, to evaluate different pharmacokinetic parameters, could be considered as one of the future perspectives.

CHAPTER II

CHAPTER II

Physico-chemical studies on the interaction of dendrimers with lipid bilaylers. 1. Effect of dendriemer generation and liposome surface charge.

Abstract: Studies on the interaction of different generation poly(amidoamine) (PAMAM) dendrimers and combinations of liposomes are reported in this paper. Second, fourth and sixth (2G, 4G, and 6G) generation PAMAM dendrimers were used, which are cationic under normal conditions. Liposomes comprised of soy lecithin + cholesterol (SLC+CHOL) (negative surface charge), DPPC+CHOL (positive surface charge), DPPG+CHOL (negative) and a biologically simulated mixture of DPPC + DPPG (7:3) + CHOL (negative) were used as model bilayers. Silica was used as a negatively charged hard sphere model to make a comparative study. Absorbance (turbidity) at 420 nm, dynamic light scattering, zeta potential measurements on liposome and finally atomic force microscope (AFM) measurements on solid supported bilayers (by vesicle fusion on freshly cleave mica) were performed to study the interactions. Maxima in absorbance and size of liposome was observed upon PAMAM addition. Charge reversal happened with the progressive addition of dendrimer. Interaction between PAMAM with liposome were found to be driven predominantly electrostatic. PAMAM activity was found to be generation dependent as 6G >4G>2G in terms of overall dendrimer concentration. But, interestingly, the order gets reverse when PAMAM activity was considered in terms of total end group concentrations. AFM studies reveal the rupture of bilayer structure upon addition of dendrimer.

1. Introduction

Recent advancements in drug therapeutics are mainly based on nanotechnology. Dendrimers are assumed to have very high potentials to act as nano-vectors for drugs. Dendrimers are basically polyionic compounds, having precise molecular weight, low polydispersity and also a core/shell like structure [1-6]. The dendrimers, due to their unique structure, different from the usual/conventional polyelectrolytes, possess special features. For example, the cavity/cage inside the dendrimer core, can host small molecules, may it be drug or metallic clusters [7]. The dendrimers have a predominant spherical/globular structure, for which they have low viscosity compared to linear polyelectrolytes. Dendrimers are now becoming one

of the priority research sectors for their potential applications in material sciences like photo sensors, catalysis [8], nanoparticle synthesis. These compounds are now considered to be highly promising in biological systems too. Dendrimers are now extensively being used in drug delivery, gene therapy, biomemitcs [8-14]. All the dendrimer actions in biological systems are basically dependent on their membrane disrupting capabilities/properties [7]. Therefore a basic understanding on the bilayer disruption, induced by dendrimers, is essential. Studies on such interaction thus have become an emerging area of scientific research. Such studies could help in a better understanding of membrane disruption by dendrimers, especially in the molecular level [15-18].

Natural cell membranes are composed of lipid bilayers. Liposomes, vesicles, and phospholipids bilayers, supported on planar substrates, are considered as useful model membrane systems [19]. Although reports on the bilayer-dendrimer interactions are gradually increasing [7, 16-18, 20], but specific choice of lipid was not clearly mentioned in most of the reports. Use of different kinds of lipids can be found in different reports. Therefore, a systematic study on the lipid charge, surface charge on the liposome and also use of a more biologically relevant/mimic system is essential. This driving force encouraged us to execute a systemic study on dendrimer-liposome interaction with the different kind of liposomes. In the present report, we have used a variety of lipids. Moreover, we have used cholesterol as one of the components, rarely used by others. Liposomes made from a pure lipid DPPC (positive surface charge), DPPG (negative), a natural mixture soy lecithin (SLC) and a 7:3 (mole/mole) of DPPC/DPPG were studied. In every case 30 wt% of cholesterol was also used. By such a study, we could vary the surface charge on the liposome and hence a better understanding of interaction was attempted.

Poly(amidoamine) (PAMAM) dendrimers are nowadays frequently being used for their excellent monodispersity, well defined shape and size and other physicochemical parameters[6, 21]. This class of dendrimers can cause bilayer disruption easily and their activity increases with generation [17, 20]. Biocompatibility of PAMAM dendrimers have encouraged several research groups to study their effect on lipid vesicles [22-26].

Interaction of liposomes with dendrimers results in the formation of bigger aggregates of various sizes. The growth can be envisaged by the dispersion turbidity change. The more effective way to visualize the growth in liposome aggregates, assisted by dendrimers, is through

measuring their hydrodynamic diameter. Dynamic light scattering measurements (DLS) experiments help in measuring the growth and subsequent changes, size distribution, upon addition of dendrimer to liposome solutions.

The driving forces during the interaction of dendrimer with liposome are basically electrostatic and / or through hydrogen bonding. Weak van der Waals types of forces also contribute the interaction. Basically dendrimers get adsorbed onto the liposome surface by virtue of electrostatic interaction [27-28]. Hence one should expect a change in the zeta potential during the dendrimer-liposome interaction, provided the interaction is predominantly electrostatic.

Atomic force microscope (AFM) has proven itself a useful toll to shed light on the membrane disruption by dendrimers in the molecular level. Such a measurement helps to point out a common underlying mechanism for bilayer disruption [16-18, 27-28].

In this paper, we have presented reports on the interaction of 2G, 4G and 6G PAMAM with liposomes of different phospholipids. Pure, as well as mixtures were used. Turbidity/absorbance, DLS, zeta potential measurements of liposome solutions were done. Solid supported bilayer, by fusion of liposome on freshly cleave mica, was used to study the bilayer disruption induced by PAMAM dendrimer, monitored by AFM measurements.

2. Materials and Methods

2.1. Materials

Soya lecithin (99% pure) was a product from BDH, England while cholesterol was purchased from Lab Chem, Australia. All other phospholipids were purchased from Avanti Lipids, AL, USA. They were used as received. PAMAM dendrimers of different generations, 2G PAMAM, 4G PAMAM and 6G PAMAM were obtained as methanolic solutions in different concentrations from Sigma Chemicals Co., USA. Sodium chloride was an E. Merck product. Milli Q water with a resistivity of 18 Ωcm was used throughout the experiments.

2.2. Methods.

2.2.1. Liposome preparation. Unilamellar vesicles were prepared by the well known thin film rehydration- sonication- extrution method [29-31]. Briefly, phospholipids, along with cholesterol of

desired combination and amount, were dissolved in chloroform-methanol (3:1, v/v) in a round bottom flask. A thin film was created in the flask using a rotary evaporator. Care was taken to prevent formation of any bubbles. Thin film was then rehydrated in 1.0 mmol dm^{-3} NaCl solution in a water bath at 75^0C with rotation (well above the transition temperature of lipids). Concentration of liposome was made 5.0 m $moldm^{-3}$ with respect to the phospholipids PC and/or PG). Hydration was done for a period of three hours after which almost all the solid materials got dispersed into aqueous phase. It was then sonicated in a bath sonicator for about 40 mins when a homogeneous solution was generated. The entire mass was then frozen and thawed for five cycles. Large multilamellar vesicles were thus broken into small unilamellar vesicles (SUV). It was then successively extruded (at least 11 times) through 800 nm and 100 nm polycarbonate membrane filters in a laboratory extruder (Liposo Fast-Pneumatic, Avestin Inc.) prior to studying dendrimer-liposome interactions. For the (DPPC+DPPG+CHOL) liposome, it was very hard to extrude, as probably due to higher transition temperature. Extrusion was done at 45^0C, although after extrusion size were found to be higher than other liposome systems. Size analysis, by DLS measurements, although revealed fair monodispersity.

2.2.2. Instrumental analysis.

Interaction of dendrimers with different liposomes were studied by measuring the absorbance at 420 nm [32]. At this wavelength, the turbidity of a solution is assumed to be proportional to absorbance. Measurements were done using a Cary1E UV-Visible spectrophotometer (Varian). Liposome solutions without dendrimer was used as blank.

Size and zeta potential of liposome solutions in presence and absence of dendrimer were measured in a dynamic light scattering spectrometer (Zeta Sizer Nano, Malvern Instruments, U. K). He-Ne laser emitting light at 632 nm was used. Size measurements were done using a quartz cell of 1.0 cm path length, while a specially designed plastic cell was used for zeta potential measurements [33]. STDEV for zeta potential was found to be ±5.0 mV.

AFM images (tapping mode) on solid supported bilayer was obtained using a Multi Mode Nanoscope III microscope (Digital Instruments, Santa Barbara, CA, USA). Images were taken in tapping mode. 100 μL of liposomal suspension was placed on a $1cm^2$ freshly cleaved mica, incubated for an hour at 37^0C. Excess lipid was then gently washed off with 1 mmol dm^{-3} NaCl

solution. It was then scanned in tapping mode using a liquid cell. Silicon nitride cantilever with a spring constant of 0.06 Nm^{-1} operating at a driving frequency of 7-9 kHz was used. Bilayer images were taken at different resolutions. Then dendrimer solutions of desired concentration was used to rinse the bilayer. After half an hour, it was again scanned to visualize the effect of dendrimers. Rinsing was done slowly with care so that the bilayer structure does not get disturbed.

All the experiments were done at 25^0C.

3. Results and Discussion

As already mentioned in the introduction that interaction effectiveness in dendrimer-liposome system can be assessed by turbidity measurements. This method has extensively been used by others [27-28, 32]. Figure 1 shows the effect of different generation PAMAM dendrimers on the absorbance enhancement of (SLC+CHOL) liposome. There is a threshold concentration after which the absorbance increase, reachs a maximum and decreases again. Similar effects are observed in case of size analysis by DLS measurements. Representative DLS data are shown in Figure 2. Initial size increment and attainment of maxima is probably due to aggregation/association of liposomes, assisted by dendrimers. Dendrimers being oppositely charged, compared to the surface charge of liposome, get easily bound/attached to the liposome surface. Sideratou et al. suggested that dendrimer acts as "glue" for liposome [27-28]. We also observed a decrease in size upon further addition of dendrimer. But unlike them our system become clear even after the maxima. This clarity was due to the formation of dendrimer-liposome soluble complexes (dendriosomes) or other soluble forms. Capability in enhancing turbidity or size in liposomes were found to be dependent on the generation of the dendrimer. From Figures 1 and 2 it is clear that activity of PAMAM dendrimer followed the order 6G>4G>2G. This trend is not unexpected when one considers the activity in terms of molar concentration of dendrimer itself. 2G, 4G and 6G PAMAM have 16, 64 and 256 end groups respectively. Higher generation dendrimers having higher number of end groups will obviously require lesser amount for effective interaction. Thus it is more meaningful to consider dendrimer activity in terms of end groups' concentration, as shown inset of Figure 2. Figure in the inset of

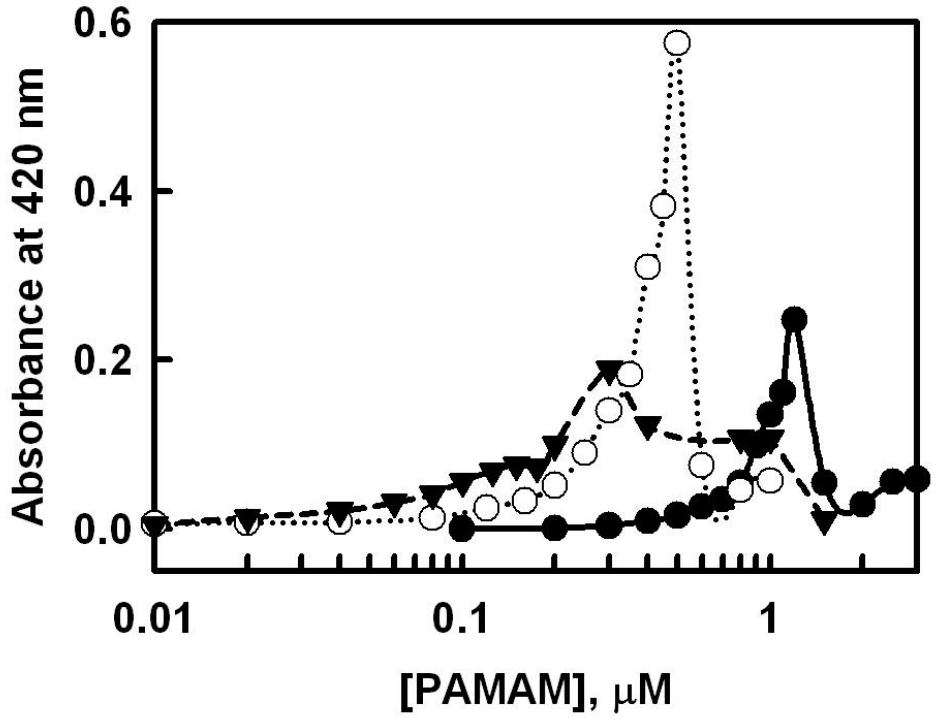


Figure 1. Effect of dendrimers on the abosorbance of the 0.1 m mol dm^{-3} (SLC+CHOL) liposome solutions (with respect to phospholipid). ●,2G PAMAM, O,4G PAMAM, ▲,6G PAMAM.

Figure 2 reveals the reverse order in dendrimer activity. Lower the generation more the end groups accessible for effective interaction. Moreover, with the increase in dendrimer generation, end groups tend to back fold. Thus for higher generation dendrimers, lesser number of end groups could actively take part in inducing liposome aggregation.

Interaction of other liposome surface with different dendrimers were also studied. DPPC did not response to any dendrimer. DPPC has a positive surface charge in its liposomal solution (as to be seen from zeta potential measurements). Hence it is not supposed to interact with the positively charged PAMAM dendrimers. On the other hand DPPG+CHOL liposome was quite responsive, even compared to SLC+CHOL liposome. DPPG being negatively charged should respond in a stronger way. But most surprisingly, when (DPPC+DPPG+CHOL) mixture was used in liposome, it showed the maximum interaction. This could better be explained by zeta

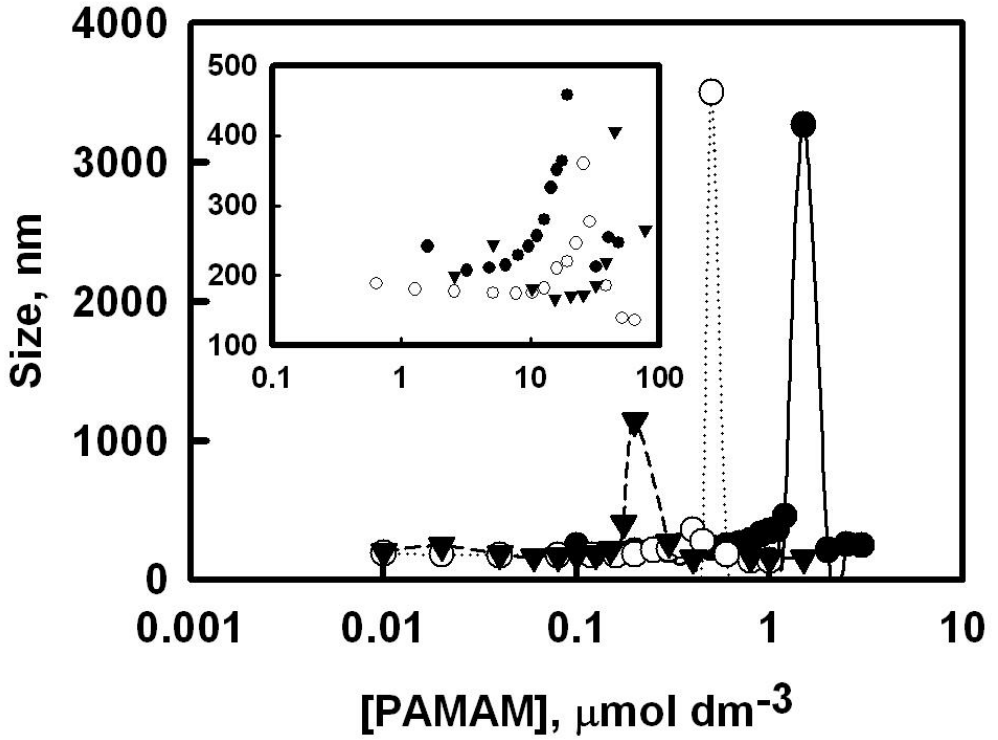


Figure 2. Effect of dendrimers on the size of 0.1 m mol dm^{-3} (SLC+CHOL) liposome solutions (with respect to phospholipid). ●,2G PAMAM, O, 4G PAMAM, ▲,6G PAMAM. Inset: Similar plots in terms of end group concentration of dendrimers.

potential measurements. Interaction between negatively charged surface (liposome bilayer) and the positively charged dendrimer were further explored by zeta potential measurements. Representative results are summarized in Figure 3. From Figure 3A it is evident that negative values of zeta potential gets decreased to the positive side upon addition of dendrimers. At a certain concentration zeta potential of the mixture attains zero value, which suggest the charge neutralization. Further increase in zeta potential suggests that it is possible for the non charged liposomal particles to further interact and aggregate with the PAMAM [34]. After a certain concentration limit, the zeta potential attains a platue, indicating the saturation of liposomes. Interaction after the charge neutralization is probably driven through hydrogen bond and/or hydrophobic interaction. Presence of secondary and tertiary amino groups might induce such interaction.

Soy lecithin is a mixture of different phospholipids, major ingredient of which is phosphatidylcholine. Therefore, it is worthwhile to study with a pure compound (along with cholesterol) towards a better understanding of dendrimer liposome interaction. Thus we have

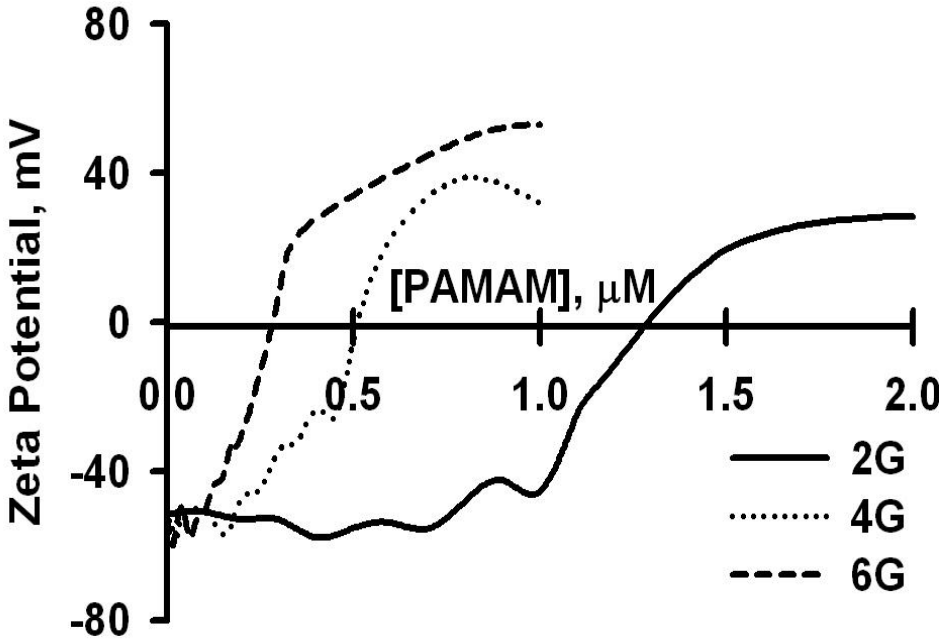


Figure 3. Variation of zeta potential upon the progressive addition of different generation dendrimers on a 0.1 m mol dm^{-3} (SLC+CHOL) liposome. Dendrimer generations are indicated in the graph.

also studied DPPG+CHOL liposome mixture. DPPG being a negatively charged lipid generates a negatively charged surface. We also observed that this liposome interacts stronger than SLC+CHOL. Stronger interaction is ascertained by a higher slope of zeta potential and lesser requirements of dendrimers for charge/zeta potential reversal (data not shown). The order also followed the same trend in terms of dendrimer generation, i.e., 6G>4G>2G. But when the dendrimer activities were expressed in terms of total end group concentration, reverse was the order. Results are summarized in Figure 4. This clearly suggested that all the end groups of dendrimers could not effectively take part during interaction. It is known that for higher generation dendrimers, the end groups get back folded. For lower generation dendrimer, it is easier for more end groups to actively take part in inducing the charge neutralisation of liposome surfaces.

To further ascertain the pre-dominancy of electrostatic interaction, similar studies with silica particles (of 500 nm diameter) was also done. Silica, having a stronger surface charge, showed instantaneous interaction with the liposome. It was considered to be a baseline. On the

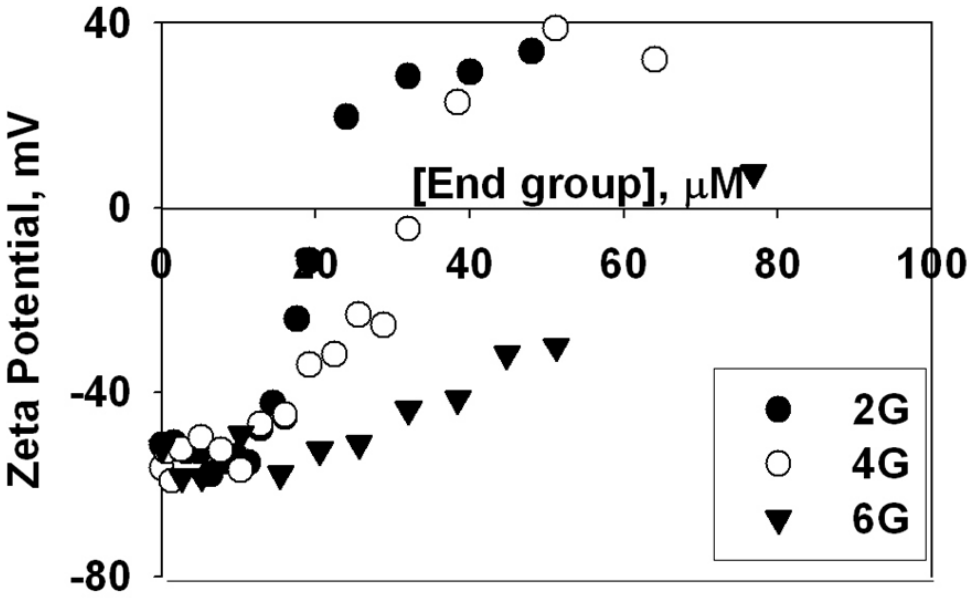


Figure 4. Effect of different generation PAMAM dendrimers on a 0.1 m mol dm^{-3} (SLC+CHOL) liposome. Dendrimer generations are indicated in the graph. Concentrations are expressed in terms of total end group concentration.

other hand when a mixture of DPPC+DPPG+CHOL (PC:PG = 7:3 mole ratio, and Phospholipid : CHOL = 3:1, weight ratio) was used, something interesting happened. This particular liposome shows better activity than the DPPG+CHOL mixture. This supports the hydrogen bonding/hydrophobic interaction between the dendrimer and liposomes. A comparative data is graphically represented in Figure 6. It is clear from the figure that the order of activity amongst the liposome/ substrate was silica>> (DPPC+DPPG+CHOL) > (DPPG+CHOL) > (SLC+CHOL) >> (DPPC+CHOL) (almost no interaction). The order was in accordance with the surface charge, except in the second one. Here predominantly hydrophobic/hydrogen bonding take part in interaction.

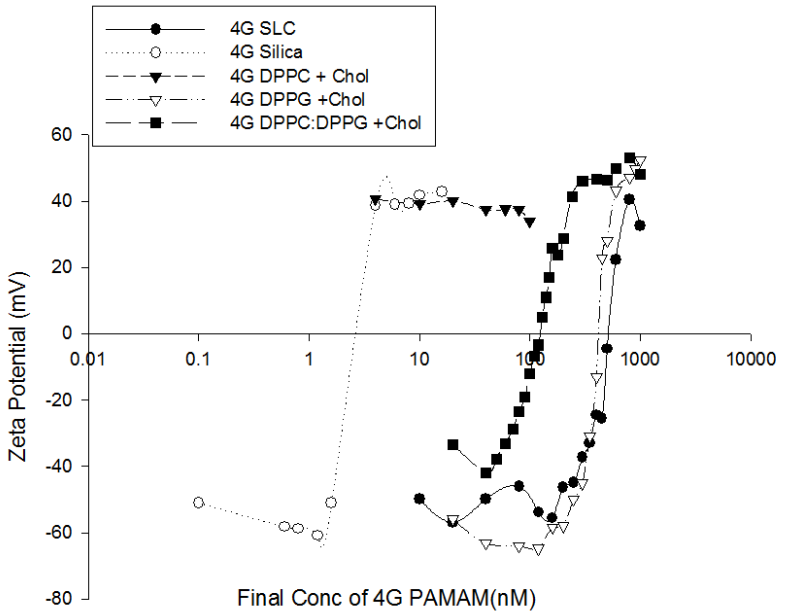


Figure 5. Comparative studies on the interaction of different substrates with 4G PAMAM by zeta potential measurements. Liposome concentration = 0.1 m mol dm^{-3} (with respect to the phospholipid). Similar strength (in terms of weight) of silica was used for a comparison.

Atomic force microscope has now become a useful tool for understanding the bilayer structure in a molecular level. Also bilayer disruption, induced by dendrimers, can also be visualized by this method. We have tried with different kinds of liposomes for such study. (SLC+CHOL) liposome did not generate any characteristic feature, (DPPC+CHOL) showed bilayer structures. Bilayer structures of vesicles, fused on freshly cleaved mica surface, were supported by height analysis. A typical bilayer should have a height profile of around 5.9 – 6.0 nm. In our systems we also observed the same. (DPPC+CHOL) liposome did not respond to any dendrimers. It was also not unexpected, as we did not observe significant interaction of dendrimers with this liposome. In figure 6 a representative AFM image of a (DPPC+DPPG+CHOL) bilayer is shown in absence of dendrimers, along with the height analysis. When dendrimers are added, the bilayer feature gets disrupted, as shown in Figure 7. Upon addition of 100 nM 6G PAMAM, as shown in Figure 7B, it is observed that the dendrimers get adsorbed onto the bilayer, preferably near the bilayer edges, while at higher dendrimer concentration, the bilayer structure gets completely disrupted. Preferential adhering of dendrimers around the bilayer edges is not uncommon[17]. Mica having a negative surface should attract positively charged dendrimers, hence such a feature is observed. At higher concentration

of dendrimers, possibly dendrimers forms some complex conjugates with the liposomes, hence the disruption takes place.

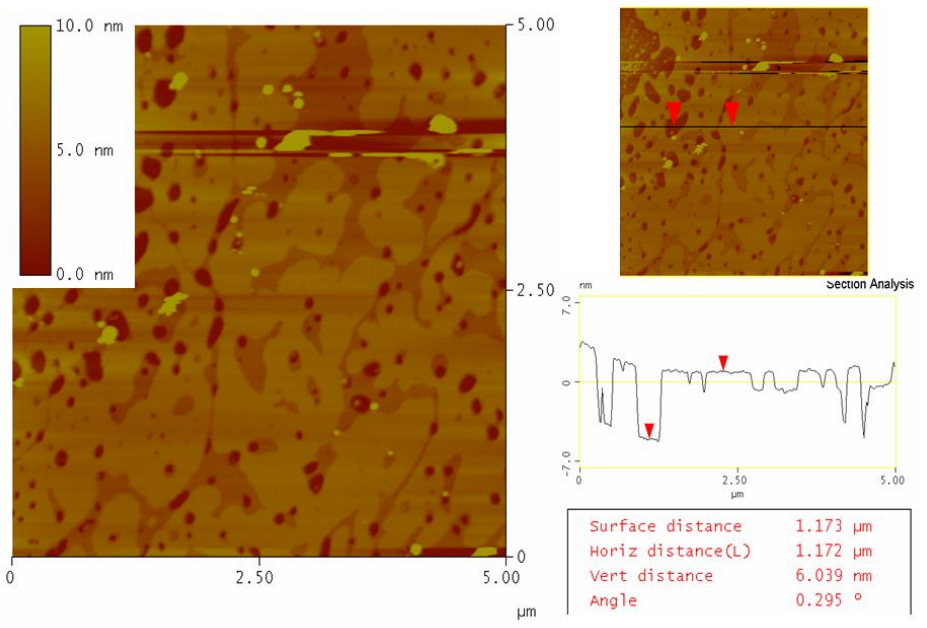


Figure 6. AFM image of a (DPPC+DPPG+CHOL) vesicle, fused on freshly cleave mica. Height analysis on the right reveals the bilayer formation.

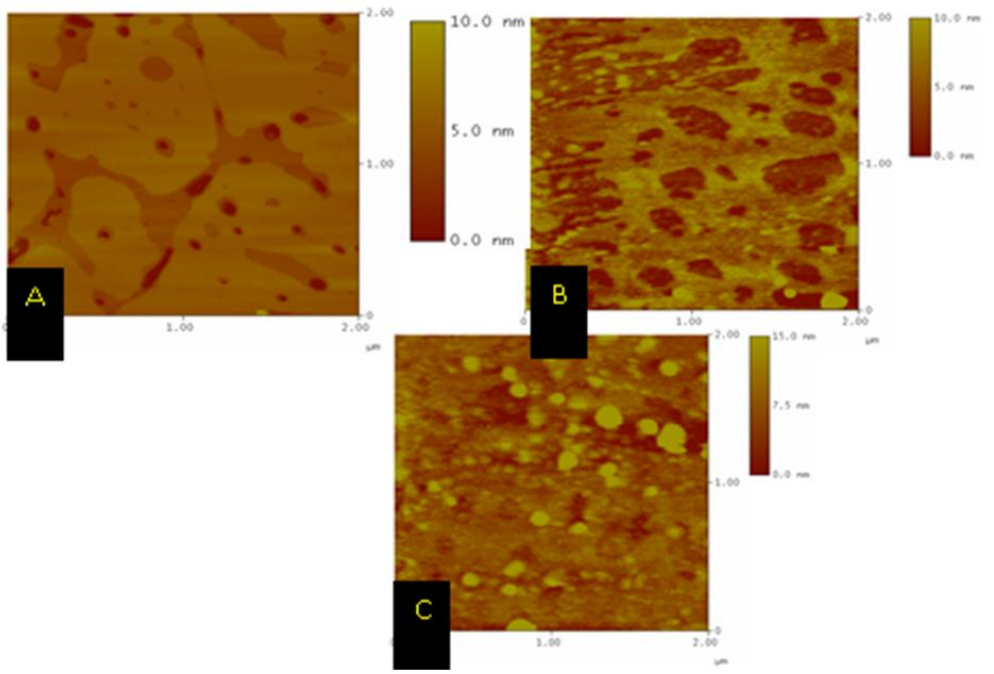


Figure 7. Effect of 6G PAMAM on the (DPPC+DPPG+CHOL) bilayer, fused on mica substrate. A. No dendrimer. B. 100 n mol dm^{-3} 6G PAMAM, C. 500 n mol dm^{-3} 6G PAMAM.

4. Conclusion

Studies on the interaction of 2G, 4G and 6G PAMAM dendrimers with different liposome substrates were done using absorbance, size analysis, zeta potential measurements and AFM measurements on solid supported bilayers. Increase in absorbance and size of the liposome is due to the adhesion of the individual liposomes, where the dendrimers acted as "glue". Maxima in the absorbance and size were due to the maximum adhesion of liposomes, after which the size decreases, due to the formation of liposome dendrimers complexes, probably "dendriosomes". Charge reversal during the zeta potential measurements reveal the electrostatic interaction among the liposome and dendrimers, which are significant when they are opposite in terms of surface charge. Further enhancement of zeta potential due to dendrimer addition was due to hydrogen bond/ hydrophobic interactions. Bilayer disruption of vesicles was observed upon addition of dendrimer to negatively charged surface of liposomes, as revealed by AFM measurements.

CHAPTER III

CHAPTER III

Biophysical correlates on the composition, functionality and structure of dendrimer-liposome aggregates.

Abstract: Interaction of liposomes carrying net negative charges with cationic polyamidoamine dendrimers (PAMAM) of different generations were investigated by combined size, zeta potential, turbidity, electron microscopy, atomic force microscopy, fluorescence spectroscopy and calorimetric studies. Stability of the liposomes comprising 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) + dihexadecyl phosphate, DPPC+1, 2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, DPPC+1, 2-dipalmitoyl-*sn*-glycero-3-phosphate and DPPC+1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanol were checked through their size and zeta potential with the variation of time. Existence of lipid bilayer and subsequent adsorption of dendrimer onto the liposome surfaces were evidenced. Interaction between the dendrimers and liposomes were electrostatic in nature, as evidenced through the charge neutralization of liposomes and its subsequent reversal with increasing dendrimer concentration. Extent of dendrimer-liposome interaction followed the sequence: generation 5 > 4 > 3 in addition to the head group charge, moiety and hydrocarbon chain length of the lipids. Fluorescence anisotropy and differential scanning calorimetry (DSC) studies suggest the fluidization of the bilayer although the surface rigidity was enhanced by the added dendrimers. Thermodynamic parameters of interaction processes were evaluated by isothermal titration and differential scanning colorimetric studies; the binding processes were exothermic in nature. Enthalpy of the transition of the chain melting of lipids decreased systematically with increasing dendrimer concentration and generation. Dendrimer-liposome aggregates were non-toxic to healthy human blood cell lymphocyte as well as in human RBCs suggesting the potential of such aggregates as drug delivery systems against microbial diseases.

1. Introduction.

The physical properties of liposomes are reliant on the type of constituents (head group charge, headgroup moiety and hydrocarbon chain length), intra-/inter- molecular interactions as well as the surrounding environments.[1-2] Due to their easy manifestation and readily flexible nature, liposomes have received substantial considerations as the simplified version of cell membrane.

All living cells have membrane bilayers; besides the sub-cellular components like mitochondria and golgi bodies also possess the membrane bilayer structure.[3] Therefore, the biophysical correlates on the composition, function and structure of membrane bilayer structure in understanding the function of cell membranes are necessary. Natural cell membranes are negatively charged; however, the choice of a mixture of lipids over the individual components (where lipids mixtures exhibit superior performance than the single components) by the nature is still not completely understood. It is therefore, considered to be worthwhile in exploring the physicochemical properties of a mixture of lipids whereby the anionic components could be varied.

1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) is a naturally occurring zwitterionic phospholipid found in pulmonary surfactant (PS). It has the capability to achieve high surface pressure; however, being solid in nature, it is unable to get fluidized by its own. In order to maintain parity in terms of charge, 30 mol% of phosphaditylglycerol is present in pulmonary surfactant. Such a combination of lipids mixtures is capable of forming liposomes with net negative charge. With the intention to mimic these combinations, we have formulated a variety of liposomes, whereby 30 mol% of different anionic lipids were used separately in combination with DPPC. The anionic lipids used herein include dihexadecyl phosphate (DHP), 1, 2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphate (DPP) and 1, 2-dipalmitoyl-*sn*-glycero-3-phospho ethanol (DPPEth). Dihexadecyl phosphate lipid is different from other phospholipids even though it can form stable liposome.[4-5] Phosphatidyl alcohols have special effects on their membrane properties. Phosphatidyl alcohols are highly potent promoters of membrane curvature, and their trans bilayer movement is three times higher than any naturally occurring phospholipid at physiological pH.[1, 6]

Although the aforementioned anionic lipids (except DMPG) are not directly relevant to biological cell membranes, however, it is expected that such combination of lipids in the term of liposome can be explored as drug delivery systems against the microbial membrane,[7] if they are used in combination with other components like dendrimers.[4, 8-9] Cholesterol is also another important component of liposome that modulates the fluidity/rigidity of membrane. 30 mol% cholesterol were used for the each combination along with the other lipids. It is known that the most active compounds (drug molecule) cannot attain therapeutically efficacy because of their inability to reach the target side by crossing the cell membrane barrier. The present set of

liposomes with negative charge are expected to be biocompatible. Here, the liposomes are expected to act as drug carrier or, more generally, as platform for theranostic.[4, 8-9]

Dendrimers are synthetically prepared hyper branched macromolecules which contain high number of active termini that describe their properties and functions.[10-11] Because of perfect branching, dendrimers have the maximum number of terminal functionalities of any polymeric material at a given molecular weight and are perfectly monodispersed. Dendrimers, compared to the corresponding linear polymers, possess architectural advantages in terms of drug delivery: (i) reproducible pharmacokinetic behavior (due to mono disperse nature);[10] ii) globular shape provides superior biological and rheological properties;[12-13] and iii) controlled multivalency can attach several molecules (drugs, imaging agents, cell-penetrating peptides, targeting groups, and solubilizing moieties *etc.*).[10,14-16]

The membrane disruption by linear or coiled polymers, or membrane disruption by peptides or detergents are well known phenomenon.[17-19] Because of the difference in the type and nature of interaction, studies involving the dendrimer and membrane bilayers are in the ascendance. Dendrimers can create holes in oppositely charged bilayer or can be incorporated into the lipid aggregates.[20,21,] Charge and size are the main parameters that governs the strength of dendrimer function.[22] Positively charged dendrimers interact more effectively with cell membranes or other model bilayer carrying net negative charge because of obvious reasons. Since the first successful production of poly(amidoamine) (PAMAM) dendrimer in the mid-1980s by Tomalia's group,[23-24] dendrimers have received much attention, in particular, the biomedical applications of dendrimers in various medical and pharmaceutical field including drug delivery, gene transfection, nanoscale drug and diagnostic tools. Such systems have been regarded as highly promising and have drawn eminent interest and studies in these few areas.The possibility to introduce several functionalities into the dendrimer structures has opened the door for their applications in theranostics. Dendrimers usually cross cell membrane barriers by endocytosis, thus they are entrapped in endosomes.[3, 25] The translocation mediated by PAMAM dendrimers in combinations with liposomes are considered as promising drug delivery systems (DDS). Although the mechanism of the spontaneous translocation of dendrimers through the bilayers is not yet well understand, such studies could motivate changes in the bilayer, which need to be taken into account in designing the drug delivery system.[23-24] To understand how a dendrimer mediates the cell membrane crossing, it is necessary to understand its interactions

with lipid bilayers. Liposomes are exceptional model systems for biological testing because of their simple and membrane like arrangement, easy preparation, biocompatibility, biodegradability and satisfactory stability above time.[10,26] Some dendrimers can interact with lipids by hydrophobic interactions between the lipid acyl chains and the hydrophobic dendrimer interior. The strength of the interaction also depends on the size and head group charge of the lipids molecule.[4-5, 27]

Adsorption of dendrimers on liposomes surface and the formation of dendrimer-liposome aggregates are the most common aspects of dendrimers-liposomes interaction. Dendrimers can act as "glue" for oppositely charged liposomes.[28] Most of the recent studies have focused on the interactions of positively charged dendrimers with cell membranes, since greater interaction potency is expected between these dendrimers with the negatively charged cell membrane or liposome compared to other neutral or negatively charged dendrimers. In addition to the surface charge and other physicochemical properties of dendrimers which influence the interaction between dendrimers and lipid bilayers that include the dendrimer type, generation and surface charge as well as the composition of lipid bilayer. Prevalence of electrostatic interaction can be evaluated through the zeta potential measurements. Calorimetric studies on liposome and dendrimers, can lead to the evaluation of the thermodynamic parameters of the interaction process like the chain melting temperature (T_m), width of the chain melting peak ($T_{1/2}$), enthalpy change of the chain melting process ($\triangle H$), heat capacity change ($\triangle C_P$) and binding constant (K), *etc*. Such studies can shed light on the effect of the PAMAM dendrimer-liposome aggregates. Size, turbidity, morphology (TEM and FF-TEM), atomic force microscopy (AFM), differential scanning calorimetry (DSC), fluorescence spectroscopy and isothermal titration calorimetry (ITC) measurements are the convenient tools to elucidate the dendrimer-liposome interactions in order to elaborate the design of new drug delivery systems that consist of dendrimers incorporating bioactive molecules. The talent of PAMAM dendrimers in forming aggregates with liposomes carrying net negative charge is expected to enlighten how dendrimers act as drug delivery vehicles across the cell membrane.

2. Materials and methods

2.1. Materials. 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), dihexadecyl phosphate (DHP),1, 2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), 1, 2-dipalmitoyl-*sn*-

glycero-3-phosphate (DPP) and 1, 2-dipalmitoyl-*sn*-glycero-3-phospho ethanol (DPPEth), 7-hydroxycoumarin *(7-HC),* 1,6-diphenyl-1,3,5-hexatriene (DPH), polyamidoamine (PAMAM) dendrimers of different generations [generation 3 (3G), generation 4 (4G) and generation 5 (5G) as methanolic solutions] were the products from Sigma-Aldrich Chemicals Pvt. Ltd. (USA). AR grade disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), sodium chloride (NaCl), HPLC grade chloroform and methanol were the products of Merck Specialties Pvt. Ltd, India. Double distilled water with specific conductance 2-4 μS (at 25°C) was used in preparing the solutions. All the chemicals were stated to be ≥99.5% pure and were used as received.

2.2. Methods.

2.2.1. Preparation of Liposome. Liposomes were prepared by the conventional thin film hydration technique.[29-30] Quantitative amount of phospholipid and cholesterol were dissolved in chloroform and methanol (3:1, V/V) in a round bottom flask. Solvent was evaporated on a rotary evaporator at 40°C. The resultant thin film was further maintained under vacuum for 6 h. It was then hydrated with 10 mM phosphate buffer saline (PBS) for 1 h at 70°C (temperature above chain melting temperature of all lipids). Ionic strength was maintained at 100 mM using sodium chloride. Then the systems were frozen at -20°C and thawed followed by sonication at 45°C. The freeze-thaw sonication process was repeated for four cycles in order to obtain small unilamellar vesicles. Liposomes were prepared separately using DHP+DPPC, DMPG+DPPC, DPP+DPPC and DPPEth+DPPC along with 30 mole% cholesterol at pH 7.4. In case of dye (7-hydroxycumarine and 1,6-diphenyl-1,3,5-hexatriene (DPH)) loaded liposomes, dyes were mixed along with the lipids in the chloroform-methanol (3:1) mixture prior to the generation of thin film. Dispersions were filtered through 0.45 μm cellulose nitrate membrane filter prior to size and zeta potential measurements.

2.2.2. Instrumental Analyses.

2.2.2.1. Turbidity, size and zeta potential measurements. Interaction of dendrimers with different liposomes was studied by measuring the % transmittance (%T) at 420 nm.[31-32] At this wavelength, the turbidity of a solution is assumed to be proportional to (100-%T). Measurements were done using a Cary1E UV-Visible spectrophotometer (UVD-2950, Labomed Inc., USA). Liposome solutions without dendrimers were used as blank.

The size and zeta potential of liposome solutions in combination with dendrimers were measured using a Zeta Sizer Nano, Malvern Instruments, U. K. He-Ne laser emitting light at 632 nm was used. The size measurements were done using a quartz cell of 1.0 cm path length, while a specially designed plastic cell was used for zeta potential measurements.

2.2.2.2. Electron microscopic studies. Morphology of the liposomes was studied by the transmission electron microscopy. A drop of dilute (10^{-4} M) liposome dispersion was placed on Formver™ carbon-coated 200 mesh copper grid and dried under air. It was then analyzed to obtain TEM images using Hitachi H-600 transmission electron microscope (Japan) operating at 80 kV. In case of FF-TEM studies, a drop of the sample was placed onto the sample holders and frozen in liquid propane. FR-7000A (Hitachi High Technologies Ltd., Japan) was used at -150 °C for the freeze fracturing. Samples were then replicated by evaporation using platinum carbon. The replica was placed on 300- mesh copper grid, dried, and examined in a transmission electron microscope (H-7650, Hitachi High Technologies Ltd., Japan) with an accelerating voltage of 120 kV.

2.2.2.3. Atomic force microscopy (AFM). AFM images on solid supported bilayer were obtained using a Multi-Mode Nanoscope III (Digital Instruments, Santa Barbara, CA, USA). 100 µL of liposomal suspension was placed on a 1cm^2 freshly cleaved mica incubated for an hour at 37ºC. Excess lipid was then gently washed off with 1 mmol dm^{-3} NaCl solution. It was then scanned in tapping mode using a liquid cell. Silicon nitride cantilever with a spring constant of 0.06 Nm^{-1} operating at a driving frequency of 7-9 kHz was used. Bilayer images were taken at different resolutions. Then dendrimer solutions of preferred concentration were used to rinse the bilayer. After half an hour, it was again scanned to visualize the effect of dendrimers. Rinsing was done slowly with care so that the bilayer structure does not get disturbed.

2.2.2.4. Steady-State Fluorescence Spectroscopy. Fluorescence anisotropy and life time studies were carried out by a bench-top spectrofluorometer (Quantummaster-40, Photon Technology International Inc., NJ, USA). 7-hydroxycoumarine (7-HC) and 1, 6-diphenyl- 1,3,5-hexatriene (DPH), used as the probe, was excited at 321 nm and 350 nm respectively. All the anisotropy data were measured at room temperature with emission wave length of 451 nm for 7-HC and 422 nm for DPH. The fluorescence anisotropy value ‘r’ was determined using the following equation:[33]

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2G.I_{VH}} \quad (1)$$

where, I_{VV} is the parallel polarized and I_{VH} is the perpendicular polarized fluorescence intensities, $G = I_{HV}/I_{HH}$ is the monochromator grating correction factor. Felix G_x software was used to calculate the anisotropy value.

Fluorescence lifetime measurements for 7-HC loaded in different liposome and dendrimer-liposome aggregates were determined with a Horiba Jobin Yvon Fluoro Max (Horiba Jobin Yvon, UK) using the time-correlated single photon counting (TCSPC) technique. Scattering was measured by using a ludox solution; excitation was performed at 288 nm with a delta diode-C1 diode controller.

The experimental results of time-resolved fluorescence decay profiles, R(t) were estimated by inbuilt Horiba EZ time software unit according to the following expression equation.[34]

$$R(t) = \sum_{i=1}^{n} \alpha_i \exp\left(-\frac{t}{\tau_i}\right) \quad (2)$$

Here, n = number of distinct decay components and τ_i and α_i are excited-state fluorescence lifetimes and the pre-exponential factors related to the i^{th} component, respectively

2.2.2.5. Differential Scanning Calorimetry (DSC). DSC studies were carried out to understand the changes in lipid membrane thermal properties upon the interaction with dendrimers. All scans were accomplished on a differential scanning calorimeter (DSC 1, STAR^e system, Mettler Toledo, Switzerland) with a scan rate of 2°C /min. The appropriate amounts of phospholipid mixture (7:3) with cholesterol were dissolved in chloroform-methanol (3:1) in a 40 µL Al pan and after that the solvent was evaporated under stream of nitrogen. The samples were placed under vacuum for removing traces of the solvent. Then the dendrimers in 10 mM phosphate buffer were added to the dry lipid film and hydrated at 70 °C. The temperature range was set in 10-70 °C. An empty pan was used as a reference. The enthalpies and characteristic temperatures were calculated using Mettler-Toledo STAR^e software.

2.2.2.6. Isothermal titration calorimetry (ITC). ITC measurements were performed in a VP-ITC titration calorimeter (Micro Cal, Northampton, MA). Before loading, the solutions were thoroughly degassed. The preformed samples were kept in a sample cell, and a syringe of

volume 300 μL was filled by the ligand in the same buffer. The ligand solution was added sequentially in 3μL aliquots (for a total of 75 injections, 15 s duration each) at 3 min intervals at 25 °C. The sequential titrations were performed to ensure full occupancy of the binding sites by loading and titrating with the same ligand without removing the samples from the cell until the titration signal was essentially constant. The respective heat of dilution was subtracted from the corresponding binding experiments prior to curve fitting. Typically three replicate titration experiments were performed.

2.2.2.7. Binding constant. Binding constant of the dendrimer-liposome aggregates were also determined colorimetrically. Different amounts of dendrimer solution were separately added to a fixed amount of liposome solution. After homogenization, the solutions were kept for two hours in attaining the equilibrium. It was then centrifuged at 20,000 rpm for one hour whereby the dendrimer-liposome aggregate got sedimented. The supernatant, which contains the fee dendrimer, were estimated colorimetrically using a UV-vis spectrophotometer (Jasco V-30, USA) at 282 nm using a matched pair of cell having 1.0 cm path length. Corresponding liposome without dendrimer was used as reference. The binding constant, K, is associated with the binding and unbinding reaction of receptor (L) and ligand (D) molecules, which is formalized as:

$$D + L \rightleftharpoons \mathrm{DL} \tag{3}$$

The reaction is characterized by the forward constant k_1 and the backward rate constant k_{-1}. In equilibrium, the forward binding transition D + L → DL should be balanced by the backward unbinding transition DL → D + L. That is, k_1 [L] [D]= k_{-1} [LD], where [L], [D] and [LD] represent the concentration of unbound free receptor (liposome), the concentration of unbound free ligand (dendrimer) and the concentration of ligand-receptor complexes. The binding constant K is defined by

$$K = \frac{k_1}{k_{-1}} = \frac{[DL]}{[D][L]} \tag{4}$$

2.2.2.8. Cytotoxicity Analyses. The blood sample was collected from the healthy human subjects (n=3) for the separation of lymphocytes as described previously.[35] 5.0 ml human blood were diluted with phosphate buffered saline (PBS) (1:1) and layered onto Histopaque 1077 as described earlier.[33] After the treatment with liposome and dendrimer-liposomes aggregates, the peripheral blood mononuclear cell (PBMCs) (2 x 10^5 cells in each set) were washed with PBS

(1x) for three times using centrifugation (2200 rpm for 3 min per wash) and were subjected to quantitative estimation for cytotoxicity by a nonradioactive, colorimetric assay systems using tetrazolium salt, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenil-tetrazolium bromide (MTT). The percentage of proliferation was calculated as described previously.[33]

2.2.2.9. Haemolysis Assay. The haemocompatibility of liposomes and dendrimer-liposome aggregates were determined in terms of the percent haemolysis as described earlier,[36] where

$$\% \, Haemolysis = \frac{(A_S - A_N)}{(A_P - A_N)} X100 \qquad (5)$$

A_S is the sample absorbance, A_N is the absorbance of the negative control and A_P is the absorbance of the positive control.

All the experiments, except the DSC studies, were carried out at ambient but controlled temperature.

3. Results and discussion

3.1. DLS and turbidity studies.

3.1.1. Characterization of liposomes. Hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta potential (Z. P.) of four different liposomes (DHP+DPPC), (DMPG+DPPC), (DPP+DPPC) and (DPPEth+DPPC) were measured at pH 7.4 as a function of time by dynamic light scattering; results are summarized in Figure 1. Initially, for most of the systems, the size increased with increasing time (up to 5-18 days) due to the structural reorganization of the lipidic components.[37] The size remained almost constant up to 60 days for all the systems, indicating their substantial stability. The lower PDI values designate that all the liposomes are fairly monodispersed.[30, 38] Size of the liposome depends on the lateral packing of lipid molecules within the membrane bilayer and is mainly determined by the van der Waals interactions between the hydrocarbon chains.[5, 10] Larger d_h values for DHP+DPPC, DPP+DPPC and DPPEth+DPPC than DMPG+DPPC are due to the fact that the fraction of the head group region of higher density decreases with increasing hydrocarbon-chain length.[39-40] This may be explained as a consequence of the orientation of polar head groups to compensate the close packing imposed by the lateral stronger van der Waals interactions of the acyl hydrocarbon chains. The PDI values follow the sequence DPPEth+DPPC> DMPG+DPPC> DPP+DPPC> DHP+DPPC. In case of DHP, phosphate group is directly connected to the long hydrocarbon chains that strongly

push the electron towards phosphate group and enhance the electron density. Thus the DHP+DPPC liposome shows highly negative zeta potential. In case of DPP+DPPC and DPPEth+DPPC liposomes, with the same kinds of dipalmitoyl groups, unlike the DPPEth (phosphatidyl alcohol), where the phosphate group is directly connected to $-CH_2CH_3$ group

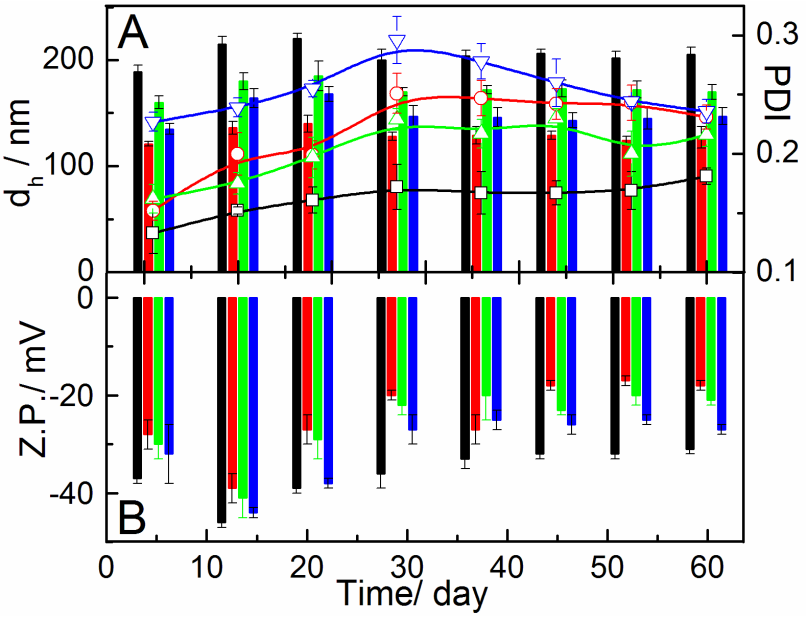


Figure 1. Variation in the hydrodynamic diameter (d_h), polydispersity index (PDI, line graphs) and zeta potential (ZP) of different liposomes with time. 30mole% cholesterol was used in each case. DPP : anionic lipid ratio is 7:3 (M/M). Bar/lines: black, DHP+DPPC; red, DMPG+DPPC; green, DPP+DPPC and blue, DPPEth+DPPC. Temperature 25°C.

which has a stronger +inductive effect. Hence the zeta potential of DPPEth liposome is higher than that of DPP liposome but not more than DHP. On the other hand, DMPG has two myristoyl groups and also the phosphate group directly connected with a glycerol moiety.[39,41] At the same time, the hydroxyl group in solution of glycerol moiety forms hydrogen bond with the phosphate group of DPPC or neighboring DMPG moiety. Thus the enhancement of the electron density on phosphate group in DMPG is less pronounced than the other systems; hence the magnitude of the zeta potential is lower in case of DMPG+DPPC liposome. The zeta potential for different liposome follows the sequence DHP+DPPC > DPPEth+DPPC > DPP+DPPC > DMPG+DPPC (Figure 1).

3.1.2. Impact of dendrimer on liposome. The interaction efficiency between dendrimers and liposomes can be assessed by the turbidity measurements. Figure 2 (panel A1) shows the concentration effect of different generation PAMAM dendrimers on the turbidity, size and zeta

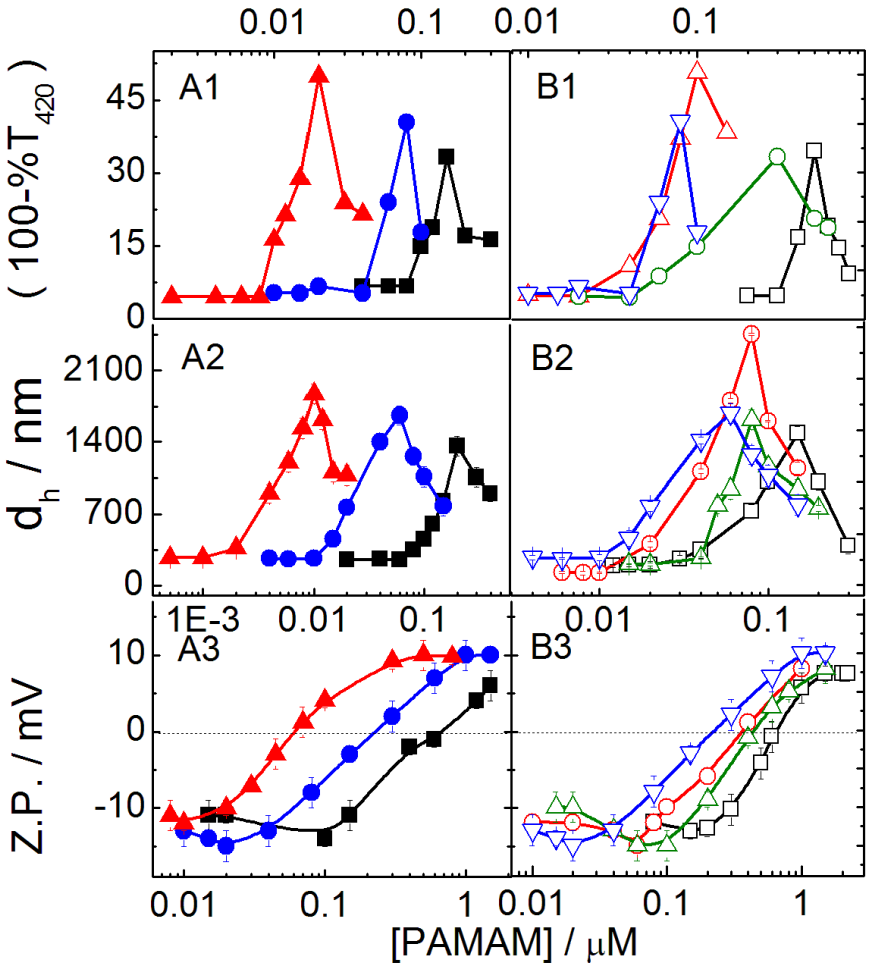


Figure 2. Effect of PAMAM dendrimer generation: (■,3G; ●,4G and ▲,5G) on the turbidity (A1), size (d_h, A2) and zeta potential (Z.P., A3) of (DPPEth+DPPC) liposome solutions and the effect of 4G dendrimer on the turbidity (B1), size (B2) and zeta potential (B3) of different liposomes (□, DHP+DPPC; ○, DMPG+DPPC; △, DPP+DPPC and ▽, DPPEth+DPPC.

potential of (DPPEth+DPPC) liposome as representative. Turbidity values of the dendrimer-liposome complexes pass through maxima likewise the size after a threshold dendrimer concentration.[32,28] Initial size or turbidity increments, and the attainment of maxima are due to the formation of liposome aggregates assisted by dendrimers.[32] The dendrimers being oppositely charged, get adsorbed on to the liposome surface.[27] Decrease in size and turbidity upon further

addition of dendrimers are due to the formation of water soluble dendrimer-liposome aggregates.[32,28] The ability in imparting turbidity or size enhancement depend on the dendrimer

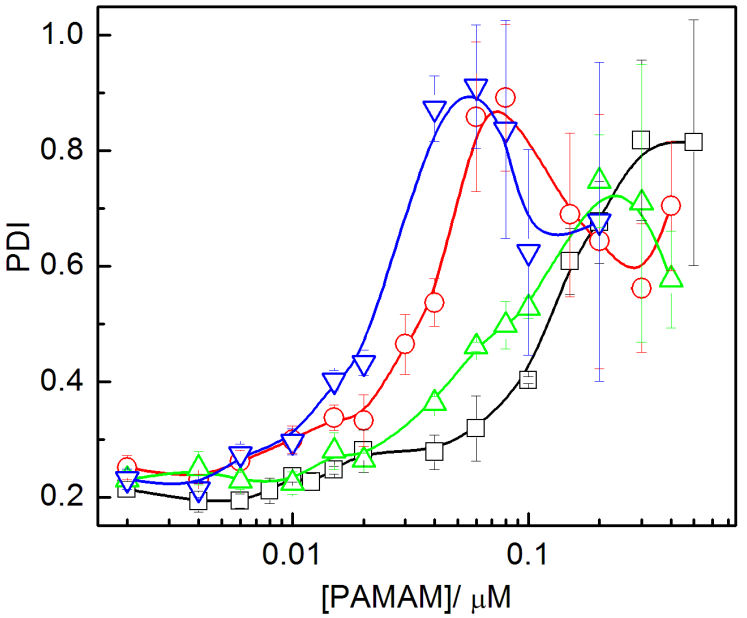


Figure 3. Polydispersity index (PDI) of different liposomes with varying concentration of 4G PAMAM dendrimer. Liposomes: □, DHP+DPPC; ○, DMPG+DPPC; Δ, DPP+DPPC and ▽, DPPEth+DPPC.

generation. PAMAM dendrimers follow the order 5G>4G>3G while considering the size and turbidity variation. These are 128, 64 and 32 end groups in the 5G, 4G and 3G PAMAM dendrimers respectively. With the increase in dendrimer generation number of end groups increases and hence lesser amount of dendrimers are required for effective interaction. It is, therefore, reasonable to consider the dendrimer activity in terms of the end groups' concentration, as shown in Figure 4. Activity of dendrimers was independent of dendrimer generation while considering the end groups activities. Earlier reports reveal that the activities follow the opposite trend $G_{\text{lower generation}} > G_{\text{higher generation}}$.[32] The lower is the generation; the higher number of end groups become accessible for effective interactions. Besides, with increasing dendrimer generation, the end groups tend to back fold.[32] Thus, for higher generation of dendrimers, the lesser number of end groups can effectively participate in the interaction process.

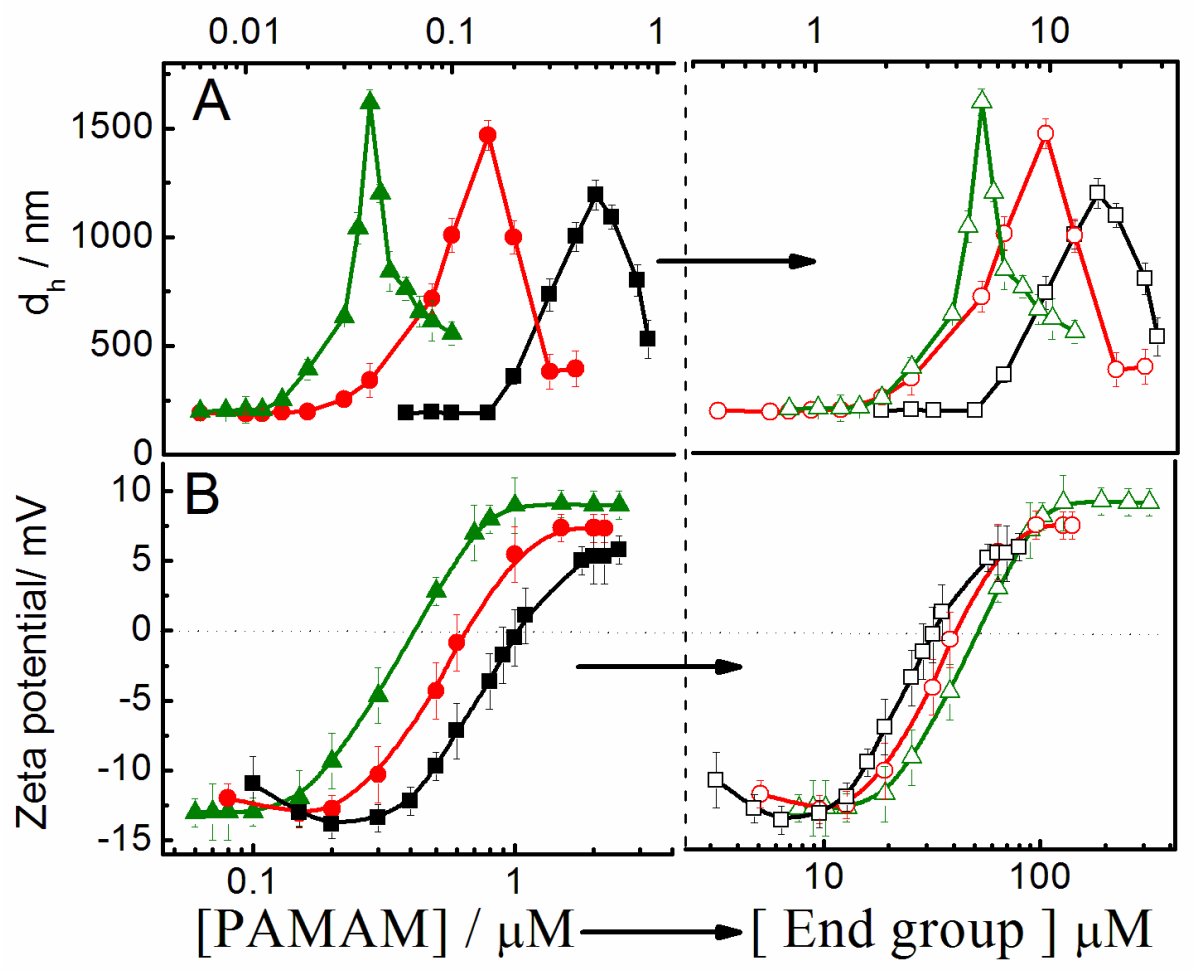


Figure 4. Effect of dendrimer generation and concentration :(G3, ■; G4, ● and G5,▲) on the size (A) and zeta potential (B) of the (DPPC+DHP+CHOL) liposome solutions (Left). Right side indicates the effect of end group concentration instead of concentration of dendrimer (□,G3; ○, G4;△,G5).

In addition to the dendrimer generation, the extent of interaction also depends on the lipid composition. Dendrimer-liposome complex is stabilized by strong electrostatic interactions and by hydrogen bonds between amine end groups of dendrimer and the phosphate moiety of the anionic lipid head groups.[2, 42] The increasing order of the size of bulky group is DHP> DMPG> DPPEth> DPP; thus the DHP comprising liposome would exhibit weaker interaction than other systems. Although, initially, the size of DMPG liposome is lower than the other systems, its size increase with increasing dendrimer concentration is significantly higher than the other liposomes due the stronger hydrogen bonding of a glycerol group with positively charged PAMAM dendrimers [DHP, -H; DMPG, $-CH_2CH(OH)CH_2OH$; DPP, -H; DPPEth, $-CH_2CH_3$].[1, 39,43] Thus,

the DMPG liposome displays stronger interactions with dendrimers than other liposomes. On the other hand, DPPEth liposome is more sensitive towards dendrimer than other liposomes due to the presence of $–CH_2CH_3$ group, which is directly connected with phosphate group.[1] The interactions between a negatively charged surface and the positively charged dendrimers were further explored by zeta potential measurements. Representative results are summarized in panel A3 and B3 of Figure 2. Magnitude of the zeta potential decreases and the potential changes sign upon further addition of dendrimers; attainment of zero zeta potential suggest charge neutralization.[32, 44] Further increase in zeta potential towards the positive directions suggests the formation of non-stoichiometric aggregates, indicating the saturation point. The post stoichiometric aggregation is governed by hydrogen bonding and/or hydrophobic interaction induced by amine groups. It was observed that the other liposome combinations also interact strongly with the dendrimers, ascertained by the higher slopes of zeta potential-dendrimer concentration profile, and require lesser concentration of dendrimers.[4-5] However, when the dendrimer activities were expressed in terms of total end group concentrations, the zeta potential tends to opposite direction and one can see the almost reverse order shown in Figure S1. This clearly suggests that all the end groups of dendrimers cannot effectively take part in the interaction process. End groups of higher generation dendrimer get back-folded. DPPEth+DPPC liposome was more sensitive towards dendrimers than the other liposomal formulations which could better be explained by further experiments.

3.2. Morphological analyses (TEM, FF-TEM and AFM). Spherical morphology with smooth surfaces were observed for all the liposomes; the sizes were comparable to the DLS data as shown in Figure 5 (panel A1). Conventional TEM analysis is associated with the drying of samples which may alter the size and morphology. To double check the morphological informing FF-TEM studies were performed. Representative are shown while considering the impact of dendrimers, it was observed that with increasing concentration of PAMAM, liposomes surfaces lost their homogeneity with the ultimate formation of aggregated heterogeneous entities. PDI values as determined by DLS, also support this phenomenon; PDI value increased non systematically with increasing dendrimer concentration added to the liposomes shown in Figure 3. It is known that the oppositely charge dendrimers can act as glue to

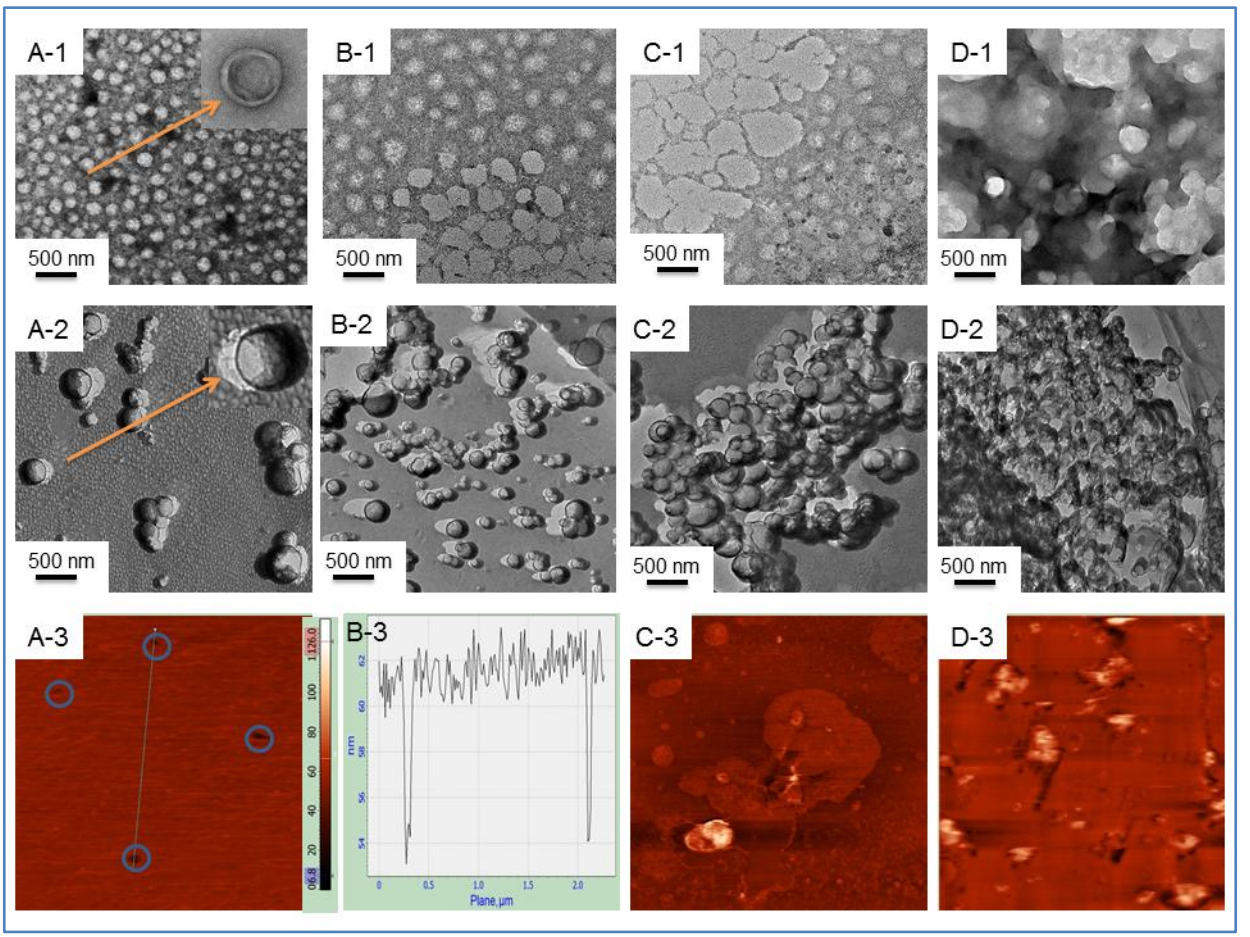


Figure 5. Effect of 4G PAMAM on the (DPPC+DPPEth+CHOL) bilayer.A, No dendrimer; B, 10 n mol dm^{-3} 4G PAMAM(except B3); C, 100 n mol dm^{-3} 4G PAMAM and D, 500 n mol dm^{-3}4G PAMAM. (Images: 1,TEM; 2, FF-TEM and 3, AFM). Panel B3, height analysis for bilayer thickness. Lipid concentration: 0.1mM.

liposomes.[28] Both the conventional TEM and FF-TEM studies support this proposition. Effect of dendrimer on the structure of lipid bilayer on solid supported (mica substrate) was further investigated by AFM studies. Solid supported lipid bilayer on freshly cleaved mica surface was generated by the standard procedure. Results are shown in the bottom panel of Figure 3. Height of the solid supported bilayer was in the range 5 to 6 nm as also reported by others.[5, 30, 40] Existence of holes in solid supported lipid bilayer (Figure 5, panel A3) is a natural phenomenon, which probably are responsible in the transport processes. At lower concentration of added dendrimer, white patches with higher height profiles were noticed (panel C3 of Figure 3). This is due to the preferential adsorption of positively charged PAMAM dendrimers on the mica and/or

the edge of the bilayer holes. At lower dendrimer concentration, homogeneity of the membrane bilayer was significantly perturbed (panel D3); holes become bigger due the disruption of bilayer through the formation of water soluble dendrimer-lipid aggregates. These results further support the proposition of the formation of dendrimer-liposome complexes are discussed in the DLS studies.

3.3. Steady State Fluorescence Anisotropy and Life-time Analyses. State of polarity of the liposome surface and the packing of bilayer are the two important parameters while considering the dendrimer-liposome interaction processes. Solvatochromic dye 7-hydroxycoumarine (7-HC) was used as the molecular probe to understand the state of polarity as well as the rigidity/fluidity of the palisade layer of the liposome and the effect of dendrimers. Additionally, the hydrophobic probe 6-diphenyl-1,3,5-hexatriene (DPH) were used to understand about the bilayer packing of dendrimer-liposome complexes. Fluorescence anisotropy value of 7-HC loaded liposomes (DHP+DPPC, 0.048; DMPG+DPPC, 0.125; DPP+DPPC, 0.1451 and DPPEth+DPPC, 0.0932) were lower than DPH loaded liposomes (DHP+DPPC, 0.078; DMPG+DPPC, 0.155; DPP+DPPC, 0.1751 and DPPEth+DPPC, 0.1232) in absence of dendrimer. DPH, being completely hydrophobic, resides inside the bilayer while the solvatochromic dye 7-HC resides on the palisade layer of the vesicles. Increase in fluorescence anisotropy of 7-HC with increasing dendrimer concentration were recorded as shown in Figure 6 (panel A and B). Dendrimers in liposomal suspension leads to significant changes of fluorescence anisotropy of 7-HC. Increase in the fluorescence anisotropy was due to the adsorption of dendrimer liposome surface; further addition of dendrimer leads to mild decrease in the fluorescence anisotropy probably due to the formation of holes in the bilayer, reflecting the fact that some dendrimers probably move through the liposome bilayer.[20-21] In addition, to understand the process of de-aggregation details of the bilayer packing for dendrimer-liposome complex, DPH was used as the molecular probe. DPH being completely hydrophobic will preferentially reside within the lipid acyl chain with parallel orientation that results in the increase in anisotropy value. Results, as summarized in Figure 4 (panel C and D) suggest that the membrane fluidity decreases with increasing concentration of dendrimer. These fluorescent probe monitors interactions between the external and internal regions of the membrane with dendrimers. The change in fluorescence anisotropy with

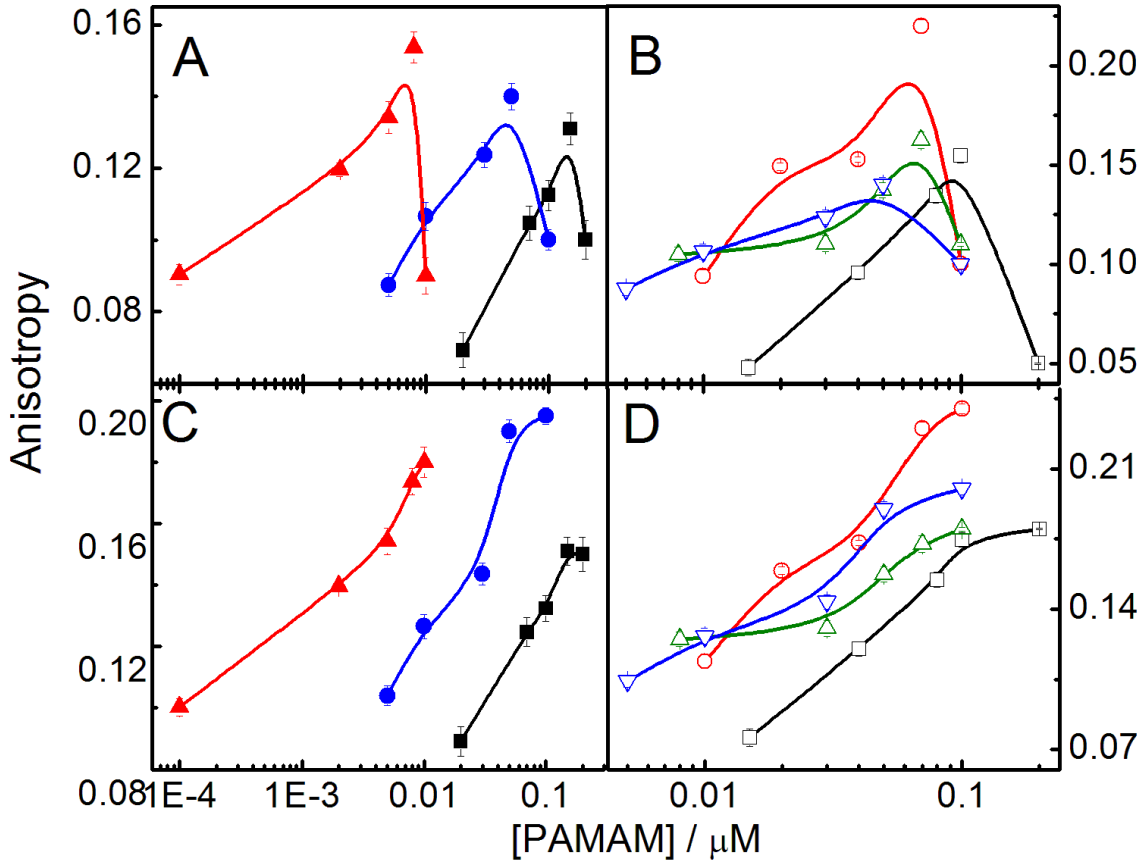


Figure 6. Variation in the fluorescence anisotropy values of 7-HC (panel A) and DPH (panel C) with dendrimer concentration. Effect of dendrimer generation (G3, ■; G4, ● and G5, ▲) on the anisotropy of DPPEth+DPPC liposome solutions and panel B (7-HC) and D (DPH): effect of dendrimer (4G) on different liposomes (DHP + DPPC, □; DMPG + DPPC, ○; DPP + DPPC, △ and DPPEth + DPPC, ▽).

increasing dendrimer concentration indicates alterations in membrane fluidity.[45] Significant changes in fluorescence anisotropy were also observed with the variation of dendrimer generation shown (Figure 6, panel A and C). It has been found that the extent of interaction between dendrimers of 3G and 4G with liposome was less than 5G. This was due to formation of the rigid structure of a bilayer for higher generations of demdrimer-liposome aggregates.[20, 41] The higher generation of dendrimers having the larger number of end groups can effectively interact with higher magnitude with liposome surface (similar reasons were noted in case of DLS and turbidity study).

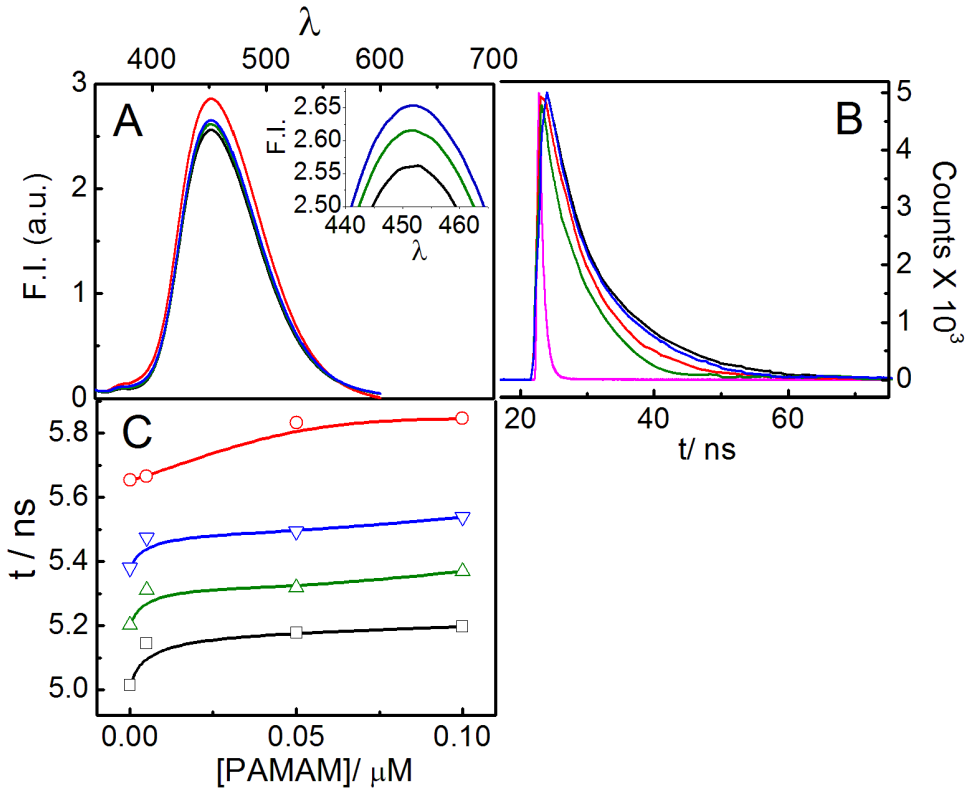


Figure 7. Steady state fluorescence spectra and life time study using 7-HC as a probe: panel A, steady state spectra of different liposomes (black, DHP+DPPC; red, DMPG+DPPC; green, DPP+DPPC and blue, DPPEth+DPPC); panel B, fluorescence intensity decay for different liposomes (black, DHP+DPPC; red, DMPG+DPPC; green, DPP+DPPC, blue, DPPEth+DPPC and pink, IRF, instrument response function) and panel C,fluorescence life timefor different liposomes (□, DHP+DPPC; ○, DMPG+DPPC; △, DPP+DPPC and Panel D, DPPEth+DPPC) with varying concentration of dendrimer.

Time-resolved fluorescence decay studies of 7-HC were carried out to further understand the emission decay parameters.[46] Fluorescence lifetime measures the duration of the excited state of a probe in the liposomal environment. It is also one of the important tools to study interaction phenomena as it gives information on the change in binding environment of the fluorophores. Figure 7 in panel C explains the variation in excited state lifetime (τ) in liposomes at different dendrimer concentrations. 7-HC in liposome exhibits the fluorescence lifetime values of 5.01, 5.65, 5.20 and 5.38 ns for DHP+DPPC, DMPG+DPPC, DPP+DPPC and DPPEth+DPPC respectively. Initially, with the progressive addition of dendrimer, fluorescence life time increased, and then attained constancy. Rotational diffusion of 7-HC decreased with increasing

dendrimer concentration due to the formation of stable dendrimer-liposome complex that lead to overall increase in the viscosity of the medium as also revealed from the anisotropy studies. The lifetime did not change significantly with the variation of dendrimer generation.

3.4. Differential scanning calorimetry (DSC) Studies. DSC studies were carried out in order to understand the thermal behavior of dendrimer-liposome interaction process.[45] DPPC, DPP and DPPEth contain two palmitoyl chains whereas DMPG has two myristoyl chains and DHP contains two hexadecyl hydrocarbon side chains.[41,47] Lipids with lower hydrocarbon chain length are expected to have lower pre-transition and main transition temperature (T_m).[41, 48] DMPG shows the main phase-transition temperature at 23.9°C, whereas for DPPC, it was 41.4°C. For the DMPG+DPPC liposome, the chain melting temperature was at 25.5°C, as shown in Figure 9. DPP+DPPC and DPPEth+DPPC liposomes show the chain melting temperature at 44.7°C and 42.0° respectively, about the same to DPPC at 41.4°C (as all of them

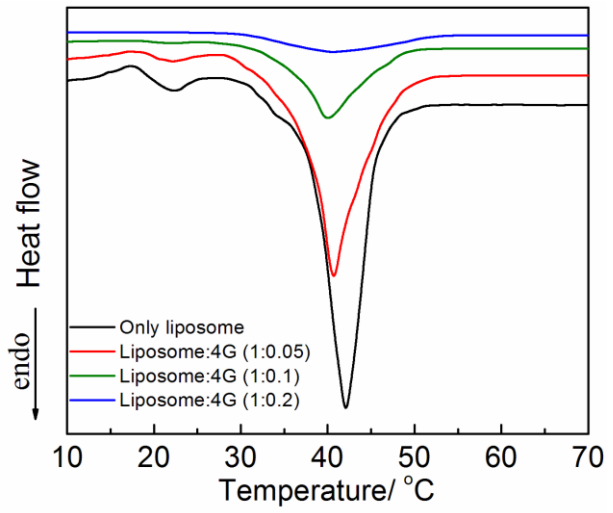


Figure 8. DSC thermogram of DPPEth+DPPC liposome at different concentrations of 4G PAMAM dendrimer. Liposome-4G PAMAM ratio is indicated in the figure (M/M) ratio.

have similar chain lengths). The chain melting temperature of DHP+DPPC liposomes were found as 57.8°C.[49] But the change in enthalpy of DMPG+DPPC liposome was found to be higher due to intra-/inter- molecular hydrogen bonding (Table 1). Phospholipids are known to exist in two different mesomorphic phases; highly ordered gel phase and more disordered liquid

crystalline fluid phase. The transition from the gel phase to the liquid-crystalline phase can be reached with increasing temperature. When the temperature increases, the intermolecular motion around C-C bonds, lateral and rotational diffusion among the lipid molecules also increase.

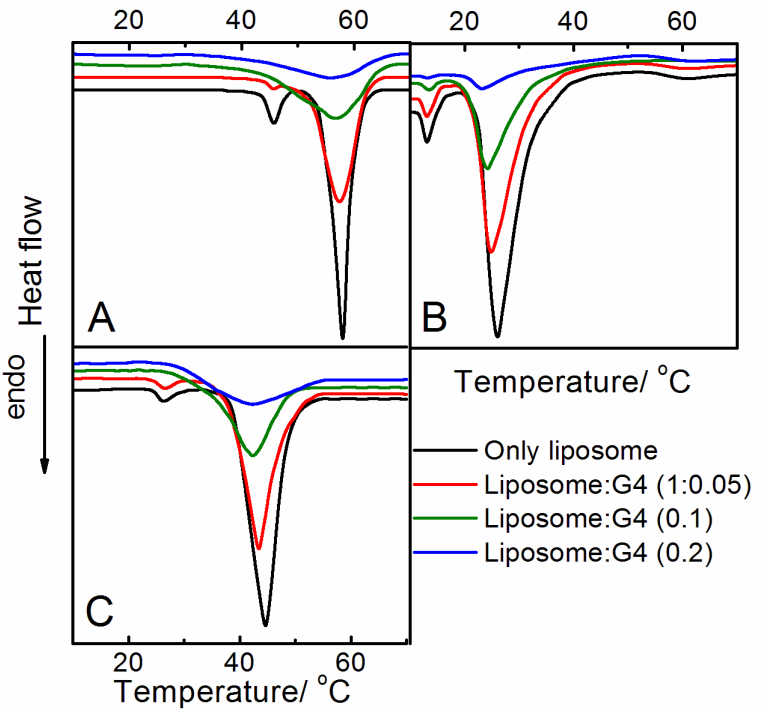


Figure 9.DSC thermogram of liposomes (panelA, DHP+DPPC; panel B, DMPG+DPPC and panel C, DPP+DPPC liposome) in presence and absence of PAMAM dendrimer of generation 4.

Thermally induced transition of liposomes were affected by dendrimers (DSC peaks are shifted towards lower enthalpies) and this perturbation concentration dependent. A downshift in the T_m values was recorded with dendrimer concentration. Pre-transition is the best approachable parameter for discovering interactions between dendrimer and phospholipid bilayers. Even at low concentrations of dendrimer pre-transition temperature was significantly affected. With increasing dendrimer concentration, the pre-transition temperature of lipids declined and the main transition peak became shorter and wider. Figure 8 shows that the high concentrations of dendrimers in DPPEth+DPPC lipid bilayer, that led to the abolishion of the transition peak indicating the perturbation of lipid bilayer. A decrease in the pre-transition peak of

Table 1. Calorimetric Data for the Interaction of Dendrimersand Liposomes.

Differential Scanning Calorimetry					
Liposome	Liposome:4G dendrimer	T_m /°C	$\triangle T_{1/2}$ / °C	$\triangle H$ /kcal.mol^{-1}	$\triangle C_P$ /kcal.mol^{-1}C^{-1}
DHP+DPPC+ CHOL	Only liposome	58.10	6.60	32.11	8.12
	1:0.05	57.37	9.51	26.45	4.64
	1:0.1	56.65	13.20	20.11	2.54
	1:0.2	55.90	15.77	10.12	1.10
DMPG+DPPC+ CHOL	Only liposome	25.55	5.97	50.22	14.76
	1:0.05	24.91	6.66	45.11	11.88
	1:0.1	24.29	7.60	40.21	9.28
	1:0.2	23.10	7.35	30.21	7.21
DPP+DPPC+ CHOL	Only liposome	44.72	11.10	29.67	4.68
	1:0.05	43.80	12.97	27.24	3.67
	1:0.1	42.84	13.30	19.11	2.51
	1:0.2	42.20	14.90	7.48	0.87
DPPEth+DPPC+CHOL	Only liposome	42.56	2.54	32.51	22.61
	1:0.05	42.00	3.96	29.53	13.14
	1:0.1	40.78	4.91	24.96	8.97
	1:0.2	40.17	5.70	17.16	5.30
Isothermal Titration Calorimetry					
	Dendrimer	K/mol^{-1} x 10^{-6}	10^{-4} x $\triangle H$ /kcal.mol^{-1}	$\triangle S$ /kcal.mol^{-1}.C^{-1}	
DHP+DPPC+CHOL	3G	9.6	-111	-11.40	
	4G	8.1	-197	-34.50	
	5G	7.6	-219	-41.90	
DMPG+DPPC+CHOL	3G	15.0	-300	-71.80	
	4G	8.0	-417	-111.00	
	5G	6.6	-513	-141.00	
DPP+DPPC+CHOL	3G	12.0	-221	-41.00	
	4G	4.2	-181	-26.00	
	5G	1.5	-300	-67.90	
DPPEth+DPPC+CHOL	3G	12.0	-225	-42.90	
	4G	4.2	-290	-67.20	
	5G	1.1	-454	-120.00	

T_m is chain melting temperature; $\triangle T_{1/2}$ is the peak width;$\triangle H$ is the enthalpy change;$\triangle C_P$is theheat capacity change;K is binding constant and $\triangle S$ is the entropy change.

DPPEth+DPPC liposome (Figure 8) suggests that dendrimers can interact with this liposome surface, whereas alteration of the main transition peak suggests that dendrimers can lead to the

generation of holes in the bilayer. The bilayer can lose its homogeneity that depends on the dendrimer concentration.[41] Interaction between the dendrimers and the bilayer occurs mainly in the palisade region.[21] Dendrimers affect the thermotropic properties of lipid bilayers in a concentration-dependent manner. With increasing the dendrimer concentration, the change in enthalpy ($\triangle$H) decreases (32.51 to 17.16 kcal.mol^{-1}, for DPPEth+DPPC liposome) leading to the abolition of the peak indicating the bilayer disruption.[50] The results of DSC studies are summarized in Table 1 along with the other data. In case of DMPG+DPPC, the carbonyl groups and glycerol backbone favor the hydrogen bonding interactions with dendrimers and it show higher enthalpy values than other systems. In aqueous medium, the hydration of the head group increases its effective volume and decreases the orderedness of hydrocarbon chains. The increase in head group volume, induced by the dendrimers (through the formation of dendrimer lipid aggregates) creates energetically unfavorable voids in the hydrocarbon region of non-interdigitated membranes. The observed decrease in T_m and broadening of the DSC transition peaks probably indicate the increased size of the DMPG+DPPC liposomes as a consequence of their interaction with PAMAM dendrimers (similar observation were found from size measurement by DLS study).[41, 50] Results further suggest that at higher dendrimer concentration, the lipid bilayer loses its homogeneity in a concentration dependent manner (similar observation was found by PDI measurement as shown in Figure 3). With increasing dendrimer concentration, the bilayer rigidity increases and reaches a maxima, then decreases further (similar to the observations in the fluorescence anisotropy studies). With increasing dendrimer concentration, the membrane fluidity increases and the endothermic heat change decreases that lead to the lowering of the enthalpy changes of the chain melting processes.

3.5. Binding constant by UV-spectroscopic analysis. Binding constant is another important parameter to quantify the extent of the interaction processes between the dendrimer and liposome. Concentration of dendrimer was varied in fixed liposome concentration whereby the concentration of the free and bound (to liposome) dendrimer were estimated colorimetrically. Results are summarized in Table 2 (Supplementary Section). The binding tendency of dendrimer on liposomes follows the order DMPG+DPPC> DPP+DPPC~ DPPEth+DPPC > DHP+DPPC shown in supplementary section Table 2. Due to formation of hydrogen bonding between dendrimer and hydroxyl group of DMPG, DMPG+DPPC liposome shows highest binding

constant value than that of other systems. With increasing dendrimer generation, less binding affinity were found. Because for higher the generation of dendrimer having higher number of end groups, thus it requires the lesser amount for effective interaction. The binding constant study by this technique is less sensitive than other process due lower concentration of dendrimer. To be acquainted with the details about binding phenomena, isothermal titration calorimetric titrations were done.

Table 2: Binding constant (K) for the formation of dendrimer-liposome complex.

Dendrimer generation / **Liposomes**	**10^{-4} x K / mol^{-1}**		
	3G	4G	5G
DHP+DPPC	8.81	6.71	5.21
DMPG+DPPC	10.74	9.72	7.21
DPP+DPPC	10.06	7.82	6.32
DPPEth+DPPC	9.98	7.81	6.11

3.6. Isothermal titration calorimetric studies. Binding properties of the dendrimer with liposomes in phosphate buffer solution were explored by employing the isothermal titration calorimetry. Negatively charged liposomal dispersions were titrated by the dendrimers of different generations. The exothermic enthalpy changes related to the interaction reached a saturation plateau quite fast as shown in Figure 10. The observed exothermic enthalpy changes include contributions from the binding of dendrimers by the lipid phosphate groups. The effect is clearly demonstrated by the titration experiments.[4-5] The control experiments for the interaction of dendrimers with liposomes have also been carried out by diluting the dendrimers into PBS without having the liposomes. Upon subtraction of the calorimetric contribution from the control experiment, a single-site binding model was applied for the interaction between amino groups of the dendrimers with liposome phosphate groups, leading to the $\triangle H$. In this case a significantly less binding enthalpy change was recorded during the titration (Figure 10) suggesting the weak binding of the dendrimers by the phosphate groups of the lipids.[51] The much weaker interaction emerges due to the competition between the liposomal and the buffer phosphate groups.[51] The apparent binding constants (K*)* are listed in Table 1.[10] Due to presence of the glycerol moiety of DMPG phospholipid liposomes, the binding constant is higher than for other liposomes (reasons

already mentioned earlier) as shown in Table 1. Dendrimers get strongly absorbed onto the liposome surface through the combined electrostatic interactions and hydrogen bonding between amino groups of the dendrimers and hydroxyl groups of DMPG.[51] Another thing is that with increasing dendrimer generation the binding constant values decreases. Due to presence of larger number of polymeric amino groups, higher generation of dendrimers interact effortlessly than that of lower generation of dendrimer.

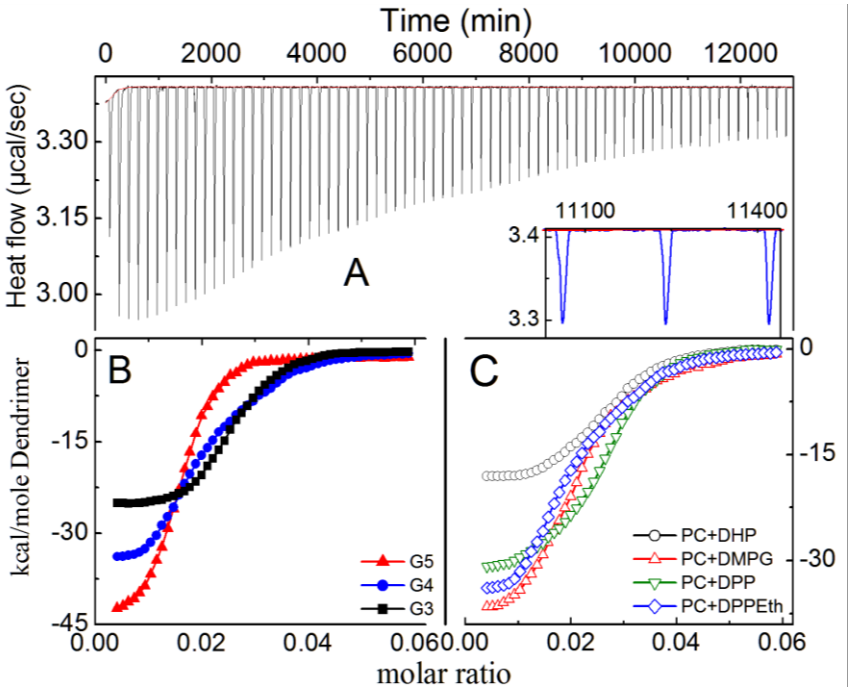


Figure 10. Isothermal titration calorimetric profiles of dendrimer - liposome interaction process. Panel A, the raw ITC data of DPPEth+DPPC liposome; PAMAM G4 dendrimer; Panel B, effect of dendrimer generation on DPPEth+DPPC liposomes and panel C, effect of lipid variation on G4 PAMAM dendrimers.

3.7. Cytotoxicity Studies. The understanding of the mechanism of dendrimer-liposome interactions is important as the cytotoxicity issue is vital for acceptance and development of dendrimer-liposome aggregates as pharmaceutical agents. The cytotoxicity results obtained from MTT assay clearly demonstrate that the all the liposome formulations and dendrimer-liposome

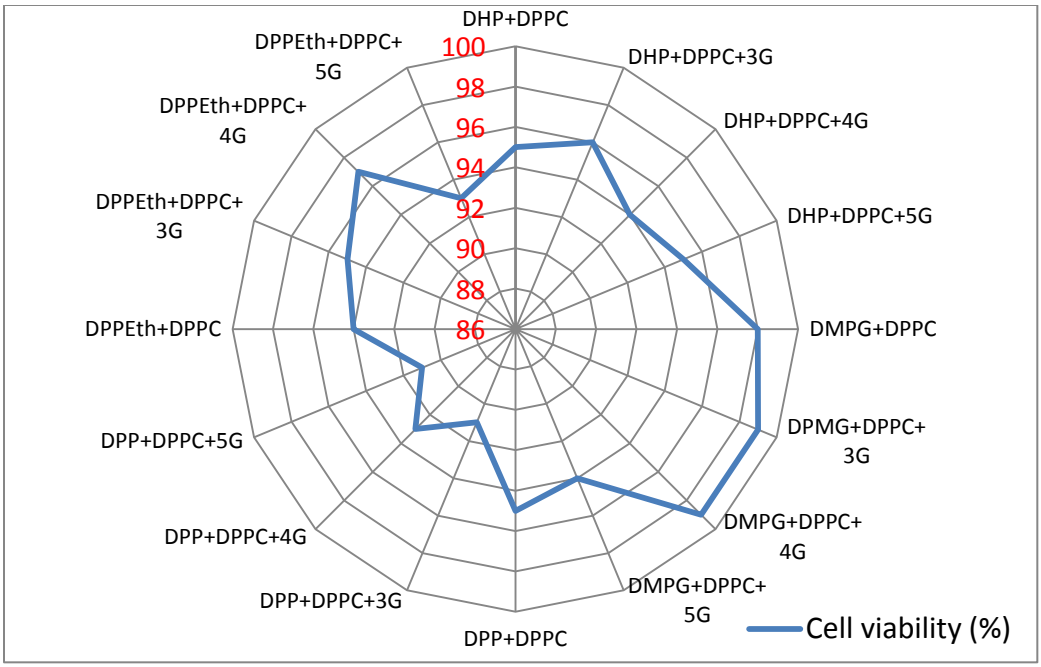


Figure 11. Dose response radar plot of various liposomes and dendrimer-liposome complexes on human blood cell lymphocyte.

aggregates were completely non-toxic towards normal human blood lymphocyte. The nontoxicity of a bioactive compound is the most important requirement for its therapeutic application. The obtained results showed that the liposome and dendrimer-liposome aggregates were almost non-toxic and possessed no effect on cell viability as shown in Figure 11. The results indicate that the dendrimer-liposome aggregates could be considered as a good drug delivery system. In PBS buffer solution the components did not affect the ionic strength of the solution. Thus the formulations could be considered safe in terms of drug delivery. However, the further in vivo studies are warranted to make final conclusions on this issue. Moreover, the haemolysis results showed that all the formulations of a liposome (0.1 mM) and dendrimer-liposome aggregates (liposome: dendrimer, M/M; 1:0.05, 1:0.1 and 1:0.2) were nontoxic towards human RBCs with only <1.1% haemolysis shown in Figure 12 (supplementary section).[52] It has been reported earlier that the materials with <5% haemolysis were regarded as haemocompatible.[53] Thus, the dosage of dendrimer-liposome complexes (liposome: dendrimer, M/M; 1:0.05, 1:0.1 and 1:0.2) were found to be hemocompatible. Hence, the formation of dendrimer-liposome aggregates enfolding by positively charge entity can acts as mimic biological simulated systems.

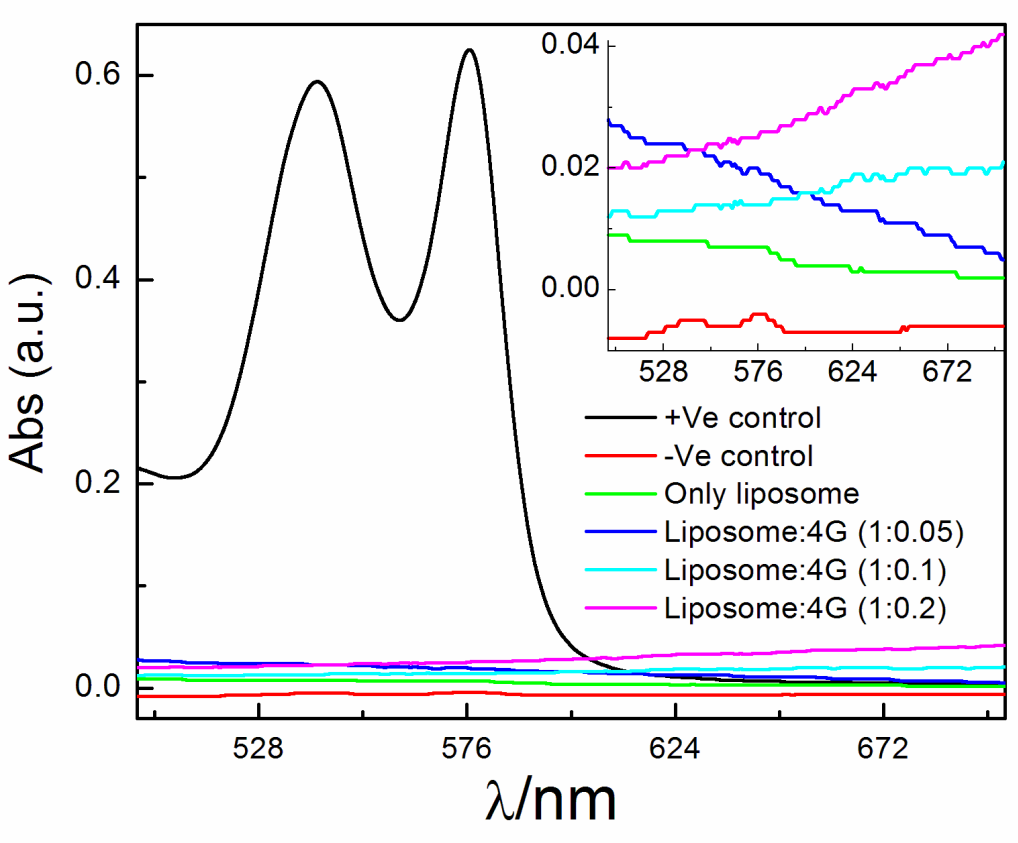


Figure 12. Hymolysis study of DPPEth liposome and dendrimer-liposome aggregates.

4. Conclusion

The manuscript describes the interaction between negatively charged liposomes with cationic PAMAM dendrimer and also to study the different biophysical properties of dendrimer-liposome aggregates. The type and strength of the interaction is dependent on charge and size of the liposomes as well as the dendrimer generation. The larger size of DHP+DPPC, DPP+DPPC and DPPEth+DPPC in the gel state than for DMPG+DPPC in the same state is rationalized through the lateral packing of lipid molecules within the membrane, due to the stronger van der Waals interactions between the hydrocarbon chains. Zeta potential of the liposome depends on the electron density on phosphate group of phospholipids and head group moietyof phosphate group [DHP, -H; DMPG, $-CH_2CH(OH)CH_2OH$; DPP, -H; DPPEth, $-CH_2CH_3$]. Here all the liposomes have net negative charges, thus the electrostatic interactions with the cationic dendrimers play an important role. The higher generation dendrimers causes greater disturbances in a lipid bilayer and interacts more effectively with liposomes. The formation of dendrimer-

liposome aggregates by higher concentrations of dendrimers were also visualized by TEM, FF-TEM and AFM studies. The increase in the fluorescence anisotropy shows that the liposomal membranes become more rigid, reflecting the fact that dendrimers had probably moved into the liposome bilayer via palisade layer. The differential scanning calorimetry and fluorescence anisotropy showed that the dendrimers interact not only with the hydrophilic part of the membranes but also the hydrocarbon chain. The binding constant for the formation of dendrimer-liposome aggregates depends on the head group moiety of the liposome and the generation of the dendrimers. The cytotoxicity and hemolysis results show that liposomes and dendrimer-liposome complexes are non-toxic in healthy human blood cell lymphocyte as well as human RBCs. In conclusion, it is clear that the exploration of the dendrimer-liposome aggregates as a potential drug carrier has significant perspectives.

CHAPTER IV

CHAPTER IV

Effect of cationic dendrimer on the physicohemistry of solvent spread and adsorbed membrane mimetic lipid monolayer.

ABSTRACT: The mutual miscibility and stability of the mixed monolayers of zwitterionic phospholipid, dipalmitoylphosphatidylcholine (DPPC) with negatively charged phospholipids (dihexadecyl phosphate (DHP), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPP) and 1,2-dipalmitoyl-sn-glycero-3-phospho ethanol (DPPEth) were investigated at the air-buffer interface. Interaction between the positively charged dendrimer with the monolayers have been studied in detail using surface pressure-area isotherms. Thermodynamic analysis indicate miscibility of the binary mixtures when spread at the air/buffer interface with synergistic interaction between the components. The surface pressure-area isotherms the binary monolayers of DPPC and negatively charged lipids at the air-water interface showed maximum deviation for DPPC : anionic lipid at 7:3 M/M ratio mixed monolayer was more stable than the monolayers individual components. Based on the regular solution theory, the miscibility and stability of the two components in the monolayer were analyzed in terms of compression modulus (C_s^{-1}) and excess Gibbs free energy ($\triangle G^0$) and these physiochemical parameters dependent on phospholipids composition. Stable liposomes were formulated by the binary mixture in 7:3 molar ratio of DPPC with negatively charged phospholipids. Subsequently adsorbed monolayers were generated through vesicles disruption technique. Effect of polyamidoamine (cationic) dendrimers on the adsorbtion kinetics at the vesicles were followed. Bylayer disintegration and subsequent interfacial adsorbtion of lipids were followed up through the surface pressure. Time analysis bylayer disintegration kinetics was govern by tht lipid head groups, chainlength as well as the dendrimer generation an concentratio

1. Introduction:

Pseudo-physiological Langmuir monolayers are frequently used to establish the necessity to mimic their counterpart of a single leaflet in cell membranes.[1] Langmuir monolayers at the air-water interface have been extensively used to elucidate the interfacial and elastic properties of natural cell membranes under a simplified and controlled physicochemical environment. [2] This simple technique has many advantages when compared with the methods employed in spherical structure such as liposomess, emulsions and micelles. Many interfacial structure of the

monolayer and phases at the micro and nano scale can be visualized by various optical techniques such as fluorescence microscopy (FM), brewster angle microscopy (BAM), ellipsometm, infrared spectroscopy and atomic force microscopy (AFM) *e.t.c.* . [3] Such studies can help in understanding the different physicochemical phenomenon at the sub-cellular level. Besides the different interaction that take place between the lipidic components and proteins, peptides and others stimulu *etc*. Phospholipids are widely found in nature either in plants or animals. Pure forms of these compounds and/ or their mixtures have important biological functions. [4] Furthermore, the physical mixtures of these compounds at definite combinations bear in its nature a rich variety of phases which are closely related to the molecular packing and the structure of cell membrane. Studies on the lipid monolayer at the air-water interface is very informative in subjects such as chain packing and lipid head group interactions prevalent in the cellular systems. The structural properties of monomolecular films have been thoroughly studied by Langmuir monolayers technique to gain insights about their natural membrane counterpart. The present work endeavors in understanding the effect of polar head group's moiety, lipid chain length[5] and the hydrophobic interactions between the monolayer components. Besides the monolayer elasticity and miscibility such studies help in judiciously choosing a simulated combination of mixed phosoholipids that can form a stable liposome. A mixture of lipids possesses higher hydrophobicity to be incorporated into the hydrophobic core of plasma cell membrane in higher eukaryotic organisms. [6] Studies on the mixed lipid monolayers are essential in understanding their roles on membrane properties at the air-water interface. [7] Owing to the fact that these mixed monolayers are too simple to mimic the real cell membrane, but nevertheless it gives an over simplified idea about the molecular interactions and/or organization of these molecules at cellular level. The DPPC lipid is a zwitterionic amphiphilic molecule with two saturated hydro carbon chains, containing 16 carbon atoms each, with a well-known phase behavior at the air-water interface. It is one of the most commonly used lipids in monolayer studies due to its occurrence in the mammalian cell membranes. 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) is a naturally occurring zwitterionic phospholipid found in pulmonary surfactant (PS). It has the capability to achieve high surface pressure; however, being solid in nature, it is unable to get fluidized by its own. In order to maintain parity in terms of charge, 30 mol% of phosphaditylglycerol is present in pulmonary surfactant. Such a combination of lipids mixtures is capable of forming monolayer and bilayer with net negative charge.[8]With the intention to mimic these combinations, we have formulated a variety of lipid mixture. The

anionic lipids used herein include dihexadecyl phosphate (DHP), 1, 2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphate (DPP) and 1, 2-dipalmitoyl-*sn*-glycero-3-phospho ethanol (DPPEth). Among them DHP, DPP, DPPEth and DPPC molecules have essentially the same hydrophobic chain length with 16C (except DMPG, 14C). Dihexadecyl phosphate lipid is different from other phospholipids even though it can form stable monolayer. Phosphatidyl alcohols have special effects on their membrane properties. Phosphatidyl alcohols are highly potent promoters of membrane curvature, and their trans bilayer movement is three times higher than any naturally occurring phospholipid at physiological pH.[9-10] The head groups of DHP, DMPG, DPP and DPPEth molecules possess dipole moment with an overall orientation greatly contributes to electrostatic interactions with the DPPC molecule head groups across the interface at which they are partially immersed in water. On the other hand, cholesterol as well as other sterols are known to influence the conformational order of the lipid acyl carbon chains and membrane fluidity. Cholesterol is also the main sterol component with a maximum of 30 mol% of the biological membranes composition. Although the aforementioned anionic lipids (except DMPG) are not directly relevant to biological cell membranes, however, it is expected that such combination of lipids in the term of monolayer/bilayer can be explored as drug delivery systems against the microbial membrane. [9] It is known that the most active compounds (drug molecule) cannot attain therapeutically efficacy because of their inability to reach the target side by crossing the cell membrane barrier. [11]The present set of lipid combinations with negative charge are expected to be biocompatible when formulating liposome. Here, the liposomes are expected to act as drug carrier or, more generally, as platform for theranostic.

The aims of this study are to clarify the interfacial behavior of mixture of phospholipids of DPPC with different kinds of negatively charged lipids (DHP, DMPG, DPP and DPPEth). With the attempt to find out how these liquid-condensed micro domains are achieved in these binary mixtures, the DPPC is mixed with the negatively charged phospholipids which may structurally mimic the driving potency to produce these LC-phases at the air-water interface. [8] Interfacial pressure-molecular area (π-A) isotherms and the corresponding thermodynamic assets of the mixing processes were evaluated at the pure air-water interface. Elastic properties of the binary mixtures were assessed by evaluating compressibility modulus extracted from π-A isotherms. [12-13] The phase behavior was examined using the Gibbs additivity rule as well as the Gibbs free energy of mixing.[14]

On the other hand, the bilayer disintegration induced by positively charged polyamidoamine PAMAM dendrimer studied understand well defined features of liposome. For the medical therapeutics, numbers of nanoparticles have been utilized as drug delivery vehicle. Understanding the type of interaction between nanoparticles and cell or plasma membrane is still a fundamental challenge as the mechanism of their interaction remains poorly understood. [15] Before employing one should judicially design nanoparticles having least cytotoxicity, biodegradability with better efficiency in transporting the drug or gene to the target tissue through the survival under the physiological conditions. For decades liposomes comprising phospholipids in different forms have been utilized in the field of drug delivery because of its fascinating hydrophilic-lipophilic nature.[16] It mimics with biological cell membranes often help to understand or predict the interaction of drug or other molecules such as dendrimers.[17] However limitation such as stability issue for the liposomes initiates researchers to develop a new class of hyper branched three dimensional polymeric macro molecules having lipophilic core and functional surface groups.

Polyamidoamine (PAMAM) dendrimers are such compounds that have drawn considerable interest in the field of vaccine carriers, drug and gene delivery etc. [18-21] The details of dendrimers structure and its synthesis have widely been reported.[22] Its structure generally consists of a core and several units of branching which help to reside number of functional moieties on the surface. Molecular shape of dendrimers depends on the generation number. The architect of dendrimers is such that its generation number, units of branching and function of the surface groups can be tuned. Its unique properties like the well define molecular weight, spherical morphology, cage like architect, lipophilic "dendritic box" and active functional groups to the surface marked it as potential candidate for the process of drug delivery.[22-23] Although, the main focus of the present work lies on the studies on the formation of biologicaly simulated monolayers of different negatively charged phospholipids with zwitterionic DPPC, yet, liposomes with net negatively charges were prepared to study the membrane disintegration through the formation of surface adsorbed monolayers subsequently the impact PAMAM dendrimer were studied on different processes, whereby the compositions of the liposomes were varied together with the anionic lipids (in combination with DPPC). Dependence of the type and strength of the interaction between dendrimer and lipid bilayer on the charge and size of the liposomes are expected to be evaluated through such studies. Impact of dendrimer concentration was investigated to understand the bilayer disintegration into surface adsorbed to monolayer.

Although there are different reports on the interaction studies between dendrimers and liposomes,[24-25] however, no comprehensive and systematic studies have been carried out previously in order to assess the impact of dendrimer concentration as well as the variation of the liposome type for bilayer disintegration kinetics.

2. Materials and methods

2.1. Materials.1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), dihexadecyl phosphate (DHP),1, 2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1, 2-dipalmitoyl-sn-glycero-3-phosphate (DPP) and 1, 2-dipalmitoyl-sn-glycero-3-phospho ethanol (DPPEth), PAMAM dendrimers of generation 4 were the products from Sigma-Aldrich Chemicals Pvt. Ltd. (USA). AR grade disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), sodium chloride (NaCl), HPLC grade chloroform and methanol were the products of Merck Specialties Pvt. Ltd, India. Double distilled water with specific conductance 2-4 μS (at 25 °C) was used in preparing the solutions. All the chemicals were stated to be ≥99.5% pure and were used as received.

2.1.1. Cell Culture: The MDA-MB-468 cell line were obtained from American Type Culture Collection (Manassas, VA) and were cultured in RPMI-1640 supplemented with 10% FBS, penicillin (100 U ml^{-1}), and streptomycin (100 mg ml^{-1}) in a humidified atmosphere of 5% CO_2 at 37°C. All cells were cultured in a humidified incubator containing 5% CO_2 at 37°C.

2.2. Methods.

2.2.1. Surface pressure(π) -area(A) measurement:

Surface pressure measurement using Langmuir surface balance.

Surface pressure (π)-area (A) isotherms of pure as well as mixed monolayers (solvent spread) were obtained using a Langmuir surface balance (Micro Trough X, Kibron, Helsinki, Finland). A monolayer was generated by gently spreading the appropriate amount of a lipid solution dissolved in a 3:1 (V/V) chloroform/methanol mixture onto the air-buffer (pH 7.4) interface using a Hamilton micro syringe, allowed to stabilize for 20 minutes to achieve solvent evaporation and film equilibration. After that, isotherms were recorded continuously under symmetric compression, at a constant barrier speed of 5 mm^2/min, using Film Ware X software.

2.2.2. Liposomal formulation:

For adsorption kinetics study, liposomes were prepared by the conventional thin film hydration technique. Quantitative amount of phospholipid and cholesterol were dissolved in chloroform and methanol (3:1, V/V) in a round bottom flask. The solvent was removed using a rotary evaporator at 40°C. The resultant thin film was further maintained under vacuum for 6 h. It was then hydrated with 10 mM phosphate buffer saline (PBS) for 1 h at 70 °C (temperature above chain melting temperature of all lipids). Ionic strength was maintained at 100mM using sodium chloride. Then the systems were frozen at -20 °C and thawed followed by sonication at 45 °C. The freeze-thaw-sonication process was repeated for four cycles in order to obtain small unilamellar liposomes. Liposomes were prepared separately using DHP+DPPC, DMPG+DPPC, DPP+DPPC and DPPEth+DPPC along with 30 mole% cholesterol at pH 7.4. Dispersions were filtered through 0.45 μm cellulose nitrate membrane. Then, PAMAM dendrimer were added to liposome and put it for 6 hour for development of dendrimer-liposome complex.

2.2.3. Bilayer disintegration kinetics studies. The process of disintegration and subsequent formation of monolayer at air-water interface was monitored by using Langmuir surface balance set up with a multiwell trough (micro trough X, Kibron, Finland). Langmuir adsorption isotherms were (π vs. t) obtained by carefully adding 500 μL of the liposome/dendrimers mixture in the trough. Plexi glass box was used which covered the stage and trough to prevent the entry of dust particles. All the π-t isotherms were recorded at. To ensure reliable result, each set of experiment was performed twice.

2.2.4. MTT reduction assay.

The in-vitro cytotoxicity of the synthesized liposome and liposome-dendrimers complexes (LDCs) on MDA-MB 468 cells were determined by the MTT assay described elsewhere.[26] The optical density (OD) at 570 nm was measured with reference at 655 nm on an ELISA reader (Bio-Red, Japan) using wells without the sample containing cells as blanks. All experiments were performed in triplicate and the % of cell viability using the following formula.

$$\%\ \text{cell viability} = \left(\frac{\mathrm{OD_{sample} - OD_{control}}}{\mathrm{OD_{control}}}\right) \mathrm{X}\ 100 \qquad (1)$$

3. Results and discussion.

The experimental investigation presented in this study was divided into two parts. Part one compares the mixture miscibility studies and devaluation of the associated thermodynamic

parameters of mixed monolayer. The second part of the work mainly described the formation of adsorbed monolayers through the disintegration of bilayer and the subsequent impact of PAMAM dendrimer on the adsorption kinetics.

3.1. Surface (π)- area (A) isotherm of the solvent spread monolayers:

The lift-off area for the components in their individual pure forms appeared at 1.04, 0.47, 1.08, 0.98 and 0.51 nm^2 $molecule^{-1}$ for DPPC, DHP, DMPG, DPP and DPPEth respectively as shown in Fig 1, panel B. The recorded isotherms for pure compound monolayers expose several characteristic features which are in line with those previously reported results.[27-29] All the monolayers collapse as liquid-condensed (LC) phases. The state of all the monolayers was confirmed by the values of compression moduli.

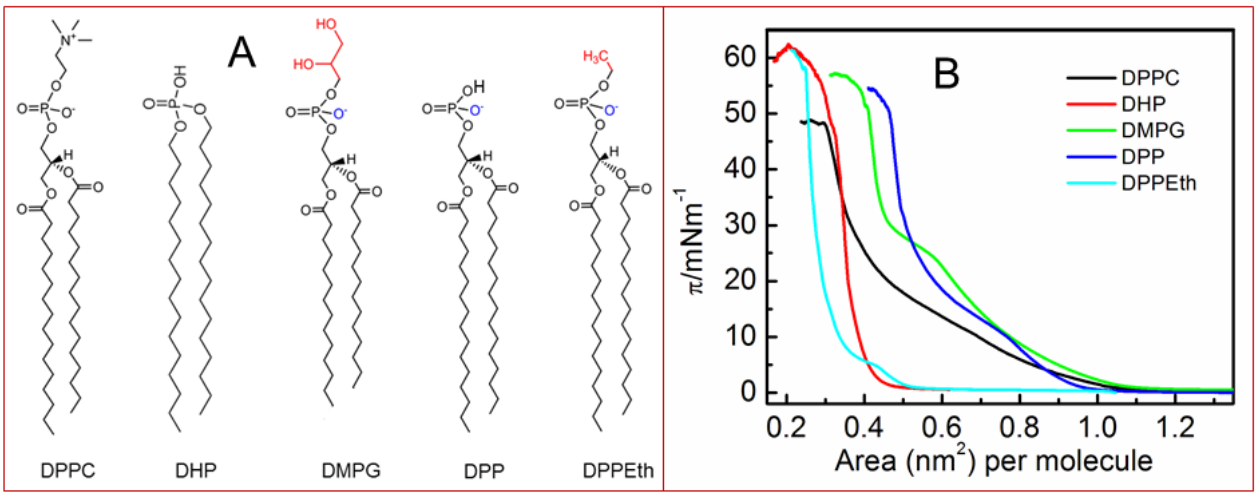


Figure 1: Panel A, molecular structure of the phospholipids; panel B, surface pressure (π)-area (A) isotherm for the monomolecular films of pure lipids (in the presence of 30 mol % cholesterol) at the air-buffer interface at 25 °C

DHP, DMPG, DPP and DPPEth are all negatively charged phospholipids, whereas DPPC is zwitterionic with the phosphocholine head group. DMPG differ from other systems in their hydrophobic chain length. Although there are of two methylene moiety differences between DMPG and other three phospholipids in term of chain length, shape of the isotherm of DMPG was significantly different from the others. There was a steep rising portion of the isotherms in the high-pressure region, where π of the DPPC, DPP, DPPEth monolayer is lower than DMPG monolayers at the same surface area, which is confined to a smaller molecular area (<1.08 $Å^2$). Evidently, in this region fewer palmitoyl chain PC molecules were needed to bring about the

same surface pressure compare to DMPG monolayers, which can be on the basis of by the stronger van der Waals interactions between hydrophobic chains of the lipid with longer aliphatic chains. It is also obvious that at the larger molecular area, the longer is the aliphatic chain more lipid molecules are needed in obtaining the same rise in surface pressure. The lift-off area, where surface pressure begins to deviate from zero, was in the sequence DHP < DPPEth < DPP < DPPC < DMPG, which is as expected since longer aliphatic chains would hinder rearrangement of the molecules from their initially flat-lying position to a more vertically oriented position at the interface. Formation of stable monolayer is highly depends on the head group moiety of a phospholipids. In DHP, two hexadecyl groups are directly covalently linked to the phosphate group unlike the other phospholipids. Hydrophobic attraction between two acyl chains result is lower lift-off area. On the other hand, in case of DPPEth, the phosphate group is connected with $-CH_2CH_3$, which has greater tendency to come out from water to decrease the lift-off area.

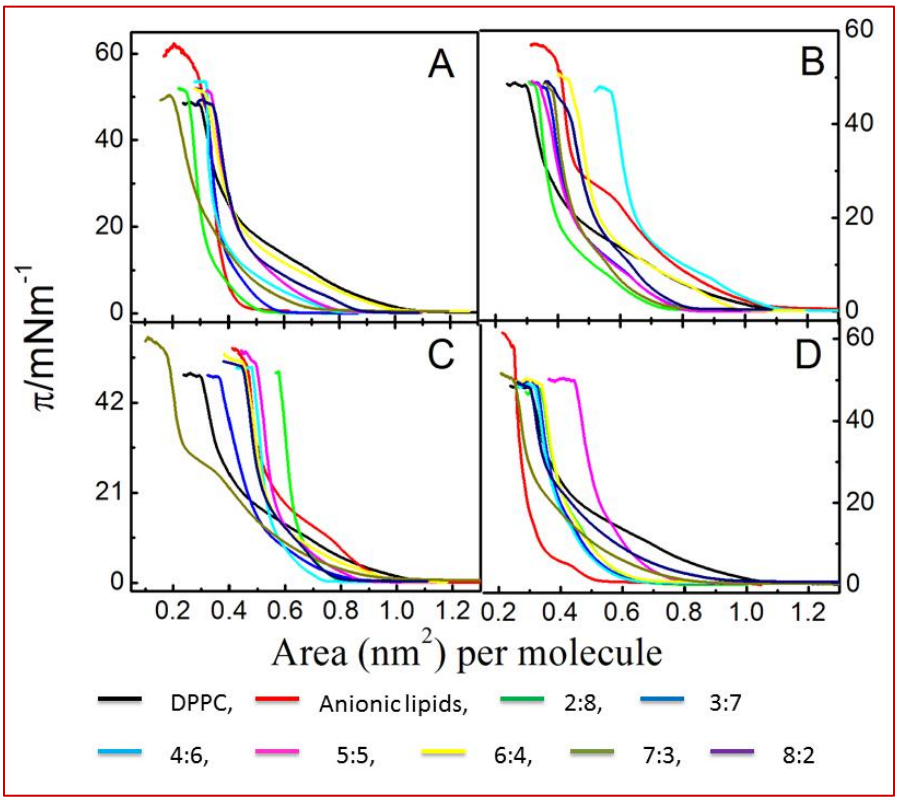


Figure 2: Surface pressure (π)-area (A) isotherm of the monomolecular films of DPPC with Anionic lipids (in the presence of 30 mol % cholesterol) at the air-buffer interface at 25 °C. Legends of DPPC, anionic lipids and their different mole ratio (DPPC:anionic lipids) are shown in bellow the figure.

The surface pressure versus area per molecule (π-A) isotherms of the mixed monolayers of different combinations of negatively charged phospholipids and DPPC along with 30 mol% cholesterol are presented in Figure 2. Measurement and analysis of the surface pressure versus molecular area curves of the mixed monolayers of DPPC and negatively charged phospholipids under various molar ratio showed that they occur associative and repulsive interaction between the components at the air-water interface. The nature and extent of deviation depend largely on the composition as well as the surface pressure. With increasing the mole ratio of DPPC the lift-off area increases for DHP:DPPC mixed monolayers. But pure DHP collapsed at higher pressure, shown in Figure 2, panel A. As the lift-off area of DPPC is higher than that of DHP, the lift-off area increases with increase the mole ratio of DPPC. But for the binary mixture of DPPC and DHP (7:3, M/M), it show moderate lift-off area with the value 0.68 nm^2/molecule. Other systems followed the same sequence.

3.2. Mutual miscibility of the lipid mixtures at the air-buffer interface:

When the two components were mixed in an organic solvent and then spread over the air-water interface to form a monolayer, it is not possible to find as whether the components miscible exist existed as a homogeneous surface phase, or whether there occur phase separation due to limited solubility of one component in the other had occurred. By analysis the π-area isotherm of the pure as well as of mixed monolayers, there are two ways to determine the miscibility of the two components at the interface. The first one is analyzed by the measuring of collapse pressures of the mixed monolayers. Each of the pure component monolayer has its own collapse pressure upon compression. If the two components are immiscible, the mixed monolayer should start to collapse at the lower one. If the mixed monolayer is further compressed, the surface pressure should rise again until the higher collapse pressure is reached. Instead, the mixed monolayer of two miscible components shows only a single collapse, probably at a pressure which is different from either of the collapse pressures of the pure component monolayers. The collapse pressure of pure components was found as DPPC, 48.1 πmNm^{-1}; DHP, 62.1 πmNm^{-1}; DMPG, 57.1 πmNm^{-1}; DPP, 54.2 πmNm^{-1} and DPPEth, 61 πmNm^{-1}.

Film functionality was found to be dependent on the composition of the lipid mixture. Isotherms of the pure components lead to calculate ideal isotherms for the mixed systems according to the additivity rule.[30]

$$A_{id} = x_1A_1 + x_2A_2 \quad (2)$$

where, A_{id} is the average theoretical area per molecule, x_1 and x_2 being mole fractions of the components 1 (DPPC+ 30 mol % cholesterol) and 2 (anionic lipid + 30 mol % cholesterol), respectively. A_1 and A_2 are the corresponding experimental area per molecule of the individual components. Deviation of the experimental value (A_{ex}) from the ideal one can be obtained through the calculation of the excess area per molecule as.[30]

$$A_{ex}=A_{12}-A_{id} \quad (3)$$

where A_{12} is the experimental area per molecule of the mixed monolayer. Any deviation from linearity or any incidence of the appearance of maximum or minimum with varying composition would produce the extent of deviation from ideality. In case of an ideal mixture, the calculated value (A_{id}) should be equal to that of the measured value (A_{12}) and should vary linearly with mole fraction of any components. A negative deviation from the ideal behavior signifies associative interaction between lipidic hydrocarbon chains of DPPC and anionic lipid, while a positive deviation signifies repulsive interaction. Representative plots for the variation of A_{ex}/A_{id} with composition are shown in the Figure 3. Initial positive deviation (for 20, 40, 50, 60 and 80 mol % of DPPC) from the linearity was the outcome of repulsive interaction between DPPC and the anionic lipid. Columbic repulsion, prevailing at this particular combination, causes the system to deviate positively from the ideality. However, associative interactions were validating for the systems with 70 mol % of DPPC for all the anionic lipids (except DMPG). Strong van der Waals force of interaction along with hydrogen bonding results in associative interaction which prevail the Columbic repulsion for these systems. In case of DPP lipid significant divergence from the trend line was observed for 40 mol % DPPC, suggesting some anomalous interactions than the other. Variation in mean molecular area and composition mole fraction was non systematic; for most of the systems and surface pressures positive deviation were noticed at two distinct values of x_{DPPC} which were due to the formation of 3D aggregated species. According to the previous report the miscibility between the different molecule at the air-water interface depends strongly on their acyl chain length, composition and degree of packing. Associative interaction occurs between the components through the formation of condensed monolayer at 70 mol% DPPC.

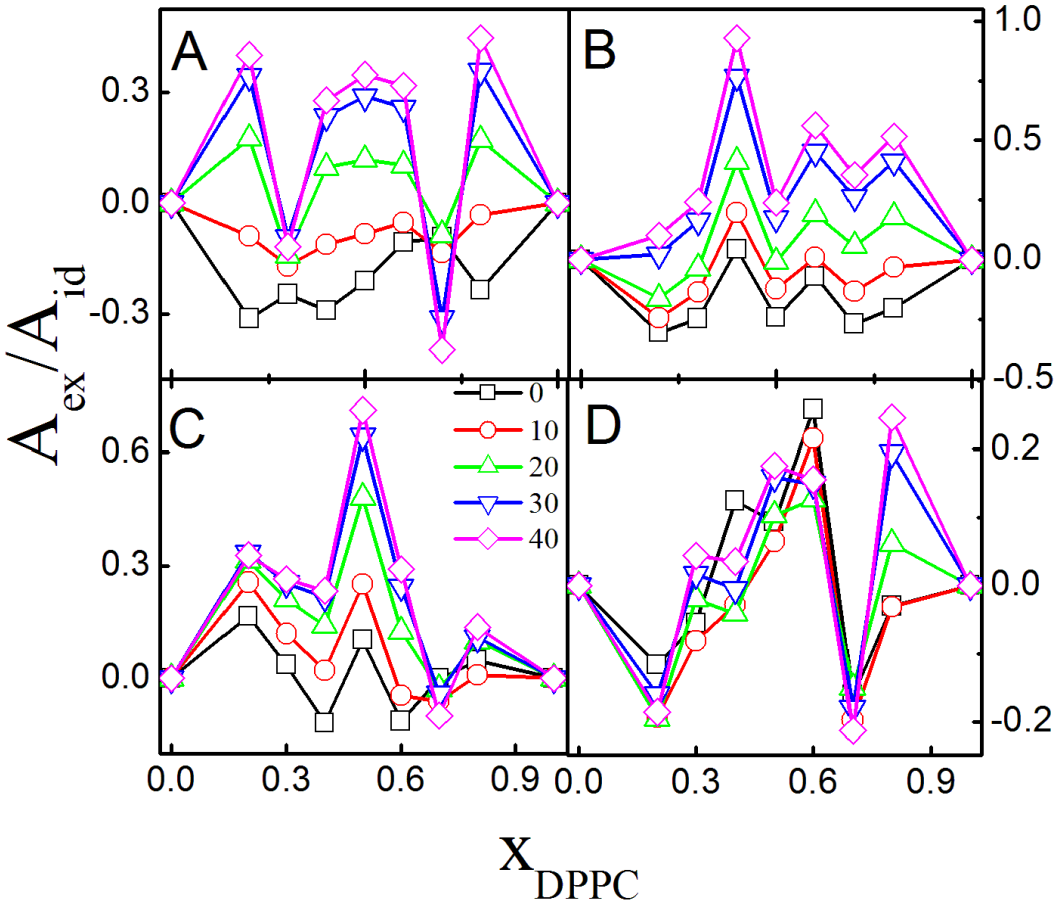


Figure 3: A_{ex}/A_{id} vs x_{DPPC} profile at different surface pressure for the mixed monolayer over 0.1 mM PBS buffer at pH 7.4. Panel A, DHP+DPPC; panel B, DMPG+DPPC; panel C, DPP+DPPC and panel D, DPPEth+DPPC. Surface pressure are indicated inside the figure.

3.3. Film stability analysis:

Condensation of mixed monolayer (area contraction) at higher DPPC mole ratio (70%) was revealed from Figure 4, and the subtle structure of the monolayer could further be scrutinized from various factors such as ordering of chain, tilting of polar head, and molecular packing. To obtain the state of the investigated films and the consequent molecular ordering, compression moduli were calculated for different mixed monolayers. Film compressibility (C_s) is a measure of the resistance of the monolayer against compression; in other words, it can be defined as the amount of pressure needed to cause a change in the molecular area. The reciprocal of compressibility, C_s^{-1} known as compression modulus, is also another route to demonstrate the phase transition variation in the C_s^{-1} with the surface pressure for different combination of lipid

mixtures are summarized in Figure 4. [30] Results suggest that the molecular packing at the interface somehow depends on the head group and hydrophobic chain length of the anionic

$$C_s^{-1} = -A\left(\frac{\delta\pi}{\delta A}\right) \quad (4)$$

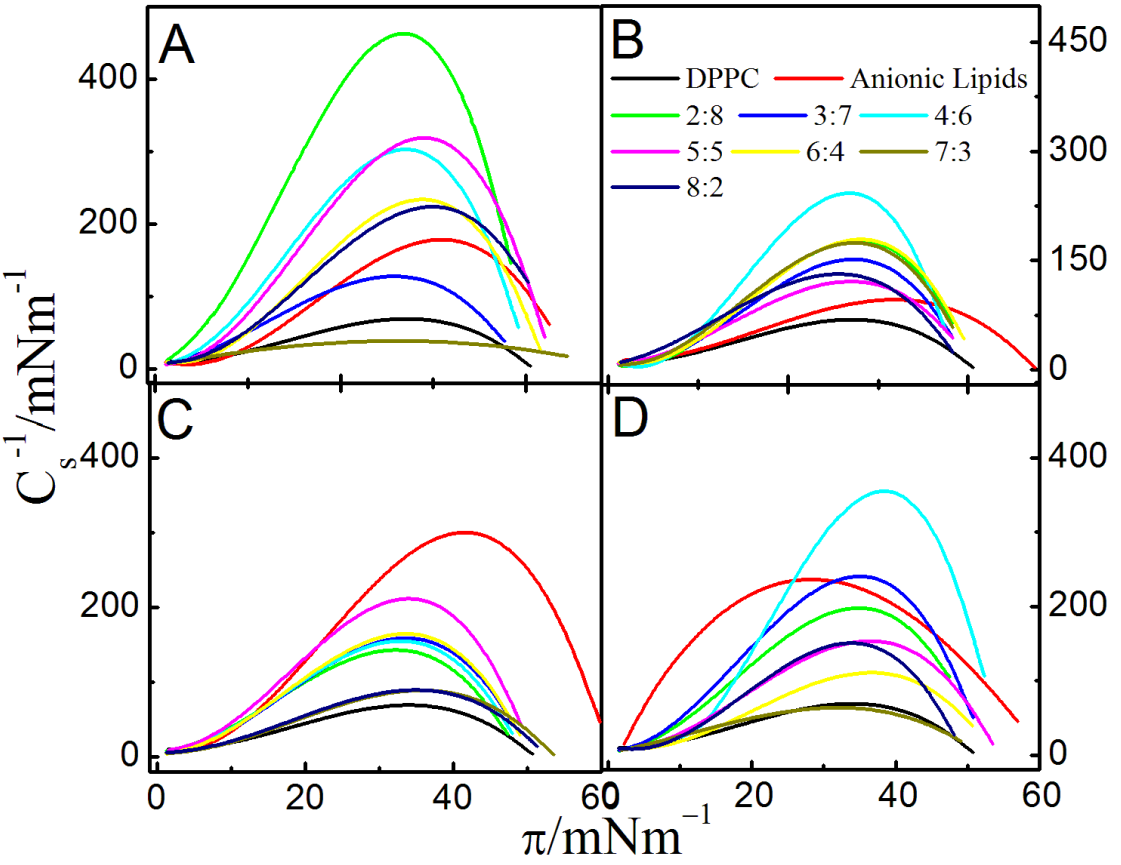


Figure 4: Variation in the compressibility moduli (Cs $^{-1}$) with the surface pressure for pseudo binary monomolecular films of DPPC + anionic lipids with 30 mol % cholesterol at 25 °C. Panel A, DHP+DPPC; panel B, DMPG+DPPC; panel C, DPP+DPPC and panel D, DPPEth+DPPC. Lipids composition are maintain inside the figure.

phospholipids. It has also been found that DMPG+DPPC monolayer was more than the other systems as the lift-off area is higher in case of pure DMPG. Shorter hydrocarbon chains leads to the formation of less condense monolayer in case of DMPG comprising systems as also reported earlier. A quantitative analysis of the interactions between molecules in the mixed DPPC/anionic

lipid can be obtained on the basis of excess free energy of mixing (ΔG_{ex}) which can be determined directly by analyzing the π-A isotherms.

Mixed monolayer study and subsequent analysis help in formulating stable liposomal dispersions by considering. The spontaneity associated with hydrophobic interactions between the hydrocarbons chains of lipids can also be viewed by evaluating the excess free energy change. Quantitative assessment of the magnitude of the mutual interaction between DPPC with the anionic lipids could be best studied by considering ΔG_{ex}^{0} values. [30]

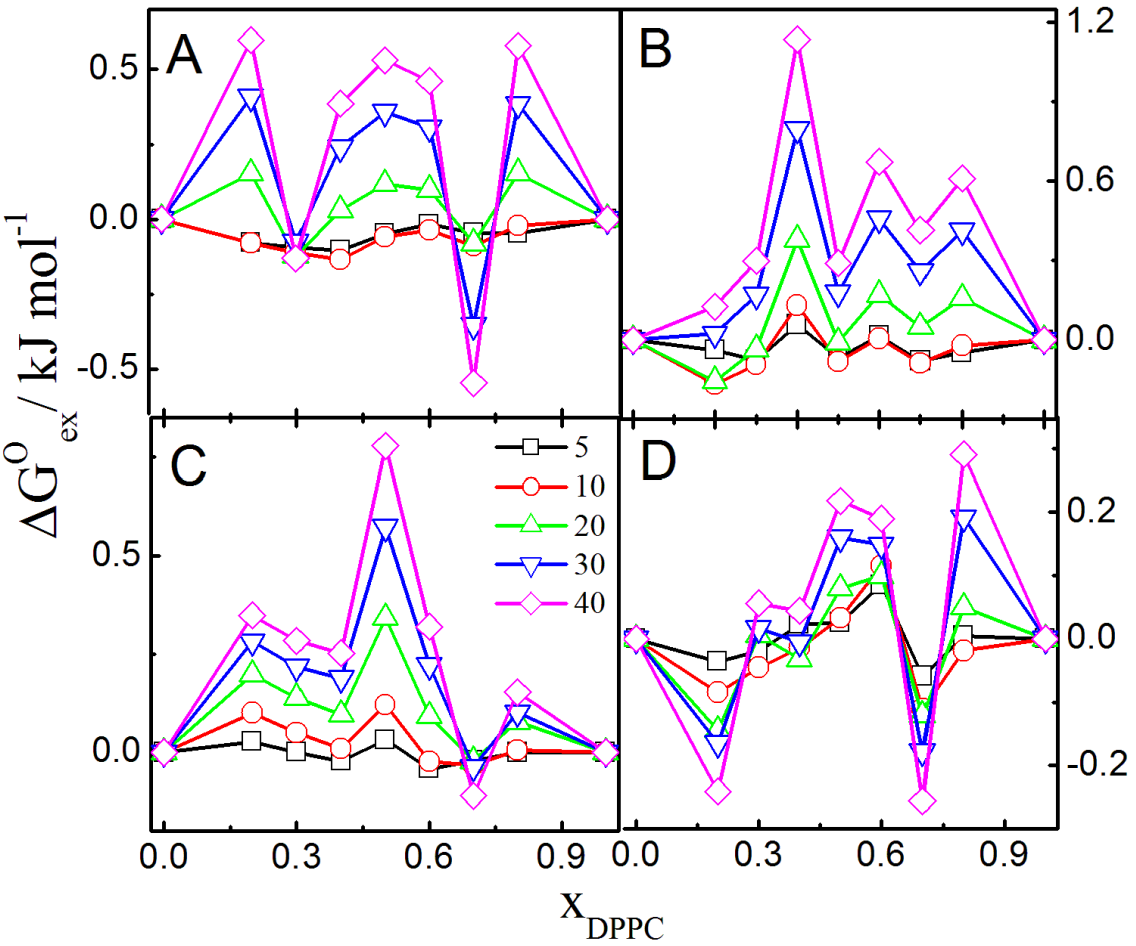


Figure 5: Variation of excess free energy (ΔG_{ex}^{0}) for the DPPC + anionic lipid monolayer systems (with 30 mol % cholesterol) with the mole fraction of DPPC(x_{DPPC}) lipid at different surface pressure at 25 °C. Panel A, DHP+DPPC; panel B, DMPG+DPPC; panel C, DPP+DPPC and panel D, DPPEth+DPPC. Surface pressure are indicated inside the figure.

$$\Delta G^{0}_{ex} = \int_{o}^{\pi} (A_{12} - A_{id}) d\pi \quad (5)$$

where, A_{id} is the average theoretical area per molecule and A_{12} is the experimental area per molecule of the mixed monolayer. Figure 5 describe the variation in the excess free energy $\triangle G^{0}_{ex}$ with the composition of mixed binary phospholipids. Non-ideal mixing behavior between DPPC and anionic lipids was confirmed from the run of the curves; positive deviation from the ideal behavior was observed for the system comprising different mol % of DPPC. Coulombic force of repulsive interaction between the polar head groups and the dissociation of lipids could be associated in the process of mixing. Negative values of ΔG_{ex}^{0} for 70 mol % of DPPC indicate that the formation of stable monolayer at their favorable arrangement was found on that compositions of associative manner.

3.4. Bilayer disintegration kinetics:

Liposomes in bulk condition can transform to monolayer at air-water interface, which motivate us to study the bilayer disintegration kinetics by monitoring the increase in surface pressure with respect to time. Due to the formation of interfacially adsorbed monolayer, one can expect rise in the surface pressure if a vesicle dispersion is allowed to adsorb. However surrounding environment and addiditives can greatly control the process of adsorbtion. The time dependent surface pressure (π) changes, eonsequence of monolayer formation at air-water interface are shown in Figure 6. Four different liposomes in the absence of dendrimers were initially checked. The aggregate constituting lipids leave the bilayer region to some extent and preferred its orientation towards air-water interface (scheme I). Dendrimers molecules tend to adsorb on the surface of the liposomes dendrimer can preferentially bind to the liposomes surface and hence can reduce the process of disintegration which eventually reduced the monolayer formation at air-buffer interface and endorse higher time in attention equilibrium the surface pressure. It has been found that due to progressive addition of dendrimer, the rate of disintegration decreases. In the dendrimer concentration range of 0.001µM to 0.01 µM, the dendrimer get adsorbed on liposome surface; that effectively retards the disintegration of liposome. After a certain concentration, (>0.01) the rate of disintegration increased. At higher concentration of dendrimer structure get subsequently disrupted the bilayer. The bilayer disintegration also depends on the liposome constituents. In case of DMPG, it has greater tendency to disintegrate from bilayer to monolayer in absence of dendrimer due to presence highly polar phosphate head group.

By suitable analysing the π-t isotherm one can obtain kinetics of the formation of the adsorption process that eventually help to understand the process of interaction between dendrimers and liposomes. The rate constant (k) of liposomes disintegration or monolayer formation kinetics for the same liposomes were calculated. Figure 6 follow Langmuir like adsorption isotherms where

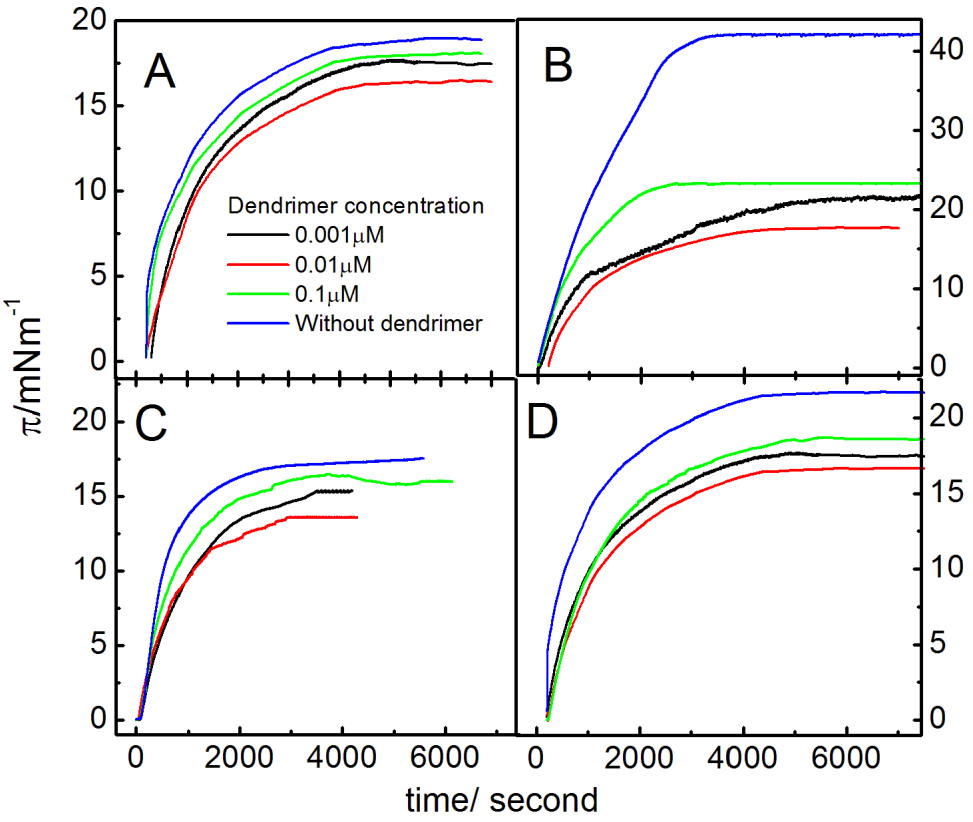


Figure 6. Surface pressure-time set of liposomes at air-buffer interface, with time. Dendrimer concentrations (μM) are indicated inside the figure. Panel, DPPC+DHP; panel B, DPPC+DMPG, panel C, DPPC+DPP and panel D, DPPC+DPPEth.

the process of monolayer formation registered constancy after some time as there were no changes in surface pressure. Hence considering the equation 1, one could easily calculate the rate constant of first order kinetics of monolayer formation from the following equation:

$$\log = \frac{\pi_\alpha - \pi_i}{\pi_\alpha - \pi_t} = \frac{kt}{2.303} \qquad (6)$$

where, t = time, k = first order rate constant, π_i = initial surface pressure, π_t = surface pressure at time t and π_α = final surface pressure. Lines are linear and passing through the origin. From slope of the plot, one could easily find out the value of first order rate constant.

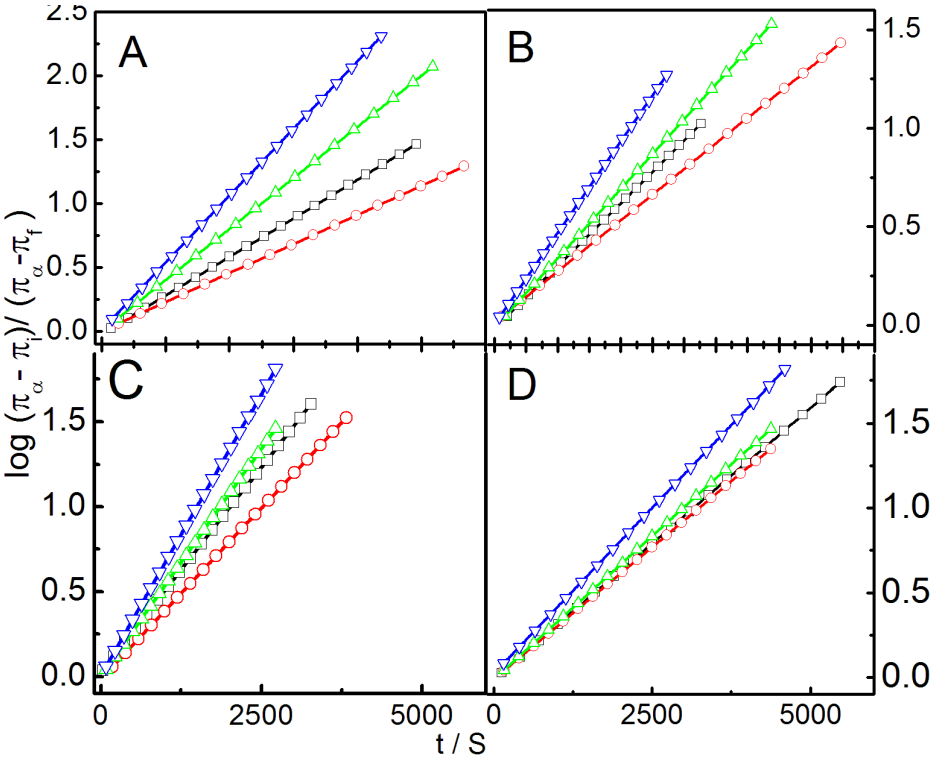


Figure 7. Kinetic of disintegration and subsequent formation adsorb monolayer kinetics for four different liposomes (panel A, DPPC+DHP; B, DPPC+DMPG, C, DPPC+DPP and D, DPPC+DPPEth) at different dendrimers concentrations ((□, 0.001; ○, 0.01 △, 0.1μM and ▽, pure systems).

As reflected from the Figure 6 for every set of formulations, blank liposome holds higher disintegration as compared to in the presence of dendrimer systems. The kinetic of disintegration slowed down from 0.001 to 0.01 μM dendrimers concentration signify modification on the surface of the bilayer that holds oppositely charged dendrimers, which restrict the lipidic tail to leave the bilayer wall. On the contrary presence of 0.1 μM dendrimers enhances the disintegration which could be viewed as bilayer disruption as described earlier. Liposome-dendrimer aggregates undergo variant architectural forms based on their stoichiometric ratio. However, the slow rate constant for the liposomes in the range of of 0.001 to 0.01 μM dendrimers concentration specify the point of interaction as a consequence of ionic interaction. Fourth generation PAMAM dendrimer is bulky molecule with positively charged head groups. Hence at fairly low concentration, 0.001 μM, it gets easily adsorbed onto the liposome surface facilitates by ionic interaction. The liposomes carrying the overall negative charges can easily

undergo electrostatics interaction whereby the dendrimer can act as a glue to the liposome/vesicle.

Table 1: Rate constant for liposomes in presence and absence of dendrimer.

Dendrimer concentration (μM) / **Liposomes**	**Rate constant X 10^{-4} (s^{-1})**			
	Only liposome	0.001	0.01	0.1
DHP+DPPC	12.11	6.96	5.24	9.22
DMPG+DPPC	10.71	7.33	5.98	8.13
DPP+DPPC	15.29	11.16	9.29	12.34
DPPEth+DPPC	8.99	7.38	7.12	7.78

Results are summarized in Table 1. Values of k dropped down at 0.001 and 0.01 μM dendrimers concentration (as compared to dendrimers free systems). At higher concentration (0.1 μM) k values increased further. At higher concentration of dendrimers the extent of ionic interaction gets predominant which subsequently facilitates the disintegration by rupturing the liposome bilayer. The rate constant values were also dependent on liposome compositions and follow the order DPP+DPPC> DHP+DPPC> DMPG+DPPC> DPPEth+DPPC.

In-vitro cytotoxicity by MTT assay: The in-vitro cytotoxicity of only liposomes (with various phospholipids), dendrimers and Liposome-Dendrimers Complexes (LDCs), (G1-G4) on breast cancer cell line (triple negative) were found to have no significant cytotoxicity in comparison to the doxorubicin treated MDA-MB-468 cell line (P>0.05). However, DMPG with G4 dendrimer-liposome complexes have 9.291% cell killing ability which is the highest among all tested LDCs. More to the point, DHP with G4 dendrimer-liposome complexes poses only 1.115% of cell killing ability and we found it the lowest among all tested LDCs (Figure 8). Drug delivery through a vehicle provides great advantages. [31] In past few years in the field of cancer therapeutic research, liposome-mediated drug delivery widely accepted as the model system for drug delivery. Liposome as a vehicle, independently capable of modulated released of the target drug.[31] However, liposome-dendrimer complexes combine offers betters therapeutics advantages than the conventional liposome-mediated drug delivery.[19] The modulated released of the drug is of with paramount important in cancer therapeutic research.[32] In our study, the low cytotoxicity

of the LDCs has wide applicability because it could be a novel drug delivery vehicle in cancer therapeutics.[33] However, it needs to be further confirmation by a detailed cytotoxicity study with drug encapsulated in LDCs.

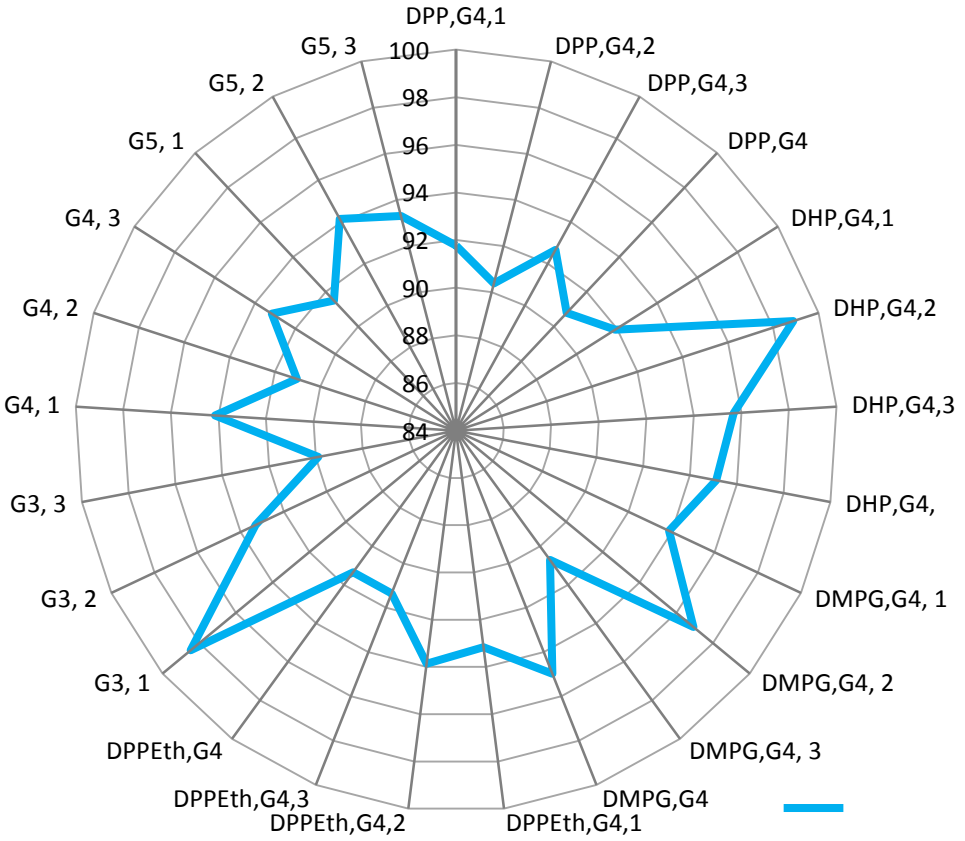


Figure 8. Radar plot depicting the dose response of various dendrimer-liposome complexes on breast cancer cell line (MDA-MB-468). Greene line indicates cell viability (%).

4. Conclusions:

DPPC and negatively charged lipids in different sets of combination were used to prepare stable liposome dispersions. Through the comprehensive investigation on the impact of negatively charged lipid on the zwitterionic DPPC were evaluated from monolayer studies where it was concluded that anionic lipids exerts prominent influence on DPPC monolayer. Associative interactions were found for some specific composition; however, the system with 30 mol % anionic lipids did respond to produce stable liposome dispersions. Other lipid combinations were

unable to form stable monolayer. This was further scrutinized by measuring as reflected through the existence of positive deviation the Gibbs free energy, found relatively less stable monolayer other than the 30 mole% comprising anionic lipids. Binary monolayer formation was also dependent on lipid composition. In case DMPG, it forms more stable mixed monolayer than the other anionic lipids. The lift-off area of DHP and DPPEth is lower than that of other lipids due directly connected with phosphate group. Also the bilayer disintegration kinetics were explored for stable liposomal systems DPPC and anionic lipids in molar ratio (7:3). Disintegration of bilayer to the interracially adsorbed monolayer also depended on lipid composition. In case of DMPG, due to presence of hydroxyl group it form intra or inter molecular hydrogen bonding. Thus DMPG lipid takes more time to disintegrate. However two shed further lights on the structure on the adsorbed monolayer, investigations like the Brewster angle microscopy (BAM) and polarization modulation infrared reflection-adsorption spectroscopy (PMIRRAS) and atomic force microscopy (AFM) studies are important. This would eventually shed lights on the structure of aggregates at the microscopic level as well as the molecular structures in these aggregates. These are considered to be the future perspectives.

SUMMARY AND CONCLUSIONS

Physicochemical properties of different liposomes, *viz.*, size, PDI, surface charge, thermal behavior, and membrane micro viscosity as well as their encapsulation efficiency and release behavior of curcumin were pronouncedly influenced by liposome composition along with pH and temperature of the medium. All the studied liposomes (SPC, DPPC, DPPG and DPPC+DPPG) were found to be stable in terms of size, PDI and zeta potential values. Size of liposomes did not vary significantly with temperature in terms of composition, albeit the minimum size was evidenced at ~40°C corresponding to phase transition temperature of the saturated phospholipids. All the systems turned out to be more rigid with increasing acidity of the medium as revealed from DSC results; this was due to increased hydrogen bonding among phospholipid molecules. Maximum anisotropy value of curcumin for SPC liposome reflected its higher binding affinity compared to the other systems. Increase in membrane microviscosity of the vesicles (except DPPC) with the rise in pH of the medium could be rationalized on the basis of the ionizing tendency of curcumin in basic environment and its ability (in neutral form) to disrupt bilayer packing at high concentration. Entrapment efficiency, however decreased with increasing pH of the medium due to the acidic nature of drug. Entrapment efficiency and release kinetics studies revealed opposite phenomenon with respect to liposome composition. SPC exhibited fastest drug release and lowest entrapment efficiency owing to its fluid nature. Curcumin loaded liposomes exhibited pronounced antibacterial activity against the Gram positive bacteria *Bacillus amyloliquefaciens*. Experimental evidences led to conclude that the optimization of the lipid composition and formulation conditions like pH is necessary to prepare liposomes with enhanced stability and efficient drug carrier property. *In vivo* studies of the curcumin loaded liposomes, to evaluate different pharmacokinetic parameters, could be considered as one of the future perspectives.

Studies on the interaction of 2G, 4G and 6G PAMAM dendrimers with different liposome substrates were done using absorbance, size analysis, zeta potential measurements and AFM measurements on solid supported bilayers. Increase in absorbance and size of the liposome is due to the adhesion of the individual liposomes, where the dendrimers acted as “glue”. Maxima in the absorbance and size were due to the maximum adhesion of liposomes, after which

the size decreases, due to the formation of liposome dendrimers complexes, probably "dendriosomes". Charge reversal during the zeta potential measurements reveal the electrostatic interaction among the liposome and dendrimers, which are significant when they are opposite in terms of surface charge. Further enhancement of zeta potential due to dendrimer addition was due to hydrogen bond/ hydrophobic interactions. Bilayer disruption of vesicles was observed upon addition of dendrimer to negatively charged surface of liposomes, as revealed by AFM measurements.

The manuscript describes the interaction between negatively charged liposomes with cationic PAMAM dendrimer and also to study the different biophysical properties of dendrimer-liposome aggregates. The type and strength of the interaction is dependent on charge and size of the liposomes as well as the dendrimer generation. The larger size of DHP+DPPC, DPP+DPPC and DPPEth+DPPC in the gel state than for DMPG+DPPC in the same state is rationalized through the lateral packing of lipid molecules within the membrane, due to the stronger van der Waals interactions between the hydrocarbon chains. Zeta potential of the liposome depends on the electron density on phosphate group of phospholipids and head group moietyof phosphate group [DHP, -H; DMPG, $-CH_2CH(OH)CH_2OH$; DPP, -H; DPPEth, $-CH_2CH_3$]. Here all the liposomes have net negative charges, thus the electrostatic interactions with the cationic dendrimers play an important role. The higher generation dendrimers causes greater disturbances in a lipid bilayer and interacts more effectively with liposomes. The formation of dendrimer-liposome aggregates by higher concentrations of dendrimers were also visualized by TEM, FF-TEM and AFM studies. The increase in the fluorescence anisotropy shows that the liposomal membranes become more rigid, reflecting the fact that dendrimers had probably moved into the liposome bilayer via palisade layer. The differential scanning calorimetry and fluorescence anisotropy showed that the dendrimers interact not only with the hydrophilic part of the membranes but also the hydrocarbon chain. The binding constant for the formation of dendrimer-liposome aggregates depends on the head group moiety of the liposome and the generation of the dendrimers. The cytotoxicity and hemolysis results show that liposomes and dendrimer-liposome complexes are non-toxic in healthy human blood cell lymphocyte as well as human RBCs. In conclusion, it is clear that the exploration of the dendrimer-liposome aggregates as a potential drug carrier has significant perspectives.

DPPC and negatively charged lipids in different sets of combination were used to prepare stable liposome dispersions. Through the comprehensive investigation on the impact of negatively charged lipid on the zwitterionic DPPC were evaluated from monolayer studies where it was concluded that anionic lipids exerts prominent influence on DPPC monolayer. Associative interactions were found for some specific composition; however, the system with 30 mol % anionic lipids did respond to produce stable liposome dispersions. Other lipid combinations were unable to form stable monolayer. This was further scrutinized by measuring as reflected through the existence of positive deviation the Gibbs free energy, found relatively less stable monolayer other than the 30 mole% comprising anionic lipids. Binary monolayer formation was also dependent on lipid composition. In case DMPG, it forms more stable mixed monolayer than the other anionic lipids. The lift-off area of DHP and DPPEth is lower than that of other lipids due directly connected with phosphate group. Also the bilayer disintegration kinetics were explored for stable liposomal systems DPPC and anionic lipids in molar ratio (7:3). Disintegration of bilayer to the interracially adsorbed monolayer also depended on lipid composition. In case of DMPG, due to presence of hydroxyl group it form intra or inter molecular hydrogen bonding. Thus DMPG lipid takes more time to disintegrate. However two shed further lights on the structure on the adsorbed monolayer, investigations like the Brewster angle microscopy (BAM) and polarization modulation infrared reflection-adsorption spectroscopy (PMIRRAS) and atomic force microscopy (AFM) studies are important. This would eventually shed lights on the structure of aggregates at the microscopic level as well as the molecular structures in these aggregates. These are considered to be the future perspectives.

BIBILOGRAPHY

References for INTRODUCTION:

1. Liechty, W. B.; Kryscio, D. R.; Slaughter, B. V.; Peppas, N. A., Polymers for Drug Delivery Systems. *Chem. Bio. Eng.* **2010,** *1* (1), 149-173.
2. Nanjwade, B. K.; Bechra, H. M.; Derkar, G. K.; Manvi, F. V.; Nanjwade, V. K., Dendrimers: Emerging polymers for drug-delivery systems. *Eur. J. Pharm. Sci.* **2009,** *38* (3), 185-196.
3. Lasic, D. D., Novel applications of liposomes. *Trend. Biotechn.* **1998,** *16* (7), 307-321.
4. Yanez Arteta, M.; Ainalem, M.-L.; Porcar, L.; Martel, A.; Coker, H.; Lundberg, D.; Chang, D. P.; Soltwedel, O.; Barker, R.; Nylander, T., Interactions of PAMAM Dendrimers with Negatively Charged Model Biomembranes. *J. Phys. Chem. B.* **2014,** *118* (45), 12892-12906.
5. Purohit, G.; Sakthivel, T.; Florence, A. T., Interaction of cationic partial dendrimers with charged and neutral liposomes. *Int. J. Pharm.* **2001,** *214* (1), 71-76.
6. Ruponen, M.; Ylä-Herttuala, S.; Urtti, A., Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. *Biochim. Biophys. Act. Biomembranes* **1999,** *1415* (2), 331-341.
7. Klajnert, B.; Janiszewska, J.; Urbanczyk-Lipkowska, Z.; Bryszewska, M.; Epand, R. M., DSC studies on interactions between low molecular mass peptide dendrimers and model lipid membranes. *Int. J. Pharm.* **2006,** *327* (1), 145-152.
8. Litster, J. D., Stability of lipid bilayers and red blood cell membranes. *Phys. Lett. A.* **1975,** *53* (3), 193-194.
9. Zhang, S.; Marini, D. M.; Hwang, W.; Santoso, S., Design of nanostructured biological materials through self-assembly of peptides and proteins. *Curr. Opin. Chem. Biol.* **2002,** *6* (6), 865-871.
10. Kozubek, A.; Tyman, J. H. P., Resorcinolic Lipids, the Natural Non-isoprenoid Phenolic Amphiphiles and Their Biological Activity. *Chem. Rev.* **1999,** *99* (1), 1-26.
11. Khalil, R. A.; Zarari, A.-h. A., Theoretical estimation of the critical packing parameter of amphiphilic self-assembled aggregates. *Appl. Surf. Sci.* **2014,** *318*, 85-89.
12. Kumar, V. V., Complementary molecular shapes and additivity of the packing parameter of lipids. *Proc. Natl. Acad. Sci.* **1991,** *88* (2), 444-448.

13. Yeagle, P. L., Lipid regulation of cell membrane structure and function. *FASEB J.* **1989,** *3* (7), 1833-1842.
14. Edidin, M., Lipids on the frontier: a century of cell-membrane bilayers. *Nat. Rev. Mole. Cell. Biol.* **2003,** *4*, 414.
15. Pigott, G. M.; Tucker, B. W., Science opens new horizons for marine lipids in human nutrition. *Food. Rev. Int.* **1987,** *3* (1-2), 105-138.
16. Mourente, G.; Bell, J. G., Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (Dicentrarchus labrax L.) over a long term growth study: Effects on muscle and liver fatty acid composition and effectiveness of a fish oil finishing diet. *Comp. Biochem.Phys. Part B: Biochem. Mole. Biol.* **2006,** *145* (3), 389-399.
17. Clarke, R.; Frost, C.; Collins, R.; Appleby, P.; Peto, R., Dietary lipids and blood cholesterol: quantitative meta-analysis of metabolic ward studies. *BMJ* .**1997,** *314* (7074), 112.
18. Fahy, E.; Cotter, D.; Sud, M.; Subramaniam, S., Lipid classification, structures and tools. *Biochim. Biophys. Act.* **2011,** *1811* (11), 637-647.
19. Fauconnot, L.; Hau, J.; Aeschlimann, J.-M.; Fay, L.-B.; Dionisi, F., Quantitative analysis of triacylglycerol regioisomers in fats and oils using reversed-phase high-performance liquid chromatography and atmospheric pressure chemical ionization mass spectrometry. *Rapid. Comm. Mass. Spect.* **2004,** *18* (2), 218-224.
20. Evershed, R. P., Biomolecular archaeology and lipids. *World. Arch.* **1993,** *25* (1), 74-93.
21. Murphy, D. J., The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog. Lipid. Res.* **2001,** *40* (5), 325-438.
22. van Meer, G.; Voelker, D. R.; Feigenson, G. W., Membrane lipids: where they are and how they behave. *Nat. Rev. Mole. Cell. Biol.* **2008,** *9*, 112.
23. Borst, P.; Evers, R.; Kool, M.; Wijnholds, J., The multidrug resistance protein family. *Biochim. Biophys. Act***. 1999,** *1461* (2), 347-357.
24. Yamaguchi, H.; Tanaka, T.; Ichioka, T.; Stoskopf, M.; Kishimoto, Y.; Gould, R., Characterization and comparison of lipids in different squid nervous tissues. *Biochim. Biophys. Act.* **1987,** *922* (1), 78-84.
25. Svennerholm, L.; Vanier, M. T., The distribution of lipids in the human nervous system. II. Lipid composition of human fetal and infant brain. *Brain. Res.* **1972,** *47* (2), 457-468.

26. Dowhan, W., Molecular Basis for Membrane Phospholipid Diversity: Why Are There So Many Lipids? *Ann. Rev. Biochem.* **1997,** *66* (1), 199-232.
27. Horvath, S. E.; Daum, G., Lipids of mitochondria. *Prog. Lipid. Res.* **2013,** *52* (4), 590-614.
28. Kotiadis, V. N.; Duchen, M. R.; Osellame, L. D., Mitochondrial quality control and communications with the nucleus are important in maintaining mitochondrial function and cell health. *Biochim. Biophys. Act***. 2014,** *1840* (4), 1254-1265.
29. Fontagné, S.; Geurden, I.; Escaffre, A.-M.; Bergot, P., Histological changes induced by dietary phospholipids in intestine and liver of common carp (Cyprinus carpio L.) larvae. *Aquaculture.* **1998,** *161* (1), 213-223.
30. Iqbal, J.; Hussain, M. M., Intestinal lipid absorption. *American Journal of Physiology-Endocr. Metabol.* **2009,** *296* (6), E1183-E1194.
31. Mueller, G. C.; Gorski, J.; Aizawa, Y., THE ROLE OF PROTEIN SYNTHESIS IN EARLY ESTROGEN ACTION. *Proceed.Nat. Acad. Sci.* **1961,** *47* (2), 164-169.
32. Farese, R. V.; Barnes, D. E.; Davis, J. S.; Standaert, M. L.; Pollet, R. J., Effects of insulin and protein synthesis inhibitors on phospholipid metabolism, diacylglycerol levels, and pyruvate dehydrogenase activity in BC3H-1 cultured myocytes. *J. Biol. Chem.* **1984,** *259* (11), 7094-7100.
33. Postic, C.; Girard, J., Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J. Clinic. Invest.* **2008,** *118* (3), 829-838.
34. Asai, A.; Miyazawa, T., Dietary Curcuminoids Prevent High-Fat Diet–Induced Lipid Accumulation in Rat Liver and Epididymal Adipose Tissue. *J. Nutr.* **2001,** *131* (11), 2932-2935.
35. Barter, P. J.; Rye, K. A., Molecular mechanisms of reverse cholesterol transport. *Curr. Opin. Lipid.* **1996,** *7* (2), 82-87.
36. Mahley, R. W.; Huang, Y.; Weisgraber, K. H., Putting cholesterol in its place: apoE and reverse cholesterol transport. *J. Clinic. Invest.* **2006,** *116* (5), 1226-1229.
37. Oliva, M. L. V.; Souza-Pinto, J. C.; Batista, I. F. C.; Araujo, M. S.; Silveira, V. F.; Auerswald, E. A.; Mentele, R.; Eckerskorn, C.; Sampaio, M. U.; Sampaio, C. A. M., Leucaena leucocephala serine proteinase inhibitor: primary structure and action on blood

coagulation, kinin release and rat paw edema11Dedicated to Prof. Hans Neurath. *Biochim. Biophys. Act***. 2000,** *1477* (1), 64-74.

38. Poole, J. C. F., FATS AND BLOOD COAGULATION. *British Medical Bulletin* **1958,** *14* (3), 253-257.
39. Hirata, F.; Axelrod, J., Phospholipid methylation and biological signal transmission. *Science.* **1980,** *209* (4461), 1082-1090.
40. G R Crabtree, a.; Clipstone, N. A., Signal Transmission Between the Plasma Membrane and Nucleus of T Lymphocytes. *Ann. Rev. Biochem.* **1994,** *63* (1), 1045-1083.
41. Hills, B. A., Analysis of eustachian surfactant and its function as a release agent. *Arch.Otolaryng.* **1984,** *110* (1), 3-9.
42. Cooper, D. G.; Zajic, J. E., Surface-Active Compounds from Microorganisms. *Adv. Appl. Microbiol.* 1980; 26, 229-253.
43. Winkenbach, F.; Wolk, C. P.; Jost, M., Lipids of membranes and of the cell envelope in heterocysts of a blue-green alga. *Planta.* **1972,** *107* (1), 69-80.
44. Sakamoto, J.; Furukawa, K.; Cordon-Cardo, C.; Yin, B. W. T.; Rettig, W. J.; Oettgen, H. F.; Old, L. J.; Lloyd, K. O., Expression of Lewis^a, Lewis^b, X, and Y Blood Group Antigens in Human Colonic Tumors and Normal Tissue and in Human Tumor-derived Cell Lines. *Canc. Res.* **1986,** *46* (3), 1553-1561.
45. Clausen, H.; Hakomori, S., ABH and Related Histo-Blood Group Antigens; Immunochemical Differences in Carrier Isotypes and Their Distribution. *Vox. Sanguinis.* **1989,** *56*, 1-20.
46. Borch, J.; Torta, F.; Sligar, S. G.; Roepstorff, P., Nanodiscs for Immobilization of Lipid Bilayers and Membrane Receptors: Kinetic Analysis of Cholera Toxin Binding to a Glycolipid Receptor. *Analyt. Chem.* **2008,** *80* (16), 6245-6252.
47. Chou, D. K.; Ilyas, A. A.; Evans, J. E.; Costello, C.; Quarles, R. H.; Jungalwala, F. B., Structure of sulfated glucuronyl glycolipids in the nervous system reacting with HNK-1 antibody and some IgM paraproteins in neuropathy. *J. Biol. Chem.* **1986,** *261* (25), 11717-11725.
48. Stein, O.; Sanger, L.; Stein, Y., colchicine-induced inhibition of lipoprotein and protein secretion into the serum and lack of interference with secretion of biliary phospholipids and cholesterol by rat liver in vivo. *J. Cell. Biol.* **1974,** *62* (1), 90-103.

49. Olson, R. E., Discovery of the Lipoproteins, Their Role in Fat Transport and Their Significance as Risk Factors1. *J. Nutr.* **1998,** *128* (2), 439S-443S.
50. Shepherd , J.; Cobbe , S. M.; Ford , I.; Isles , C. G.; Lorimer , A. R.; Macfarlane , P. W.; McKillop , J. H.; Packard , C. J., Prevention of Coronary Heart Disease with Pravastatin in Men with Hypercholesterolemia. *New. Eng. J. Med.* **1995,** *333* (20), 1301-1308.
51. Panagiotakos, D. B.; Pitsavos, C.; Arvaniti, F.; Stefanadis, C., Adherence to the Mediterranean food pattern predicts the prevalence of hypertension, hypercholesterolemia, diabetes and obesity, among healthy adults; the accuracy of the MedDietScore. *Prevent. Med.* **2007,** *44* (4), 335-340.
52. Brown, D. A.; London, E., Structure and Function of Sphingolipid- and Cholesterol-rich Membrane Rafts. *J. Biol. Chem.* **2000,** *275* (23), 17221-17224.
53. Ikonen, E., Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mole. Cell. Biol.* **2008,** *9*, 125.
54. and, J. J. R.; Mangelsdorf, D. J., The Role of Orphan Nuclear Receptors in the Regulation of Cholesterol Homeostasis. *Ann.Rev. Cell. Develop. Biol.* **2000,** *16* (1), 459-481.
55. Miettinen, H. E.; Rayburn, H.; Krieger, M., Abnormal lipoprotein metabolism and reversible female infertility in HDL receptor (SR-BI)–deficient mice. *J. Clinic. Invest.* **2001,** *108* (11), 1717-1722.
56. Gordon, D. J.; Probstfield, J. L.; Garrison, R. J.; Neaton, J. D.; Castelli, W. P.; Knoke, J. D.; Jacobs, D. R.; Bangdiwala, S.; Tyroler, H. A., High-density lipoprotein cholesterol and cardiovascular disease. *Circulation.* **1989,** *79* (1), 8-15.
57. de Lorgeril, M.; Salen, P.; Abramson, J.; et al., Cholesterol lowering, cardiovascular diseases, and the rosuvastatin-jupiter controversy: A critical reappraisal. *Arch. Int. Med.* **2010,** *170* (12), 1032-1036.
58. DeLuca, H. F., Overview of general physiologic features and functions of vitamin D. *The American Journal of Clinical Nutrition* **2004,** *80* (6), 1689S-1696S.
59. Hughes, M.; Brumbaugh, P.; Hussler, M.; Wergedal, J.; Baylink, D., Regulation of serum 1alpha,25-dihydroxyvitamin D3 by calcium and phosphate in the rat. *Science.* **1975,** *190* (4214), 578-580.
60. Lucy, J. A.; Glauert, A. M., Structure and assembly of macromolecular lipid complexes composed of globular micelles. *J. Mole. Biol.* **1964,** *8* (5), 727-IN22.

61. Delie, F.; Rubas, W., A Human Colonic Cell Line Sharing Similarities With Enterocytes as a Model to Examine Oral Absorption: Adv. Limit. the Caco-2 Model. **1997,** *14* (3), 66.
62. Carey, M. C.; Small, D. M., Micelle formation by bile salts: Physical-chemical and thermodynamic considerations. *Arch.Int. Med.* **1972,** *130* (4), 506-527.
63. Carey, M. C.; Small, D. M., The characteristics of mixed micellar solutions with particular reference to bile. *American. J. Med. 49* (5), 590-608.
64. Baoukina, S.; Monticelli, L.; Risselada, H. J.; Marrink, S. J.; Tieleman, D. P., The molecular mechanism of lipid monolayer collapse. *Proceed. Nat. Acad. Sci.* **2008,** *105* (31), 10803-10808.
65. Daniels, C. B.; Orgeig, S., Pulmonary Surfactant: The Key to the Evolution of Air Breathing. *Physiology.* **2003,** *18* (4), 151-157.
66. Bagatolli, L. A.; Ipsen, J. H.; Simonsen, A. C.; Mouritsen, O. G., An outlook on organization of lipids in membranes: Searching for a realistic connection with the organization of biological membranes. *Prog. Lipid. Res.* **2010,** *49* (4), 378-389.
67. White, S. H.; Wimley, W. C., Hydrophobic interactions of peptides with membrane interfaces. *Biochim. Biophys. Act***. 1998,** *1376* (3), 339-352.
68. Dathe, M.; Schümann, M.; Wieprecht, T.; Winkler, A.; Beyermann, M.; Krause, E.; Matsuzaki, K.; Murase, O.; Bienert, M., Peptide Helicity and Membrane Surface Charge Modulate the Balance of Electrostatic and Hydrophobic Interactions with Lipid Bilayers and Biological Membranes. *Biochemistry.* **1996,** *35* (38), 12612-12622.
69. van Rooijen, B. D.; Claessens, M. M. A. E.; Subramaniam, V., Lipid bilayer disruption by oligomeric α-synuclein depends on bilayer charge and accessibility of the hydrophobic core. *Biochim. Biophys. Act***. 2009,** *1788* (6), 1271-1278.
70. O'Brien, J. S., Cell membranes—Composition: Structure: Function. *Journal of Theoretical Biology* **1967,** *15* (3), 307-324.
71. Seelig, J.; MacDonald, P. M.; Scherer, P. G., Phospholipid head groups as sensors of electric charge in membranes. *Biochemistry* **1987,** *26* (24), 7535-7541.
72. Moll, F.; Cross, T. A., Optimizing and characterizing alignment of oriented lipid bilayers containing gramicidin D. *Biophys. J.* **1990,** *57* (2), 351-362.
73. Perez-Camacho, M. I.; Ruiz-Suarez, J. C., Propagation of a thermo-mechanical perturbation on a lipid membrane. *Soft. Matter.* **2017,** *13* (37), 6555-6561.

74. Larsson, K., Aqueous dispersions of cubic lipid–water phases. *Curr. Opin. Colloid. Int. Sci.* **2000,** *5* (1), 64-69.
75. Zhang, J. A.; Anyarambhatla, G.; Ma, L.; Ugwu, S.; Xuan, T.; Sardone, T.; Ahmad, I., Development and characterization of a novel Cremophor® EL free liposome-based paclitaxel (LEP-ETU) formulation. *Eur. J. Pharm. Biopharm.* **2005,** *59* (1), 177-187.
76. Mohammed, A. R.; Weston, N.; Coombes, A. G. A.; Fitzgerald, M.; Perrie, Y., Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. *Int. J. Pharm.* **2004,** *285* (1), 23-34.
77. Gulati, M.; Grover, M.; Singh, S.; Singh, M., Lipophilic drug derivatives in liposomes. *Int. J. Pharm.* **1998,** *165* (2), 129-168.
78. Han, M.; Watarai, S.; Kobayashi, K.; Yasuda, T., Application of Liposomes for Development of Oral Vaccines: Study of In Vitro Stability of Liposomes and Antibody Response to Antigen Associated with Liposomes after Oral Immunization. *J. Veterinary. Med. Sci.* **1997,** *59* (12), 1109-1114.
79. Gregoriadis, G., Liposomes for drugs and vaccines. *Trend. Biotech.* **1985,** *3* (9), 235-241.
80. Gregoriadis, G., [17] Enzyme entrapment in liposomes. *Method. Enzym.* 1976; Vol. 44, pp 218-227.
81. Torchilin, V. P., Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug. Discov.* **2005,** *4*, 145.
82. Vemuri, S.; Rhodes, C. T., Preparation and characterization of liposomes as therapeutic delivery systems: a review. *Pharm. Act. Helvet.* **1995,** *70* (2), 95-111.
83. Pettit, D. K.; Gombotz, W. R., The development of site-specific drug-delivery systems for protein and peptide biopharmaceuticals. *Trend. Biotech.* **1998,** *16* (8), 343-349.
84. Mitra, A.; Dey, B., Chitosan Microspheres in Novel Drug Delivery Systems. *Ind. J. Pharm. Sci.* **2011,** *73* (4), 355-366.
85. Bharti, C.; Nagaich, U.; Pal, A. K.; Gulati, N., Mesoporous silica nanoparticles in target drug delivery system. *Int. J. Pharm. Invest.* **2015,** *5* (3), 124-133.
86. Batzri, S.; Korn, E. D., Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Act.* **1973,** *298* (4), 1015-1019.

87. Szoka, F.; Papahadjopoulos, D., Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proceed. Nat. Academ. Sci.* **1978,** *75* (9), 4194-4198.
88. F Szoka, J., and; Papahadjopoulos, D., Comparative Properties and Methods of Preparation of Lipid Vesicles (Liposomes). *Ann. Rev.Biophys.Bioeng.* **1980,** *9* (1), 467-508.
89. Varona, S.; Martín, Á.; Cocero, M. J., Liposomal Incorporation of Lavandin Essential Oil by a Thin-Film Hydration Method and by Particles from Gas-Saturated Solutions. *Ind. Eng. Chem. Res.* **2011,** *50* (4), 2088-2097.
90. Deamer, D.; Bangham, A. D., Large volume liposomes by an ether vaporization method. *Biochim. Biophys. Act.* **1976,** *443* (3), 629-634.
91. Kremer, J. M. H.; Van der Esker, M. W.; Pathmamanoharan, C.; Wiersema, P. H., Vesicles of variable diameter prepared by a modified injection method. *Biochemistry.* **1977,** *16* (17), 3932-3935.
92. Maitani, Y.; Igarashi, S.; Sato, M.; Hattori, Y., Cationic liposome (DC-Chol/DOPE=1:2) and a modified ethanol injection method to prepare liposomes, increased gene expression. *Int. J. Pharm.* **2007,** *342* (1), 33-39.
93. Pons, M.; Foradada, M.; Estelrich, J., Liposomes obtained by the ethanol injection method. *Int. J. Pharm.* **1993,** *95* (1), 51-56.
94. Szoka, F.; Olson, F.; Heath, T.; Vail, W.; Mayhew, E.; Papahadjopoulos, D., Preparation of unilamellar liposomes of intermediate size (0.1–0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. *Biochim. Biophys. Act***. 1980,** *601*, 559-571.
95. Nagata, T.; Okada, K.; Takebe, I.; Matsui, C., Delivery of tobacco mosaic virus RNA into plant protoplasts mediated by reverse-phase evaporation vesicles (Liposomes). *Molecul. Gen. Genet.* **1981,** *184* (2), 161-165.
96. Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S. W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K., Liposome: classification, preparation, and applications. *Nanoscale. Res. Lett.* **2013,** *8* (1), 102.
97. Budai, M.; Szógyi, M., [Liposomes as drug carrier systems. Preparation, classification and therapeutic advantages of liposomes]. *Act. Pharm. Hung.* **2001,** *71* (1), 114-118.

98. Liu, J.; Jiang, X.; Ashley, C.; Brinker, C. J., Electrostatically Mediated Liposome Fusion and Lipid Exchange with a Nanoparticle-Supported Bilayer for Control of Surface Charge, Drug Containment, and Delivery. *J. Am. Chem. Soc.* **2009,** *131* (22), 7567-7569.
99. Abbasi, E.; Aval, S. F.; Akbarzadeh, A.; Milani, M.; Nasrabadi, H. T.; Joo, S. W.; Hanifehpour, Y.; Nejati-Koshki, K.; Pashaei-Asl, R., Dendrimers: synthesis, applications, and properties. *Nanoscale. Res. Lett.* **2014,** *9* (1), 247-247.
100. Maiti, P. K.; Çağın, T.; Wang, G.; Goddard, W. A., Structure of PAMAM Dendrimers: Generations 1 through 11. *Macromolecules.* **2004,** *37* (16), 6236-6254.
101. Tomalia, D. A., Birth of a new macromolecular architecture: dendrimers as quantized building blocks for nanoscale synthetic polymer chemistry. *Prog. Polym. Sci.* **2005,** *30* (3), 294-324.
102. Wolinsky, J. B.; Grinstaff, M. W., Therapeutic and diagnostic applications of dendrimers for cancer treatment. *Adv. Drug. Deliv.Rev.* **2008,** *60* (9), 1037-1055.
103. Luo, D.; Saltzman, W. M., Synthetic DNA delivery systems. *Nature. Biotech.* **2000,** *18*, 33.
104. Wiesler, U. M.; Berresheim, A. J.; Morgenroth, F.; Lieser, G.; Müllen, K., Divergent Synthesis of Polyphenylene Dendrimers: The Role of Core and Branching Reagents upon Size and Shape. *Macromolecules.* **2001,** *34* (2), 187-199.
105. Urbani, C. N.; Bell, C. A.; Whittaker, M. R.; Monteiro, M. J., Convergent Synthesis of Second Generation AB-Type Miktoarm Dendrimers Using "Click" Chemistry Catalyzed by Copper Wire. *Macromolecules.* **2008,** *41* (4), 1057-1060.
106. Esfand, R.; Tomalia, D. A., Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. *Drug. Discov. Today.* **2001,** *6* (8), 427-436.
107. Tomalia, D. A.; Reyna, L. A.; Svenson, S., Dendrimers as multi-purpose nanodevices for oncology drug delivery and diagnostic imaging. *Biochem.Soci. Transaction.* **2007,** *35* (1), 61-67.
108. Gillies, E. R.; Fréchet, J. M. J., Dendrimers and dendritic polymers in drug delivery. *Drug. Discov. Today.* **2005,** *10* (1), 35-43.
109. Liu, M.; Fréchet, J. M. J., Designing dendrimers for drug delivery. *Pharm. Sci. Tech. Today.* **1999,** *2* (10), 393-401.
110. Dufès, C.; Uchegbu, I. F.; Schätzlein, A. G., Dendrimers in gene delivery. *Advanced Drug. Deliv. Rev.* **2005,** *57* (15), 2177-2202.

111. Dutta, T.; Jain, N. K.; McMillan, N. A. J.; Parekh, H. S., RETRACTED: Dendrimer nanocarriers as versatile vectors in gene delivery. *Nanotech. Biol. Med.* **2010,** *6* (1), 25-34.
112. Yang, J.; Zhang, Q.; Chang, H.; Cheng, Y., Surface-Engineered Dendrimers in Gene Delivery. *Chem. Rev.* **2015,** *115* (11), 5274-5300.
113. Margerum, L. D.; Campion, B. K.; Koo, M.; Shargill, N.; Lai, J.-J.; Marumoto, A.; Christian Sontum, P., Gadolinium(III) DO3A macrocycles and polyethylene glycol coupled to dendrimers Effect of molecular weight on physical and biological properties of macromolecular magnetic resonance imaging contrast agents. *J. Alloy. Comp.* **1997,** *249* (1), 185-190.
114. Ng, L. G.; Hsu, A.; Mandell, M. A.; Roediger, B.; Hoeller, C.; Mrass, P.; Iparraguirre, A.; Cavanagh, L. L.; Triccas, J. A.; Beverley, S. M.; Scott, P.; Weninger, W., Migratory Dermal Dendritic Cells Act as Rapid Sensors of Protozoan Parasites. *PLOS Patho.* **2008,** *4* (11), e1000222.
115. Xu, M.-H.; Lin, J.; Hu, Q.-S.; Pu, L., Fluorescent Sensors for the Enantioselective Recognition of Mandelic Acid: Signal Amplification by Dendritic Branching. *J. Am. Chem. Soci.* **2002,** *124* (47), 14239-14246.
116. Yiyun, C.; Tongwen, X.; Rongqiang, F., Polyamidoamine dendrimers used as solubility enhancers of ketoprofen. *Eur. J. Med. Chem.* **2005,** *40* (12), 1390-1393.
117. Milhem, O. M.; Myles, C.; McKeown, N. B.; Attwood, D.; D'Emanuele, A., Polyamidoamine Starburst® dendrimers as solubility enhancers. *Int. J. Pharm.* **2000,** *197* (1), 239-241.
118. Morgan, M. T.; Nakanishi, Y.; Kroll, D. J.; Griset, A. P.; Carnahan, M. A.; Wathier, M.; Oberlies, N. H.; Manikumar, G.; Wani, M. C.; Grinstaff, M. W., Dendrimer-Encapsulated Camptothecins: Increased Solubility, Cellular Uptake, and Cellular Retention Affords Enhanced Anticancer Activity In vitro. *Cancer. Res.* **2006,** *66* (24), 11913-11921.
119. Nishiyama, N.; Stapert, H. R.; Zhang, G.-D.; Takasu, D.; Jiang, D.-L.; Nagano, T.; Aida, T.; Kataoka, K., Light-Harvesting Ionic Dendrimer Porphyrins as New Photosensitizers for Photodynamic Therapy. *Biocon. Chem.* **2003,** *14* (1), 58-66.

120. Battah, S.; Balaratnam, S.; Casas, A.; O'Neill, S.; Edwards, C.; Batlle, A.; Dobbin, P.; MacRobert, A. J., Macromolecular delivery of 5-aminolaevulinic acid for photodynamic therapy using dendrimer conjugates. *Mol. Cancer. Ther.* **2007,** *6* (3), 876-885.
121. Cho, Y. H.; Kim, S.; Bae, E. K.; Mok, C. K.; Park, J., Formulation of a cosurfactant-free O/W microemulsion using nonionic surfactant mixtures. *J. Food. Sci.* **2008,** *73* (3), 1750-3841.
122. Ahmed, E. M., Hydrogel: Preparation, characterization, and applications: A review. *J. Adv. Res.* **2015,** *6* (2), 105-121.
123. Bowman, K.; Leong, K. W., Chitosan nanoparticles for oral drug and gene delivery. *Int. J. Nanomed.* **2006,** *1* (2), 117-128.
124. Shaji, J.; Patole, V., Protein and Peptide Drug Delivery: Oral Approaches. *Ind. J. Pharm. Sci.* **2008,** *70* (3), 269-277.
125. Karmakar, G.; Nahak, P.; Guha, P.; Roy, B.; Chettri, P.; Sapkota, M.; Koirala, S.; Misono, T.; Torigoe, K.; Ghosh, S.; Panda, A. K., Effects of Fatty Acids on the Interfacial and Solution Behavior of Mixed Lipidic Aggregates Called Solid Lipid Nanoparticles. *J. Oleo. Sci.* **2016,** *65* (5), 419-30.
126. Pencer, J.; White, G. F.; Hallett, F. R., Osmotically induced shape changes of large unilamellar vesicles measured by dynamic light scattering. *Biophys. J.* **2001,** *81* (5), 2716-2728.
127. Layne, E., [73] Spectrophotometric and turbidimetric methods for measuring proteins. *Method. Enzym.* 1957; Vol. 3, pp 447-454.
128. Mondain-Monval, O., Freeze fracture TEM investigations in liquid crystals. *Curr. Opin. Colloid. Inter. Sci.* **2005,** *10* (5), 250-255.
129. Ruozi, B.; Tosi, G.; Forni, F.; Fresta, M.; Vandelli, M. A., Atomic force microscopy and photon correlation spectroscopy: Two techniques for rapid characterization of liposomes. *Eur. J. Pharm. Sci.* **2005,** *25* (1), 81-89.
130. Egawa, H.; Furusawa, K., Liposome Adhesion on Mica Surface Studied by Atomic Force Microscopy. *Langmuir.* **1999,** *15* (5), 1660-1666.
131. Jass, J.; Tjärnhage, T.; Puu, G., From Liposomes to Supported, Planar Bilayer Structures on Hydrophilic and Hydrophobic Surfaces: An Atomic Force Microscopy Study. *Biophys. J.* **2000,** *79* (6), 3153-3163.

132. Kawato, S.; Kinosita, K.; Ikegami, A., Dynamic structure of lipid bilayers studied by nanosecond fluorescence techniques. *Biochemistry.* **1977,** *16* (11), 2319-2324.
133. Guo, J.; Ping, Q.; Jiang, G.; Huang, L.; Tong, Y., Chitosan-coated liposomes: characterization and interaction with leuprolide. *Int. J. Pharm.* **2003,** *260* (2), 167-173.
134. Haran, G.; Cohen, R.; Bar, L. K.; Barenholz, Y., Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. *Biochim.Biophys. Act.* **1993,** *1151* (2), 201-215.
135. Mourtas, S.; Fotopoulou, S.; Duraj, S.; Sfika, V.; Tsakiroglou, C.; Antimisiaris, S. G., Liposomal drugs dispersed in hydrogels: Effect of liposome, drug and gel properties on drug release kinetics. *Colloid. Surf. B.* **2007,** *55* (2), 212-221.
136. Haidar, Z. S.; Hamdy, R. C.; Tabrizian, M., Protein release kinetics for core–shell hybrid nanoparticles based on the layer-by-layer assembly of alginate and chitosan on liposomes. *Biomaterials.* **2008,** *29* (9), 1207-1215.
137. Henriksen, I.; Sande, S. A.; Smistad, G.; Ågren, T.; Karlsen, J., In vitro evaluation of drug release kinetics from liposomes by fractional dialysis. *Int. J. Pharm.* **1995,** *119* (2), 231-238.
138. Bhattarai, R.; Sutradhar, T.; Roy, B.; Guha, P.; Chettri, P.; Mandal, A. K.; Bykov, A. G.; Akentiev, A. V.; Noskov, B. A.; Panda, A. K., Double-Tailed Cystine Derivatives as Novel Substitutes of Phospholipids with Special Reference to Liposomes. *J. Phys. Chem. B.* **2016,** *120* (41), 10744-10756.
139. Baoukina, S.; Monticelli, L.; Marrink, S. J.; Tieleman, D. P., Pressure−Area Isotherm of a Lipid Monolayer from Molecular Dynamics Simulations. *Langmuir.* **2007,** *23* (25), 12617-12623.
140. Heath, J. R.; Knobler, C. M.; Leff, D. V., Pressure/Temperature Phase Diagrams and Superlattices of Organically Functionalized Metal Nanocrystal Monolayers: The Influence of Particle Size, Size Distribution, and Surface Passivant. *J. Phys. Chem. B.* **1997,** *101* (2), 189-197.

References for CHAPTER I

1. Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S. W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K., Liposome: classification, preparation, and applications. *Nanoscale. Res. Lett.* **2013,** *8* (1), 102.
2. Pignatello, R.; Musumeci, T.; Basile, L.; Carbone, C.; Puglisi, G., Biomembrane models and drug-biomembrane interaction studies: Involvement in drug design and development. *J. Pharm. Bioallied. Scie.* **2011,** *3* (1), 4.
3. Sabın, J.; Prieto, G.; Ruso, J. M.; Hidalgo-Álvarez, R.; Sarmiento, F., Size and stability of liposomes: A possible role of hydration and osmotic forces. *Eur. Phys. J. E* **2006,** *20* (4), 401-408.
4. Sawant, R. R.; Torchilin, V. P., Liposomes as 'smart'pharmaceutical nanocarriers. *Soft Matter* **2010,** *6* (17), 4026-4044.
5. Al-Jamal, W. T.; Kostarelos, K., Liposome-nanoparticle hybrids for multimodal diagnostic and therapeutic applications. *Nanomedicine.* **2007,** *2* (1), 85-98.
6. Tseng, L.; Liang, H.; Chung, T.; Huang, Y.; Liu, D., Liposomes incorporated with cholesterol for drug release triggered by magnetic field. *J. Med. Bio. Eng.* **2007,** *27* (1), 29.
7. Allen, T. M.; Cullis, P. R., Liposomal drug delivery systems: From concept to clinical applications. *Adv. Drug. Deliv. Rev.* **2013,** *65* (1), 36-48.
8. Sułkowski, W. W.; Pentak, D.; Nowak, K.; Sułkowska, A., The influence of temperature, cholesterol content and pH on liposome stability. *J. Mol. Str.* **2005,** *744–747* (0), 737-747.
9. Lakowicz, J. R., *Principles of fluorescence spectroscopy*. Springer Science & Business Media: 2007.
10. Ulrich, A. S., Biophysical aspects of using liposomes as delivery vehicles. *Bioscience. Reports.* **2002,** *22* (2), 129-150.
11. Bensikaddour, H.; Snoussi, K.; Lins, L.; Van Bambeke, F.; Tulkens, P. M.; Brasseur, R.; Goormaghtigh, E.; Mingeot-Leclercq, M.-P., Interactions of ciprofloxacin with DPPC and DPPG: Fluorescence anisotropy, ATR-FTIR and 31P NMR spectroscopies and conformational analysis. *Biochim. Biophys. Act.* **2008,** *1778* (11), 2535-2543.
12. Garidel, P.; Johann, C.; Mennicke, L.; Blume, A., The mixing behavior of pseudobinary phosphatidylcholine-phosphatidylglycerol mixtures as a function of pH and chain length. *Eur. Biophys. J.* **1997,** *26* (6), 447-459.

13. Zhang, J.-a. A.; Pawelchak, J., Effect of pH, ionic strength and oxygen burden on the chemical stability of EPC/cholesterol liposomes under accelerated conditions. Part 1: Lipid hydrolysis. *Eur. J. Pharmaceut. Biopharm.* **2000,** *50* (3), 357-364.
14. Began, G.; Sudharshan, E.; Udaya Sankar, K.; Appu Rao, A., Interaction of curcumin with phosphatidylcholine: a spectrofluorometric study. *J. Agri.Food. Chem.* **1999,** *47* (12), 4992-4997.
15. Niu, Y.; Wang, X.; Chai, S.; Chen, Z.; An, X.; Shen, W., Effects of curcumin concentration and temperature on the spectroscopic properties of liposomal curcumin. *J. Agri.Food. Chem.* **2012,** *60* (7), 1865-1870.
16. Selvam, C.; Jachak, S. M.; Thilagavathi, R.; Chakraborti, A. K., Design, synthesis, biological evaluation and molecular docking of curcumin analogues as antioxidant, cyclooxygenase inhibitory and anti-inflammatory agents. *Bioorg.Med. Chem. Lett.* **2005,** *15* (7), 1793-1797.
17. Priyadarsini, K. I.; Maity, D. K.; Naik, G.; Kumar, M. S.; Unnikrishnan, M.; Satav, J.; Mohan, H., Role of phenolic OH and methylene hydrogen on the free radical reactions and antioxidant activity of curcumin. *Free. Rad. Bio.Med.* **2003,** *35* (5), 475-484.
18. Basnet, P.; Skalko-Basnet, N., Curcumin: an anti-inflammatory molecule from a curry spice on the path to cancer treatment. *Molecules.* **2011,** *16* (6), 4567-4598.
19. Chattopadhyay, I.; Biswas, K.; Bandyopadhyay, U.; Banerjee, R. K., Turmeric and curcumin: Biological actions and medicinal applications. *Curr. Sci.* **2004,** *87* (1), 44-53.
20. Canamares, M.; Garcia-Ramos, J.; Sanchez-Cortes, S., Degradation of Curcumin Dye in Aqueous Solution and on Ag Nanoparticles Studied by Ultraviolet–Visible Absorption and Surface-Enhanced Raman Spectroscopy. *Appl. Spect.* **2006,** *60* (12), 1386-1391.
21. Tønnesen, H. H.; Másson, M.; Loftsson, T., Studies of curcumin and curcuminoids. XXVII. Cyclodextrin complexation: solubility, chemical and photochemical stability. *Int. J. Pharm.* **2002,** *244* (1), 127-135.
22. Tapal, A.; Tiku, P. K., Complexation of curcumin with soy protein isolate and its implications on solubility and stability of curcumin. *Food. Chem.* **2012,** *130* (4), 960-965.
23. Basniwal, R. K.; Buttar, H. S.; Jain, V.; Jain, N., Curcumin nanoparticles: preparation, characterization, and antimicrobial study. *J. Agri. Food Chem.* **2011,** *59* (5), 2056-2061.

24. Kunwar, A.; Barik, A.; Pandey, R.; Priyadarsini, K. I., Transport of liposomal and albumin loaded curcumin to living cells: an absorption and fluorescence spectroscopic study. *Biochim. Biophys. Acta*. **2006,** *10* (20), 8.
25. Takahashi, M.; Uechi, S.; Takara, K.; Asikin, Y.; Wada, K., Evaluation of an oral carrier system in rats: bioavailability and antioxidant properties of liposome-encapsulated curcumin. *J. Agr.Food. Chem.* **2009,** *57* (19), 9141-9146.
26. Kurzrock, R.; Li, L. In *Liposome-encapsulated curcumin: in vitro and in vivo effects on proliferation, apoptosis, signaling, and angiogenesis*. Ann. Meet. Proc. 2005; p 4091.
27. Pandelidou, M.; Dimas, K.; Georgopoulos, A.; Hatziantoniou, S.; Demetzos, C., Preparation and characterization of lyophilised EGG PC liposomes incorporating curcumin and evaluation of its activity against colorectal cancer cell lines. *J. Nanosci. Nanotechnol.* **2011,** *11* (Copyright (C) 2015 Ame. Chem. Soci. 1259-1266.
28. Niu, Y.; Ke, D.; Yang, Q.; Wang, X.; Chen, Z.; An, X.; Shen, W., Temperature-dependent stability and DPPH scavenging activity of liposomal curcumin at pH 7.0. *Food. Chem.* **2012,** *135*, 1377-1382.
29. Zhu, M.; Li, J.; Fink, A. L., The association of α-synuclein with membranes affects bilayer structure, stability, and fibril formation. *J. Biol. Chem.* **2003,** *278* (41), 40186-40197.
30. Pencer, J.; White, G. F.; Hallett, F. R., Osmotically induced shape changes of large unilamellar vesicles measured by dynamic light scattering. *Biophys. J.* **2001,** *81* (5), 2716-2728.
31. Ruozi, B.; Belletti, D.; Tombesi, A.; Tosi, G.; Bondioli, L.; Forni, F.; Vandelli, M. A., AFM, ESEM, TEM, and CLSM in liposomal characterization: a comparative study. *Int. J. Nanomed.* **2011,** *6*, 557.
32. Demetzos, C., Differential scanning calorimetry (DSC): a tool to study the thermal behavior of lipid bilayers and liposomal stability. *J. Liposome. Res.* **2008,** *18* (3), 159-173.
33. Chakraborty, M.; Panda, A. K., Spectral behaviour of eosin Y in different solvents and aqueous surfactant media. *Spectrochim. Act.* **2011,** *81* (1), 458-465.
34. Lakowicz, J. R., *Principles of fluorescence spectroscopy*. Sci. Bus. Med. 2013.
35. Dhule, S. S.; Penfornis, P.; Frazier, T.; Walker, R.; Feldman, J.; Tan, G.; He, J.; Alb, A.; John, V.; Pochampally, R., Curcumin-loaded gamma-cyclodextrin liposomal nanoparticles as delivery vehicles for osteosarcoma. *Nanomedicine.* **2012,** *8* (4), 440-51.

36. Chen, Y.; Wu, Q.; Zhang, Z.; Yuan, L.; Liu, X.; Zhou, L., Preparation of Curcumin-Loaded Liposomes and Evaluation of Their Skin Permeation and Pharmacodynamics. *Molecules.* **2012,** *17* (5), 5972-5987.
37. Hua, S., Comparison of in vitro dialysis release methods of loperamide-encapsulated liposomal gel for topical drug delivery. *Int. J. Nanomed.* **2014,** *9*, 735.
38. Hu, G.; Xu, Q.; Liu, B.; Zhang, Z.; Chen, B.; Xu, Q.; Huang, W.; Zhang, H.; Huang, S., [Synthesis and antibacterial activity of 2-(3-pyridyl)-5-([(5-aryl-1, 3, 4-oxadiazol-2-yl) methylene] thio)-1, 3, 4-oxadiazoles]. *Act. Pharmaceut. Sinica.* **2004,** *39* (4), 263-265.
39. Lakowicz, J. R.; Masters, B. R., Principles of fluorescence spectroscopy. *J. Biomed. Optic.* **2008,** *13* (2), 9901.
40. Andersson, M.; Hammarstroem, L.; Edwards, K., Effect of bilayer phase transitions on vesicle structure, and its influence on the kinetics of viologen reduction. *J. Phys. Chem.* **1995,** *99* (39), 14531-14538.
41. Moulik, S. P.; De, G. C.; Bhowmik, B. B.; Panda, A. K., Physicochemical studies on microemulsions. 6. Phase behavior, dynamics of percolation, and energetics of droplet clustering in water/AOT/n-heptane system influenced by additives (sodium cholate and sodium salicylate). *J. Phys. Chem. B.* **1999,** *103* (34), 7122-7129.
42. Chattopadhyay, S.; Ehrman, S. H.; Bellare, J.; Venkataraman, C., Morphology and bilayer integrity of small liposomes during aerosol generation by air-jet nebulisation. *J. Nanoparticle. Res.* **2012,** *14* (4), 1-15.
43. Taylor, T. M.; Gaysinsky, S.; Davidson, P. M.; Bruce, B. D.; Weiss, J., Characterization of antimicrobial-bearing liposomes by ζ-potential, vesicle size, and encapsulation efficiency. *Food. Biophys.* **2007,** *2* (1), 1-9.
44. Chakraborty, M.; Bardhan, S.; Saha, S. K.; Panda, A. K., Effect of colloidal silica on the spectral behaviour of 7-hydroxycoumarin in aqueous medium. *Molecul.Biomolecul. Spect.* **2012,** *97*, 722-727.
45. Dutta, A. K.; Ikiki, E., Novel drug delivery systems to improve bioavailability of curcumin. *J. Bioequivalence. Bioavailability.* **2014,** *6* (Copyright (C) 2015 American Chemical Society (ACS). All Rights Reserved.), 172/1-172/9, 9.

46. Halling, K. K.; Slotte, J. P., Membrane properties of plant sterols in phospholipid bilayers as determined by differential scanning calorimetry, resonance energy transfer and detergent-induced solubilization. *Biochim. Biophys. Act.* **2004,** *30* (2), 161-71.
47. Furuike, S.; Levadny, V. G.; Li, S. J.; Yamazaki, M., Low pH induces an interdigitated gel to bilayer gel phase transition in dihexadecylphosphatidylcholine membrane. *Biophys. J.* **1999,** *77* (4), 2015-2023.
48. Koynova, R.; Caffrey, M., Phases and phase transitions of the phosphatidylcholines. *Biochim. Biophys. Act.* **1998,** *1376* (1), 91-145.
49. de Sousa, I. S.; Castanheira, E. M.; Rocha Gomes, J. I.; Real Oliveira, M. E. C., Study of the release of a microencapsulated acid dye in polyamide dyeing using mixed cationic liposomes. *J. Lipo. Res.* **2011,** *21* (2), 151-157.
50. de Oliveira, M. C.; Rosilio, V.; Lesieur, P.; Bourgaux, C.; Couvreur, P.; Ollivon, M.; Dubernet, C., pH-sensitive liposomes as a carrier for oligonucleotides: a physico-chemical study of the interaction between DOPE and a 15-mer oligonucleotide in excess water. *Biophys.Chem.* **2000,** *87* (2), 127-137.
51. Lopes, S. C. A.; Novais, M. V. M.; Ferreira, D. S.; Braga, F. C.; Magalhães-Paniago, R.; Malachias, A.; Oliveira, M. C., Ursolic Acid Incorporation Does Not Prevent the Formation of a Nonlamellar Phase in pH-Sensitive and Long-Circulating Liposomes. *Langmuir*. **2014,** *30*, 15083-15090.
52. Rankin, M. A.; Wagner, B. D., Fluorescence enhancement of curcumin upon inclusion into cucurbituril. *Supramol. Chem.* **2004,** *16* (7), 513-519.
53. Priyadarsini, K. I., Photophysics, photochemistry and photobiology of curcumin: Studies from organic solutions, bio-mimetics and living cells. *Journal of Photochemistry and Photobiology C: Photochem. Rev.* **2009,** *10* (2), 81-95.
54. Wang, Y. J.; Pan, M. H.; Cheng, A. L.; Lin, L. I.; Ho, Y. S.; Hsieh, C. Y.; Lin, J. K., Stability of curcumin in buffer solutions and characterization of its degradation products. *J.Pharm. Biomed. Anal.* **1997,** *15* (12), 1867-76.
55. Karewicz, A.; Bielska, D.; Gzyl-Malcher, B.; Kepczynski, M.; Lach, R.; Nowakowska, M., Interaction of curcumin with lipid monolayers and liposomal bilayers. *Colloids. Surf. B.* **2011,** *88* (1), 231-9.

56. Zsila, F.; Bikádi, Z.; Simonyi, M., Molecular basis of the cotton effects induced by the binding of curcumin to human serum albumin. *Tetrahedron.* **2003,** *14* (16), 2433-2444.
57. Gidwani, A.; Holowka, D.; Baird, B., Fluorescence Anisotropy Measurements of Lipid Order in Plasma Membranes and Lipid Rafts from RBL-2H3 Mast Cells†. *Biochem.* **2001,** *40* (41), 12422-12429.
58. Barry, J.; Fritz, M.; Brender, J. R.; Smith, P. E.; Lee, D.-K.; Ramamoorthy, A., Determining the effects of lipophilic drugs on membrane structure by solid-state NMR spectroscopy: the case of the antioxidant curcumin. *J. Ame. Chem. Soci.* **2009,** *131* (12), 4490-4498.
59. Lozano, H. R.; Martínez, F., Thermodynamics of partitioning and solvation of ketoprofen in some organic solvent: buffer and liposome systems. *Revista. Brasileira. Ciências. Farmacêut.* **2006,** *42* (4), 601-613.
60. Marsh, D., Polarity and permeation profiles in lipid membranes. *Proceedings of the Nat. Academ. Sci.* **2001,** *98* (14), 7777-7782.
61. Hadjidemetriou, M.; Pippa, N.; Pispas, S.; Demetzos, C., Incorporation of dimethoxycurcumin into charged liposomes and the formation kinetics of fractal aggregates of uncharged vectors. *J. Liposome. Res.* **2013,** *23* (2), 94-100.
62. Leung, M. H.; Colangelo, H.; Kee, T. W., Encapsulation of curcumin in cationic micelles suppresses alkaline hydrolysis. *Langmuir.* **2008,** *24* (11), 5672-5675.
63. Nounou, M. M.; El-Khordagui, L. K.; Khalafallah, N. A.; Khalil, S. A., In vitro release of hydrophilic and hydrophobic drugs from liposomal dispersions and gels. *Acta. Pharm.* **2006,** *56* (3), 311-24.
64. Ullrich, M.; Hanuš, J.; Dohnal, J.; Štěpánek, F., Encapsulation stability and temperature-dependent release kinetics from hydrogel-immobilised liposomes. *J. Colloid. Int. Sci.* **2013,** *394* (0), 380-385.
65. Zhang, Y.; Huo, M.; Zhou, J.; Zou, A.; Li, W.; Yao, C.; Xie, S., DDSolver: An Add-In Program for Modeling and Comparison of Drug Dissolution Profiles. *AAPS. J.* **2010,** *12* (3), 263-271.
66. Dash, S.; Murthy, P. N.; Nath, L.; Chowdhury, P., Kinetic modeling on drug release from controlled drug delivery systems. *Act. Pol. Pharm.* **2010,** *67* (3), 217-23.
67. Liu, C.-H.; Huang, H.-Y., In vitro anti-propionibacterium activity by curcumin containing vesicle system. *Chem.Pharmaceut. Bulletin.* **2013,** *61* (4), 419-425.

References for CHAPTER II

1. Chauhan, A. S.; Diwan, P. V.; Jain, N. K.; Tomalia, D. A., Unexpected In Vivo Anti-Inflammatory Activity Observed for Simple, Surface Functionalized Poly(amidoamine) Dendrimers. *Biomacromolecules.* **2009,** 1195-1202.

2. Christensen, J. B.; Tomalia, D. A. In *Dendrimers as quantized nano-modules in the nanotechnology field*. John Wiley & Sons, Inc.: **2012**, 1-33.

3. Majoros, I. J.; Williams, C. R.; Tomalia, D. A.; Baker, J. R., New Dendrimers: Synthesis and Characterization of POPAM-PAMAM Hybrid Dendrimers. *Macromolecules.* **2008,** *41*, 8372-8379.

4. Menjoge, A. R.; Kannan, R. M.; Tomalia, D. A., Dendrimer-based drug and imaging conjugates: design considerations for nanomedical applications. *Drug Discovery Today* **2010,** *15*, 171-185.

5. Tomalia, D. A., Dendrons/dendrimers: quantized, nano-element like building blocks for soft-soft and soft-hard nano-compound synthesis. *Soft. Matter.* **2010,** *6*, 456-474.

6. Tomalia, D. A.; Diallo, M. S. In *Dendrimers: synthetic science to controlled organic nanostructures and a window to a new systematic framework for unifying nanoscience*. *C.R.C.* **2012**, 413-467.

7. Khopade, A. J.; Shenoy, D. B.; Khopade, S. A.; Jain, N. K., Phase Structures of a Hydrated Anionic Phospholipid Composition Containing Cationic Dendrimers and Pegylated Lipids. *Langmuir.* **2004,** *20* (18), 7368-7373.

8. Beezer, A. E.; King, A. S. H.; Martin, I. K.; Mitchel, J. C.; Twymanb, L. J.; Wainb, C. F., Dendrimers as potential drug carriers; encapsulation of acidic hydrophobes within water soluble PAMAM derivatives. *Tetrahedron. Lett.* **2003,** *59*, 3873–3880.

9. Bielinska, A. U.; Chen, C.; Johnson, J.; Baker, J. R., DNA Complexing with Polyamidoamine Dendrimers: Implications for Transfection. *Bioconjugate. Chem.* **1999,** *10* (5), 843-850.

10. Dvornic, P. R.; Tomalia, D. A., Molecules that grow like treess.endrimers.The fourth class of macromoleular architecture. *Sci. Spectra*. **1996,** *5*, 36.

11. Esfand, R.; Tomalia, D. A., Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. *Drug. Discovery. Today.* **2001,** *6*, 427-436.

12. Liu, M.; Fréchet, M. J. J., Designing dendrimers for drug delivery. *Pharm. Sci. Tech. Today.* **1999,** *2* (10), 393-401.

13. Tomlinson, E.; Rolland, A. P., Controllable gene therapy. Pharmaceutics of non-viral gene delivery systems. *J. Controlled. Release.* **1996,** *39*, 357-372.

14. Zinselmeyer, B. H.; Mackay, S. P.; Schatzlein, A. G.; Uchegbu, I. F., The lower-generation polypropylenimine dendrimers are effective gene-transfer agents. *Pharm. Res.* **2002,** *19* (7), 960-967.

15. Hong, S.; Bielinska, A. U.; Mecke, A.; Keszler, B.; Beals, J. L.; Shi, X.; Balogh, L.; Orr, B. G.; Baker, J. R., Jr.; Banaszak Holl, M. M., Interaction of Poly(amidoamine) Dendrimers with Supported Lipid Bilayers and Cells: Hole Formation and the Relation to Transport. *Bioconjugate. Chem.* **2004,** *15*, 774-782.

16. Mecke, A.; Lee, D.-K.; Ramamoorthy, A.; Orr, B. G.; Banaszak Holl, M. M., Synthetic and Natural Polycationic Polymer Nanoparticles Interact Selectively with Fluid-Phase Domains of DMPC Lipid Bilayers. *Langmuir*. **2005,** *21*, 8588-8590.

17. Mecke, A.; Majoros, I. J.; Patri, A. K.; Baker, J. R., Jr.; Banaszak Holl, M. M.; Orr, B. G., Lipid Bilayer Disruption by Polycationic Polymers: The Roles of Size and Chemical Functional Group. *Langmuir*. **2005,** *21*, 10348-10354.

18. Mecke, A.; Uppuluri, S.; Sassanella, T. M.; Lee, D.-K.; Ramamoorthy, A.; Baker, J. R.; Orr, B. G.; Banaszak Holl, M. M., Direct observation of lipid bilayer disruption by poly(amidoamine) dendrimers. *Chem. Phys. Lipids.* **2004,** *132*, 3-14.

19. Richter, R.; Mukhopadhyay, A.; Brisson, A., Pathways of Lipid Vesicle Deposition on Solid Surfaces: A Combined QCM-D and AFM Study. *Biophys. J.* **2003,** *85* (5), 3035-3047.

20. Feng, Z. V.; Granick, S.; Gewirth, A. A., Modification of a Supported Lipid Bilayer by Polyelectrolyte Adsorption. *Langmuir.* **2004,** *20* (20), 8796-8804.

21. Bosman, A. W.; Janssen, H. M.; Meijer, E. W., About Dendrimers: Structure, Physical Properties, and Applications. *Chem. Rev.* **1999,** *99* (7), 1665-1688.

22. Karoonuthaisiri, N.; Titiyevskiy, K.; Thomas, J. L., Destabilization of fatty acid-containing liposomes by polyamidoamine dendrimers. *Colloid. Surf. B:* **2003,** *27*, 365-375.

23. Ottaviani, M. F.; Brustolon, M.; Turro, N. J.; Jockusch, S.; Tomalia, D. A. In *Formation of supramolecular structures by starburst dendrimers and vesicles,* Am. Chem. Soci.**1998**; 130.

24. Ottaviani, M. F.; Daddi, R.; Brustolon, M.; Turro, N. J.; Tomalia, D. A., Structural Modifications of DMPC Vesicles upon Interaction with Poly(amidoamine) Dendrimers Studied by CW-Electron Paramagnetic Resonance and Electron Spin−Echo Techniques. *Langmuir.* **1999,** *15* (6), 1973-1980.

25. Ottaviani, M. F.; Favuzza, P.; Sacchi, B.; Turro, N. J.; Jockusch, S.; Tomalia, D. A., Interactions between Starburst Dendrimers and Mixed DMPC/DMPA-Na Vesicles Studied by the Spin Label and the Spin Probe Techniques, Supported by Transmission Electron Microscopy. *Langmuir.* **2002,** *18* (6), 2347-2357.

26. Zhang, Z.-Y.; Smith, B. D., High-Generation Polycationic Dendrimers Are Unusually Effective at Disrupting Anionic Vesicles: Membrane Bending Model. *Bioconjugate Chemistry* **2000,** *11* (6), 805-814.

27. Sideratou, Z.; Foundis, J.; Tsiourvas, D.; Nezis, I. P.; Papadimas, G.; Paleos, C. M., A Novel Dendrimeric "Glue" for Adhesion of Phosphatidyl Choline-Based Liposomes. *Langmuir.* **2002,** *18* (13), 5036-5039.

28. Sideratou, Z.; Tsiourvas, D.; Paleos, C. M.; Tsortos, A.; Nounesis, G., Molecular Recognition of Complementary Liposomes: The Enhancing Role of Cholesterol. *Langmuir* **2000,** *16* (24), 9186-9191.

29. Bangham, A. D.; Standish, M. M.; Watkins, J. C., Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **1965,** *13*, 238-52.

30. Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R., Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta* **1985,** *812*, 55-65.

31. Olson, F.; Hunt, C. A.; Szoka, F. C.; Vail, W. J.; Papahadjopoulos, D., Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochim. Biophys. Acta, Biomembr.* **1979,** *557* (Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.), 9-23.

32. Panda, A. K.; Chakraborty, A. K., Interaction of mixed surfactants with bacterial lipopolysaccharide. *J. Colloid. Int. Sci.* **1998,** *203*, 260-264.

33. Manna, K.; Chang, C.-H.; Panda, A. K., Physicochemical studies on the catanionics of alkyltrimethylammonium bromides and bile salts in aqueous media. *Colloid. Surf. A* **2012,** *415*, 10-21.

34. Pantos, A.; Tsiourvas, D.; Nounesis, G.; Paleos, C. M., Interaction of Functional Dendrimers with Multilamellar Liposomes: Design of a Model System for Studying Drug Delivery. *Langmuir.* **2005,** *21* (16), 7483-7490.

References for CHAPTER III

1. Jaikishan, S.; Björkbom, A.; Slotte, J. P. Phosphatidyl alcohols: effect of head group size on domain forming properties and interactions with sterols. *Biochim. Biophys. Acta, Biomembr.* **2010,** *1798*, 1615-1622.

2. Morini, M. A.; Sierra, M. B.; Pedroni, V. I.; Alarcon, L. M.; Appignanesi, G. A.; Disalvo, E. A. Influence of temperature, anions and size distribution on the zeta potential of DMPC, DPPC and DMPE lipid vesicles. *Colloid. Surf. B, Biointerfaces.* **2015,** *131*, 54-58.

3. Kitchens, K. M.; Kolhatkar, R. B.; Swaan, P. W.; Ghandehari, H. Endocytosis inhibitors prevent poly (amidoamine) dendrimer internalization and permeability across Caco-2 cells. *Mol. Pharm.***2008,** *5*, 364-369.

4. Pantos, A.; Tsiourvas, D.; Nounesis, G.; Paleos, C. M. Interaction of Functional Dendrimers with Multilamellar Liposomes: Design of a Model System for Studying Drug Delivery. *Langmuir.* **2005,** *21*, 7483-7490.

5. Tsogas, I.; Tsiourvas, D.; Nounesis, G.; Paleos, C. M. Interaction of poly-L-arginine with dihexadecyl phosphate/phosphatidylcholine liposomes. *Langmuir.* **2005,** *21*, 5997-6001.

6. Lee, Y. C.; Taraschi, T. F.; Janes, N., Support for the shape concept of lipid structure based on a headgroup volume approach. *Biophys. J.* **1993,** *65*, 1429-1432.

7. Rukavina, Z.; Vanić, Ž. Current Trends in Development of Liposomes for Targeting Bacterial Biofilms. *Pharm.* **2016,** *8*, 18.

8. Evans, K. O.; Laszlo, J. A.; Compton, D. L. Carboxyl-terminated PAMAM dendrimer interaction with 1-palmitoyl-2-oleoyl phosphocholine bilayers. *Biochim. Biophys. Act. Biomembr.* **2014,** *1838*, 445-455.

9. Papagiannaros, A.; Dimas, K.; Papaioannou, G. T.; Demetzos, C. Doxorubicin-PAMAM dendrimer complex attached to liposomes: cytotoxic studies against human cancer cell lines. *Int. J. Pharm.* **2005,** *302*, 29-38.

10. Falanga, A.; Tarallo, R.; Carberry, T.; Galdiero, M.; Weck, M.; Galdiero, S. Elucidation of the interaction mechanism with liposomes of gH625-peptide functionalized dendrimers. *Plos. One.* **2014,** *9*, 112128.

11. Frechet, J. M. Functional polymers and dendrimers: reactivity, molecular architecture, and interfacial energy. *Science-AAAS-Weekly Paper Edition-include. Guide. Sci. Inform.* **1994,** *263*, 1710-1714.

12. Klajnert½, B.; Bryszewska, M. Dendrimers: properties and applications. **2001**.

13. Nunez, C. M.; Chiou, B.-S.; Andrady, A. L.; Khan, S. A. Solution rheology of hyperbranched polyesters and their blends with linear polymers. *Macromolecules.* **2000,** *33*, 1720-1726.

14. Lee, C. C.; MacKay, J. A.; Fréchet, J. M.; Szoka, F. C. Designing dendrimers for biological applications. *Nat. biotechnol.* **2005,** *23*, 1517-1526.

15. Liu, M.; Fréchet, J. M. Designing dendrimers for drug delivery. *Pharm. Sci. Tech.* **1999,** *2*, 393-401.

16. Qiu, L. Y.; Bae, Y. H. Polymer architecture and drug delivery. *Pharm. Res.* **2006,** *23*, 1-30.

17. Kragh-Hansen, U.; le Maire, M.; Møller, J. V. The Mechanism of Detergent Solubilization of Liposomes and Protein-Containing Membranes. *Biophys. J.* **1998,** *75*, 2932-2946.

18. Lichtenberg, D.; Ahyayauch, H.; Goni, F. M. The mechanism of detergent solubilization of lipid bilayers. *Biophys. J.* **2013,** *105*, 289-99.

19. Sato, H.; Feix, J. B. Peptide-membrane interactions and mechanisms of membrane destruction by amphipathic alpha-helical antimicrobial peptides. *Biochim. Biophys. Acta.* **2006,** *9*, 12.

20. Wrobel, D.; Kłys, A.; Ionov, M.; Vitovic, P.; Waczulikowa, I.; Hianik, T.; Gomez-Ramirez, R.; de la Mata, J.; Klajnert, B.; Bryszewska, M. Cationic carbosilane dendrimers–lipid membrane interactions. *Chem. Phys.Lipids.* **2012,** *165*, 401-407.

21. Ionov, M.; Gardikis, K.; Wróbel, D.; Hatziantoniou, S.; Mourelatou, H.; Majoral, J.-P.; Klajnert, B.; Bryszewska, M.; Demetzos, C. Interaction of cationic phosphorus dendrimers (CPD) with charged and neutral lipid membranes. *Colloid. Surf. B, Biointerfaces.* **2011,** *82*, 8-12.

22. Yu, S.; Larson, R. G. Monte-Carlo simulations of PAMAM dendrimer-DNA interactions. *Soft Matter.* **2014,** *10*, 5325-36.

23. Abbasi, E.; Aval, S. F.; Akbarzadeh, A.; Milani, M.; Nasrabadi, H. T.; Joo, S. W.; Hanifehpour, Y.; Nejati-Koshki, K.; Pashaei-Asl, R. Dendrimers: synthesis, applications, and properties. *Nanoscale. Res. Lett.* **2014,** *9*, 247-247.

24. Otto, D. P.; de Villiers, M. M. Poly(amidoamine) Dendrimers as a Pharmaceutical Excipient. Are We There yet? *J. Pharm. Sci.* **2018,** *107*, 75-83.

25. Najlah, M.; D'Emanuele, A. Crossing cellular barriers using dendrimer nanotechnologies. *Curr.Opin. Pharmacol.***2006,***6* (5), 522-527.

26. Simons, K.; Vaz, W. L. Model systems, lipid rafts, and cell membranes. *Annu. Rev. Biophys. Biomol. Struct.* **2004,** *33*, 269-295.

27. Yanez Arteta, M.; Ainalem, M.-L.; Porcar, L.; Martel, A.; Coker, H.; Lundberg, D.; Chang, D. P.; Soltwedel, O.; Barker, R.; Nylander, T. Interactions of PAMAM dendrimers with negatively charged model biomembranes. *J. Phys. Chem. B.* **2014,** *118*, 12892-12906.

28. Sideratou, Z.; Foundis, J.; Tsiourvas, D.; Nezis, I. P.; Papadimas, G.; Paleos, C. M. A novel dendrimeric "glue" for adhesion of phosphatidyl choline-based liposomes. *Langmuir.* **2002,** *18*, 5036-5039.

29. Ghanbarzadeh, S.; Valizadeh, H.; Zakeri-Milani, P. Application of response surface methodology in development of sirolimus liposomes prepared by thin film hydration technique. *BioImpacts. BI.* **2013,** *3*, 75.

30. Roy, B.; Guha, P.; Bhattarai, R.; Nahak, P.; Karmakar, G.; Chettri, P.; Panda, A. K. Influence of Lipid Composition, pH, and Temperature on Physicochemical Properties of Liposomes with Curcumin as Model Drug. *J. Oleo. Sci.* **2016,** *65*, 399-411.

31. Lichtenthaler, H. K.; Buschmann, C. Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. *Current. Protocol. Food. Analytical. Chem.*, John Wiley & Sons, Inc. 2001.

32. Roy, B.; Panda, A. K.; Parimi, S.; Ametov, I.; Barnes, T.; Prestidge, C. A. Physico-chemical studies on the interaction of dendrimers with lipid bilayers. 1. Effect of dendrimer generation and liposome surface charge. *J. Oleo. Sci.* **2014,** *63*, 1185-1193.

33. Koirala, S.; Roy, B.; Guha, P.; Bhattarai, R.; Sapkota, M.; Nahak, P.; Karmakar, G.; Mandal, A. K.; Kumar, A.; Panda, A. K. Effect of double tailed cationic surfactants on the physicochemical behavior of hybrid vesicles. *RSC. Adv.* **2016,** *6*, 13786-13796.

34. Liu, S.; Lv, P.; Li, D.; Guo, X.; Zhang, B.; Yu, M.; Li, D.; Xiong, Y.; Zhang, L.; Tian, C. K+ preference at the NaK channel entrance revealed by fluorescence lifetime and anisotropy analysis of site-specifically incorporated (7-hydroxycoumarin-4-yl)ethylglycine. *Chem. Comm.* **2015,** *51*, 15971-15974.

35 Mandal, A. K.; Sen, I. K.; Maity, P.; Chattopadhyay, S.; Chakraborty, R.; Roy, S.; Islam, S. S. Structural elucidation and biological studies of a novel exopolysaccaride from Klebsiella pneumoniae PB12. *Int. J. Bio. Macromolecul.* **2015,** *79*, 413-422.

36. Jana, S. K.; Mandal, A. K.; Kumar, A.; Puschmann, H.; Hossain, M.; Dalai, S. Sensing of tryptophan by a non-toxic cobalt (II) complex. *RSC. Adv.* **2016,** *6*, 95888-95896.

37. Guha, P.; Roy, B.; Karmakar, G.; Nahak, P.; Koirala, S.; Sapkota, M.; Misono, T.; Torigoe, K.; Panda, A. K. Ion-Pair Amphiphile: A Neoteric Substitute That Modulates the Physicochemical Properties of Biomimetic Membranes. *J. Phys. Chem. B.* **2015,** *119*, 4251-4262.

38. Giustini, M.; Bellinazzo, C.; Galantini, L.; Mallardi, A.; Palazzo, G.; Sennato, S.; Bordi, F.; Rissanen, K. Incorporation of the bacterial reaction centre into dendrimersomes. *Colloid. Surf. A.* **2012,** *413*, 38-43.

39. Winther, L. R. The Phase Transition of DMPG and its Dependence on pH. *Bachelor. Thesis.* **2008**.

40. Wrobel, D.; Appelhans, D.; Signorelli, M.; Wiesner, B.; Fessas, D.; Scheler, U.; Voit, B.; Maly, J. Interaction study between maltose-modified PPI dendrimers and lipidic model membranes. *Biochim. Biophys. Acta.* **2015,** *7*, 3.

41. Wrobel, D.; Ionov, M.; Gardikis, K.; Demetzos, C.; Majoral, J.-P.; Palecz, B.; Klajnert, B.; Bryszewska, M. Interactions of phosphorus-containing dendrimers with liposomes. *Biochim. Biophys. Act.* **2011,** *1811*, 221-226.

42. Singler, R. E.; Sennett, M. S.; Willingham, R. A. Phosphazene polymers: synthesis, structure, and properties. ACS. Pub. **1988**.

43. Lozano, N.; Pinazo, A.; La Mesa, C.; Perez, L.; Andreozzi, P.; Pons, R. Catanionic vesicles formed with arginine-based surfactants and 1, 2-dipalmitoyl-sn-glycero-3-phosphate monosodium salt. *J. Phys. Chem. B.* **2009,** *113*, 6321-6327.

44. Tsogas, I.; Tsiourvas, D.; Nounesis, G.; Paleos, C. M. Modeling cell membrane transport: interaction of guanidinylated poly (propylene imine) dendrimers with a liposomal membrane consisting of phosphate-based lipids. *Langmuir*. **2006,** *22*, 11322-11328.

45. Wrobel, D.; Appelhans, D.; Signorelli, M.; Wiesner, B.; Fessas, D.; Scheler, U.; Voit, B.; Maly, J. Interaction study between maltose-modified PPI dendrimers and lipidic model membranes. *Biochim. Biophys. Act,Biomembr.* **2015,** *1848*, 1490-1501.

46. Pinto da Silva, L.; Simkovitch, R.; Huppert, D.; Esteves da Silva, J. C. G. Combined experimental and theoretical study of the photochemistry of 4- and 3-hydroxycoumarin. *J. Photochem. Photobio. A. Chem.* **2017,** *338*, 23-36.

47. Smith, E. A.; Dea, P. K. *Differential scanning calorimetry studies of phospholipid membranes: the interdigitated gel phase*. INTECH Open. Acc. Pub. **2013**.

48. Feitosa, E.; Jansson, J.; Lindman, B. The effect of chain length on the melting temperature and size of dialkyldimethylammonium bromide vesicles. *Chem. Phys. Lipids.* **2006,** *142*, 128-132.

49. Ringsdorf, H.; Schlarb, B.; Tyminski, P. N.; O'Brien, D. F. Permeability characteristics of liposomes in a net-membranes of dihexadecyl phosphate with polymerizable gegenions. *Macromolecules.* **1988,** *21*, 671-677.

50. Ionov, M.; Wróbel, D.; Gardikis, K.; Hatziantoniou, S.; Demetzos, C.; Majoral, J.-P.; Klajnert, B.; Bryszewska, M. Effect of phosphorus dendrimers on DMPC lipid membranes. *Chem. Phys. Lipids.* **2012,** *165*, 408-413.

51. Pantos, A.; Tsiourvas, D.; Nounesis, G.; Paleos, C. M. Interaction of functional dendrimers with multilamellar liposomes: design of a model system for studying drug delivery. *Langmuir.* **2005,** *21*, 7483-7490.

52. Lovelock, J. The haemolysis of human red blood-cells by freezing and thawing. *Biochim. Biophys. Act.* **1953,** *10*, 414-426.

53. Lin, J.-J.; Lin, W.-C.; Dong, R.-X.; Hsu, S.-h. The cellular responses and antibacterial activities of silver nanoparticles stabilized by different polymers. *Nanotechnology.* **2012,** *23*, 065102.

References for CHAPTER IV

1. Khattari, Z.; Al-Abdullah, T.; Maghrabi, M.; Khasim, S.; Roy, A.; Fasfous, I., Interaction Study of Lipopeptide Biosurfactant viscosin with DPPC and Cholesterol by Langmuir Monolayer Technique. *Soft Mate.* **2015,** *13* (4), 254-262.
2. Boisselier, É.; Calvez, P.; Demers, É.; Cantin, L.; Salesse, C., Influence of the Physical State of Phospholipid Monolayers on Protein Binding. *Langmuir.* **2012,** *28* (25), 9680-9688.
3. Pechkova, E.; Nicolini, C., Langmuir-Blodgett nanotemplates for protein crystallography. *Nat. Protoc.* **2017,** *12* (12), 2570-2589.
4. Lagace, T. A.; Ridgway, N. D., The role of phospholipids in the biological activity and structure of the endoplasmic reticulum. *Biochim.Biophys. Act.* **2013,** *1833* (11), 2499-2510.

5. Zhao, L.; Feng, S.-S., Effects of lipid chain length on molecular interactions between paclitaxel and phospholipid within model biomembranes. *J. Colloid.Int. Sci.* **2004,** *274* (1), 55-68.
6. Khattari, Z.; Sayyed, M. I.; Qashou, S. I.; Fasfous, I.; Al-Abdullah, T.; Maghrabi, M., Interfacial behavior of Myristic acid in mixtures with DMPC and Cholesterol. *Chem. Phys.* **2017,** *490*, 106-114.
7. Hadicke, A.; Schwieger, C.; Blume, A., Cospreading of Anionic Phospholipids with Peptides of the Structure (KX)4K at the Air-Water Interface: Influence of Lipid Headgroup Structure and Hydrophobicity of the Peptide on Monolayer Behavior. *Langmuir.* **2017,** *33* (43), 12204-12217.
8. López-Oyama, A.; Taboada, P.; G Burboa, M.; Rodríguez, E.; Mosquera, V. c.; Valdez, M., *Interaction of the cationic peptide bactenecin with mixed phospholipid monolayers at the air–water interface*. 2011; Vol. 359, 279-88.
9. Jaikishan, S.; Björkbom, A.; Slotte, J. P., Phosphatidyl alcohols: Effect of head group size on domain forming properties and interactions with sterols. *Biochim.Biophys. Act.s* **2010,** *1798* (8), 1615-1622.
10. Lee, Y. C.; Taraschi, T. F.; Janes, N., Support for the shape concept of lipid structure based on a headgroup volume approach. *Biophys. J.* **1993,** *65* (4), 1429-1432.
11. Rukavina, Z.; Vanic, Z., Current Trends in Development of Liposomes for Targeting Bacterial Biofilms. *Pharmaceutics.* **2016,** *8* (2).
12. Brown, R. E.; Brockman, H. L., Using Monomolecular Films to Characterize Lipid Lateral Interactions. *Meth. Molecul. .Biol.* **2007,** *398*, 41-58.
13. Miyoshi, T.; Kato, S., Detailed Analysis of the Surface Area and Elasticity in the Saturated 1,2-Diacylphosphatidylcholine/Cholesterol Binary Monolayer System. *Langmuir.* **2015,** *31* (33), 9086-9096.
14. Karmakar, G.; Nahak, P.; Roy, B.; Guha, P.; Tsuchiya, K.; Torigoe, K.; Nath, R. K.; Panda, A. K., Use of ion pair amphiphile as an alternative of natural phospholipids in enhancing the stability and anticancer activity of oleanolic acid loaded nanostructured lipid carriers. *Colloid. Surf. A.*. **2018**.
15. Al-Jabari, M., Kinetic models for adsorption on mineral particles comparison between Langmuir kinetics and mass transfer. *Env.Tech.Innov.* **2016,** *6*, 27-37.

16. Bozzuto, G.; Molinari, A., Liposomes as nanomedical devices. *Int. J. Nanomed.***2015,** *10*, 975-999.
17. Yanez Arteta, M.; Ainalem, M.-L.; Porcar, L.; Martel, A.; Coker, H.; Lundberg, D.; Chang, D. P.; Soltwedel, O.; Barker, R.; Nylander, T., Interactions of PAMAM Dendrimers with Negatively Charged Model Biomembranes. *J.Phys. Chem. B.* **2014,** *118* (45), 12892-12906.
18. Prajapati, S.; Maurya, S.; Kumar Das, M.; Kumar Tilak, V.; Verma, K.; Dhakar, R. C., *Dendrimers in drug delivery, diagnosis and therapy: basics and potential applications*. 2016; Vol. 6, p 67-92.
19. Prieto, M. J.; del Rio Zabala, N. E.; Marotta, C. H.; Carreño Gutierrez, H.; Arévalo Arévalo, R.; Chiaramoni, N. S.; Alonso, S. d. V., Optimization and In Vivo Toxicity Evaluation of G4.5 Pamam Dendrimer-Risperidone Complexes. *PLoS. One.* **2014,** *9* (2), e90393.
20. Thuy le, T.; Mallick, S.; Choi, J. S., Polyamidoamine (PAMAM) dendrimers modified with short oligopeptides for early endosomal escape and enhanced gene delivery. *Int. J. Pharm.* **2015,** *492* (1-2), 233-43.
21. Wang, H. U. I.; Shi, H.-B.; Yin, S.-K., Polyamidoamine dendrimers as gene delivery carriers in the inner ear: How to improve transfection efficiency. *Exp. Therap. Med.* **2011,** *2* (5), 777-781.
22. Abbasi, E.; Aval, S. F.; Akbarzadeh, A.; Milani, M.; Nasrabadi, H. T.; Joo, S. W.; Hanifehpour, Y.; Nejati-Koshki, K.; Pashaei-Asl, R., Dendrimers: synthesis, applications, and properties. *Nano. Res. Lett.* **2014,** *9* (1), 247-247.
23. Mariyam, M.; Ghosal, K.; George, A.; Thomas, S.; Kalarikkal, N.; S, L., *Dendrimers: General Aspects, Applications and Structural Exploitations as Prodrug/ Drug-delivery Vehicles in Current Medicine*. 2017; Vol. 17.
24. Duarte, A. A.; Filipe, S. L.; Abegão, L. M. G.; Gomes, P. J.; Ribeiro, P. A.; Raposo, M., Adsorption Kinetics of DPPG Liposome Layers: A Quantitative Analysis of Surface Roughness. *Micros.Microan.* **2013,** *19* (4), 867-875.
25. Pantos, A.; Tsiourvas, D.; Nounesis, G.; Paleos, C. M., Interaction of Functional Dendrimers with Multilamellar Liposomes: Design of a Model System for Studying Drug Delivery. *Langmuir* **2005,** *21* (16), 7483-7490.

26. Looi, C. Y.; Arya, A.; Cheah, F. K.; Muharram, B.; Leong, K. H.; Mohamad, K.; Wong, W. F.; Rai, N.; Mustafa, M. R., Induction of apoptosis in human breast cancer cells via caspase pathway by vernodalin isolated from Centratherum anthelminticum (L.) seeds. *PLoS. One.* **2013,** *8* (2), 20.
27. Baoukina, S.; Monticelli, L.; Marrink, S. J.; Tieleman, D. P., Pressure−Area Isotherm of a Lipid Monolayer from Molecular Dynamics Simulations. *Langmuir.* **2007,** *23* (25), 12617-12623.
28. Duncan, S. L.; Larson, R. G., Comparing Experimental and Simulated Pressure-Area Isotherms for DPPC. *Biophys. J.* **2008,** *94* (8), 2965-2986.
29. Hall, R. A.; Kajikawa, K.; Hara, M.; Knoll, W., Spectral properties of aggregates in adsorbed dye monolayer films. *Thin. Solid. Film.* **1997,** *295* (1), 266-270.
30. Bhattarai, R.; Sutradhar, T.; Roy, B.; Guha, P.; Chettri, P.; Mandal, A. K.; Bykov, A. G.; Akentiev, A. V.; Noskov, B. A.; Panda, A. K., Double-Tailed Cystine Derivatives as Novel Substitutes of Phospholipids with Special Reference to Liposomes. *J. Phys. Chem. B.* **2016,** *120* (41), 10744-10756.
31. Sercombe, L.; Veerati, T.; Moheimani, F.; Wu, S. Y.; Sood, A. K.; Hua, S., Advances and Challenges of Liposome Assisted Drug Delivery. *Front. Pharm.* **2015,** *6* (286).
32. Qi, R.; Zhang, H.; Xu, L.; Shen, W.; Chen, C.; Wang, C.; Cao, Y.; Wang, Y.; van Dongen, M. A.; He, B.; Wang, S.; Liu, G.; Banaszak Holl, M. M.; Zhang, Q., G5 PAMAM dendrimer versus liposome: a comparison study on the in vitro transepithelial transport and in vivo oral absorption of simvastatin. *Nanomedicine.* **2015,** *11* (5), 1141-51.
33. Madaan, K.; Kumar, S.; Poonia, N.; Lather, V.; Pandita, D., Dendrimers in drug delivery and targeting: Drug-dendrimer interactions and toxicity issues. *J.Pharm.Bioallied. Sci.* **2014,** *6* (3), 139-150.

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