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

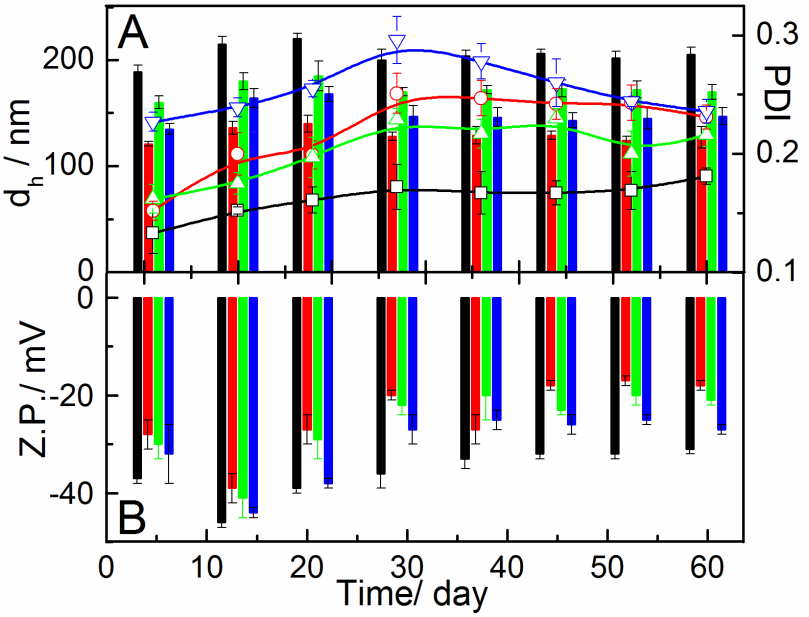

Figure 1. Variation in the hydrodynamic diameter (d_h), polydispersity index (PDI, line graphs) and zeta potential (ZP) of different liposomes with time. 30mole% cholesterol was used in each case. DPP : anionic lipid ratio is 7:3 (M/M). Bar/lines: black, DHP+DPPC; red, DMPG+DPPC; green, DPP+DPPC and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

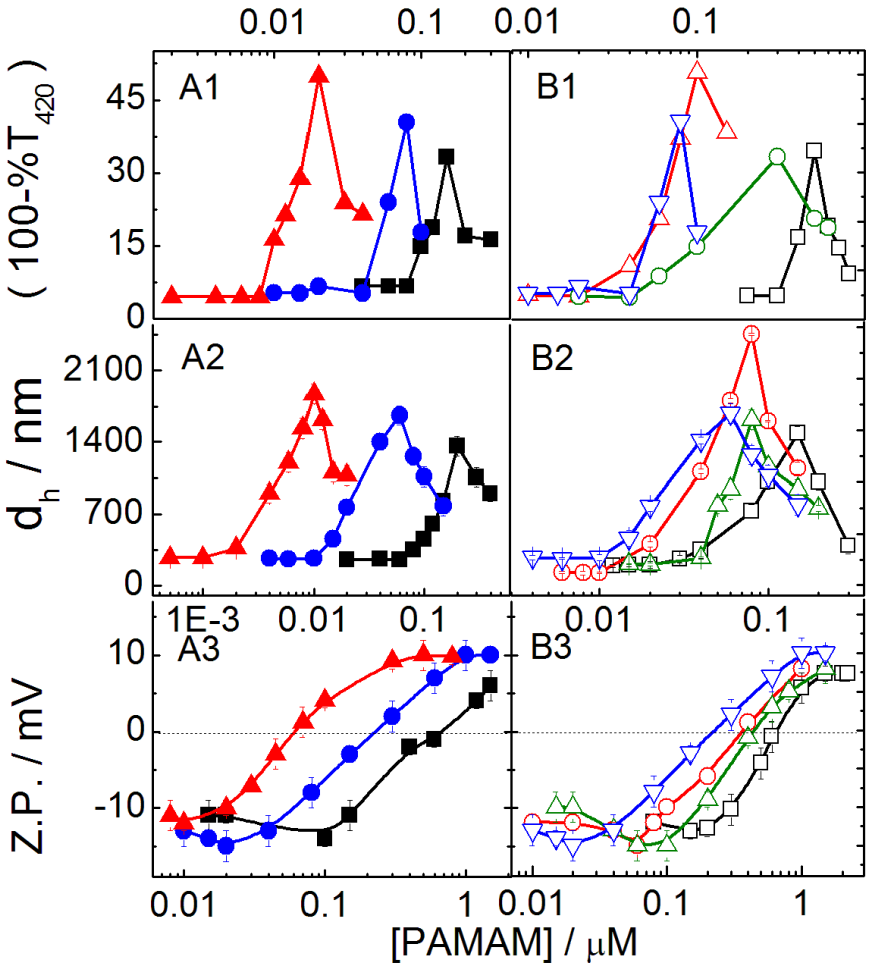

Figure 2. Effect of PAMAM dendrimer generation: (■,3G; ●,4G and ▲,5G) on the turbidity (A1), size (d_h, A2) and zeta potential (Z.P., A3) of (DPPEth+DPPC) liposome solutions and the effect of 4G dendrimer on the turbidity (B1), size (B2) and zeta potential (B3) of different liposomes (□, DHP+DPPC; ○,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

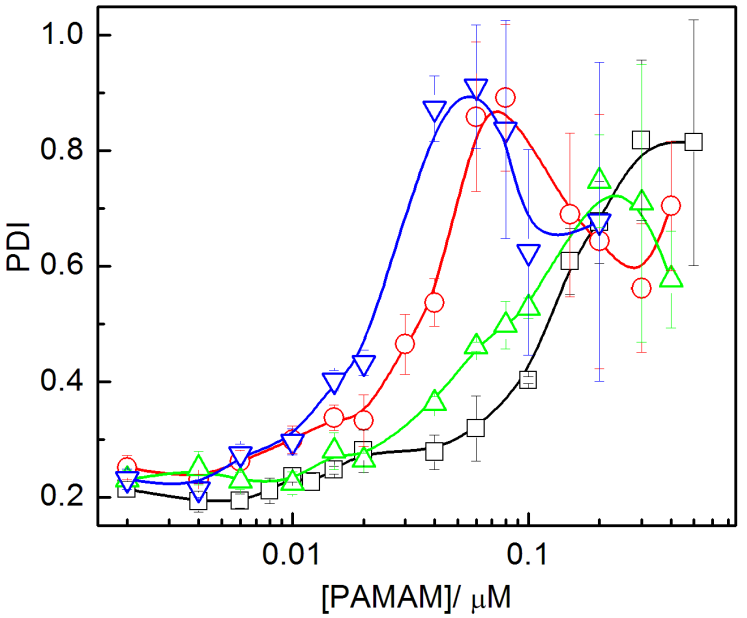

Figure 3. Polydispersity index (PDI) of different liposomes with varying concentration of 4G PAMAM dendrimer. Liposomes: □, DHP+DPPC; ○, DMPG+DPPC; Δ, DPP+DPPC and ▽, DPPEth+DPPC.

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.

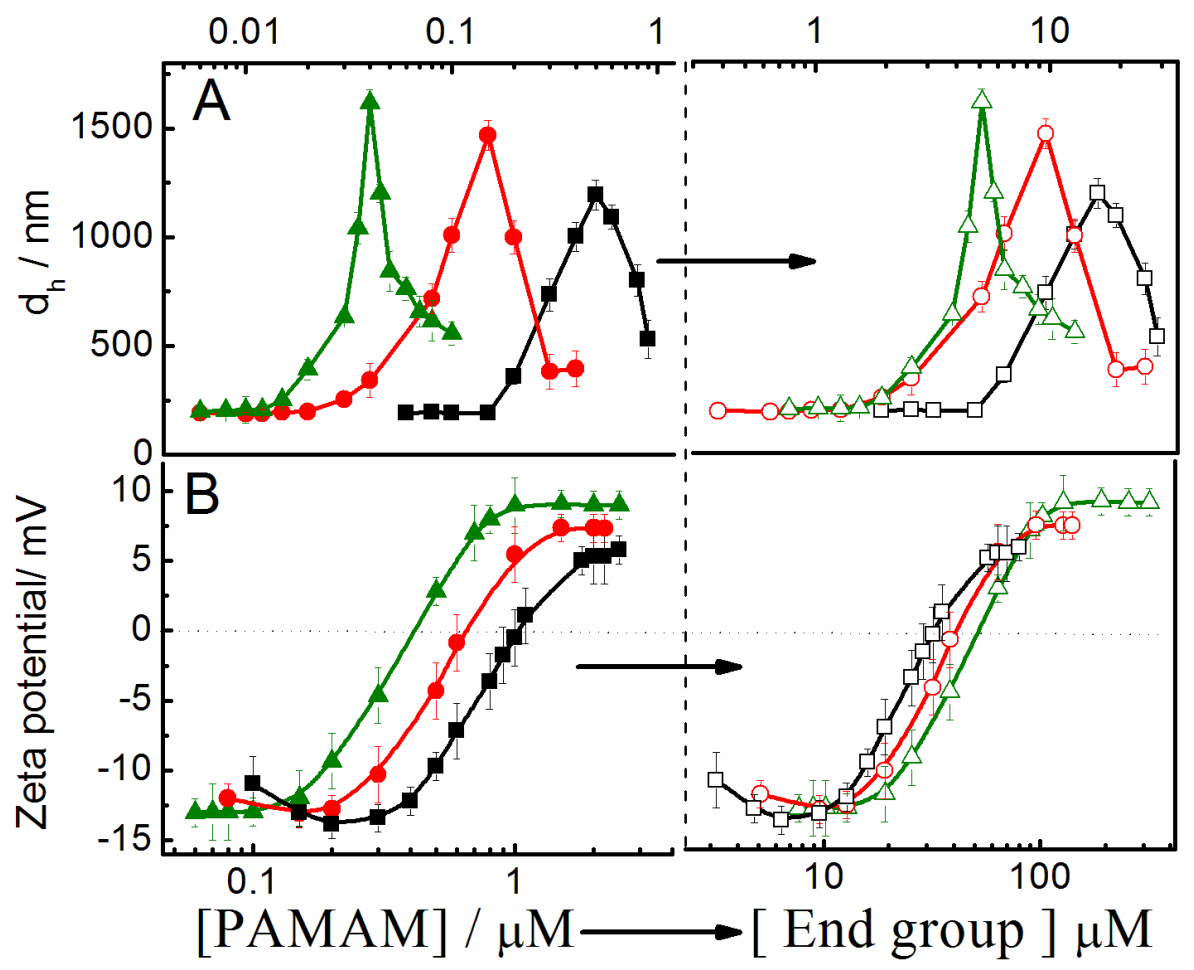

Figure 4. Effect of dendrimer generation and concentration :(G3, ■; G4, ● and G5,▲) on the size (A) and zeta potential (B) of the (DPPC+DHP+CHOL) liposome solutions (Left). Right side indicates the effect of end group concentration instead of concentration of dendrimer (□,G3; ○, G4;△,G5).

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

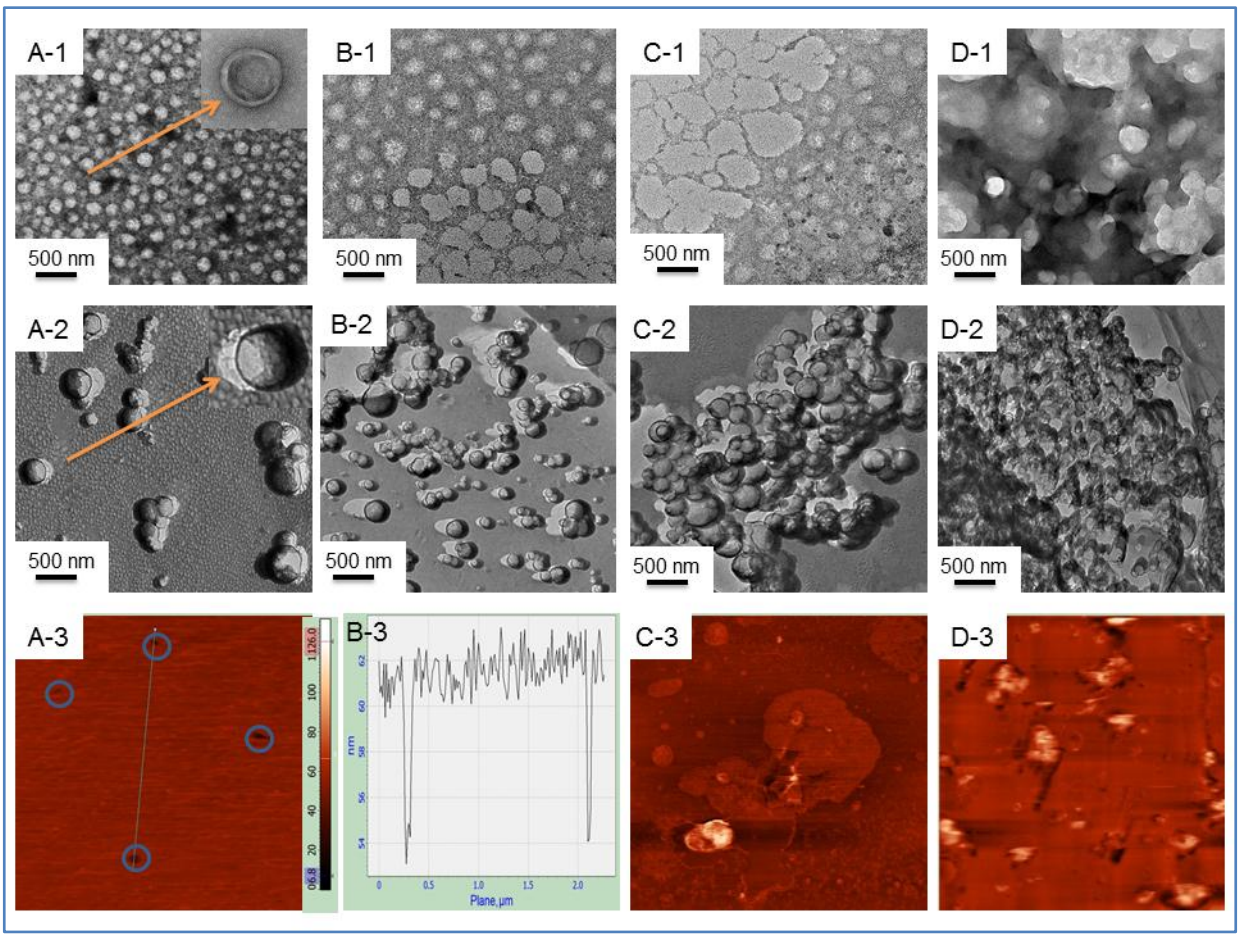

Figure 5. Effect of 4G PAMAM on the (DPPC+DPPEth+CHOL) bilayer.A, No dendrimer; B, 10 n mol dm^{-3} 4G PAMAM(except B3); C, 100 n mol dm^{-3} 4G PAMAM and D, 500 n mol dm^{-3}4G PAMAM. (Images: 1,TEM; 2, FF-TEM and 3, AFM). Panel B3, height analysis for bilayer thickness. Lipid concentration: 0.1mM.

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

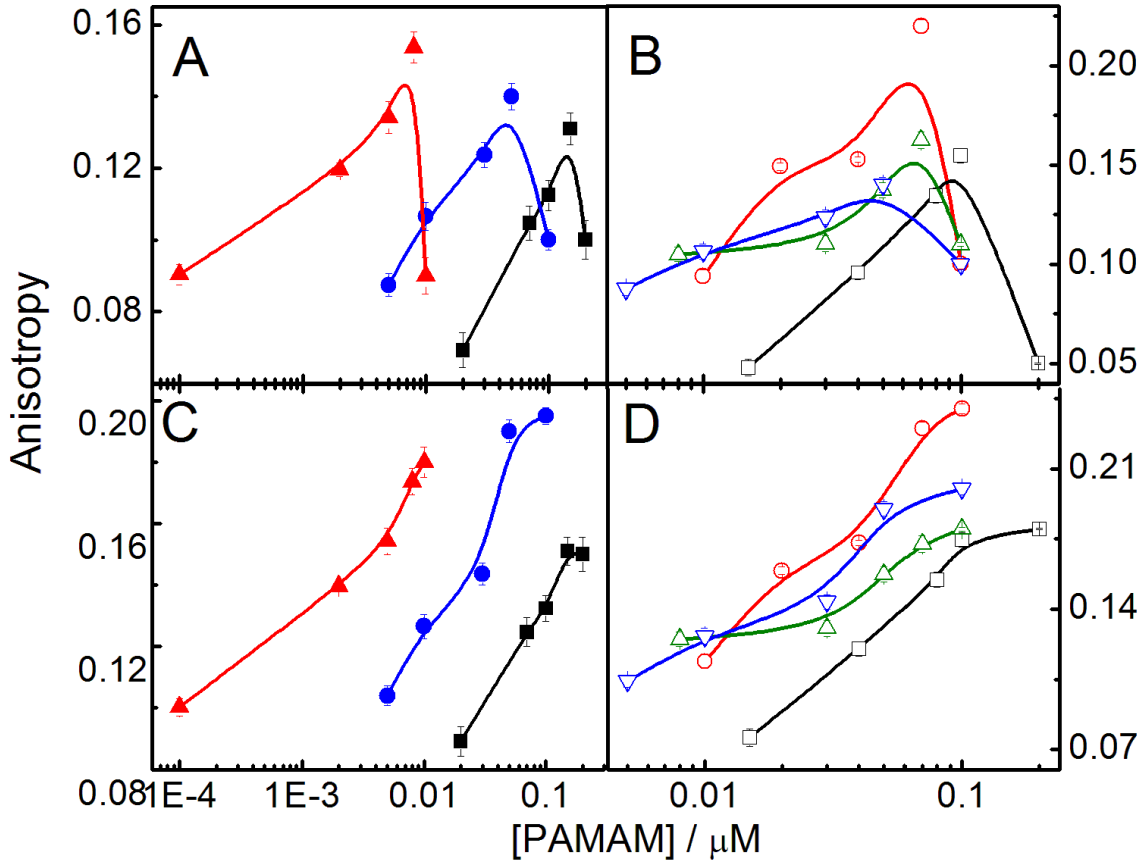

Figure 6. Variation in the fluorescence anisotropy values of 7-HC (panel A) and DPH (panel C) with dendrimer concentration. Effect of dendrimer generation (G3, ■; G4, ● and G5, ▲) on the anisotropy of DPPEth+DPPC liposome solutions and panel B (7-HC) and D (DPH): effect of dendrimer (4G) on different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

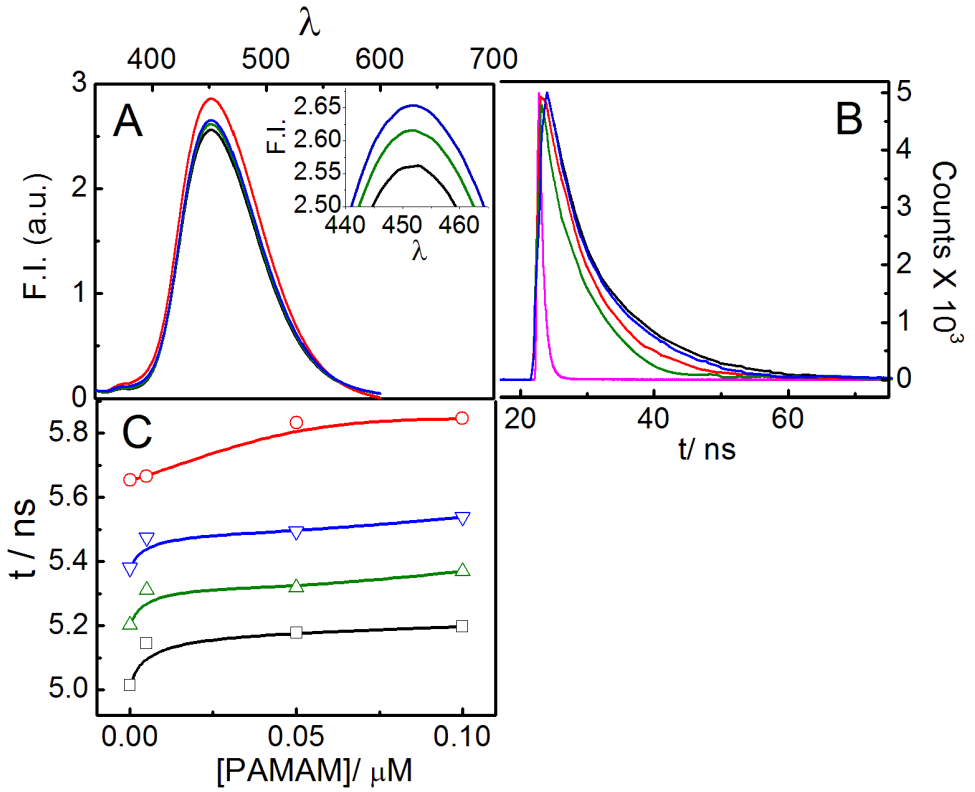

Figure 7. Steady state fluorescence spectra and life time study using 7-HC as a probe: panel A, steady state spectra of different liposomes (black, DHP+DPPC; red, DMPG+DPPC; green, DPP+DPPC and blue, DPPEth+DPPC); panel B, fluorescence intensity decay for different liposomes (black, DHP+DPPC; red, DMPG+DPPC; 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

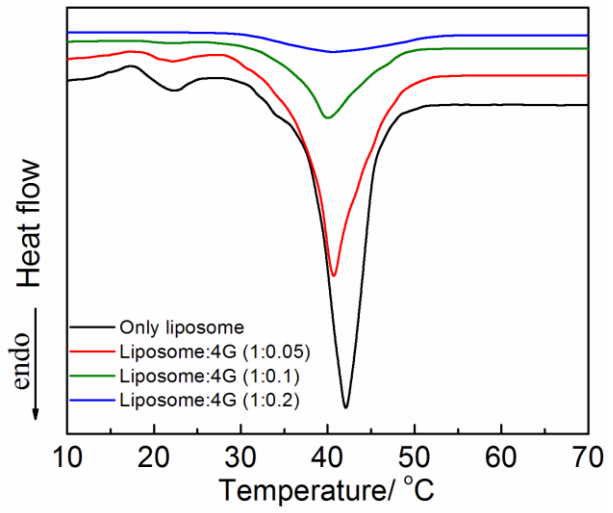

Figure 8. DSC thermogram of DPPEth+DPPC liposome at different concentrations of 4G PAMAM dendrimer. Liposome-4G PAMAM ratio is indicated in the figure (M/M) ratio.

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.

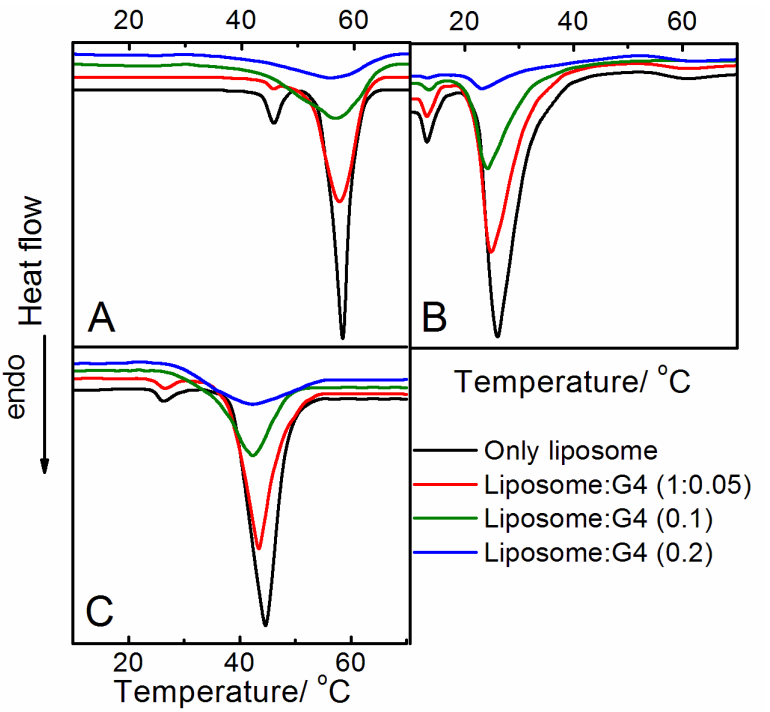

Figure 9.DSC thermogram of liposomes (panelA, DHP+DPPC; panel B, DMPG+DPPC and panel C, DPP+DPPC liposome) in presence and absence of PAMAM dendrimer of generation 4.

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.

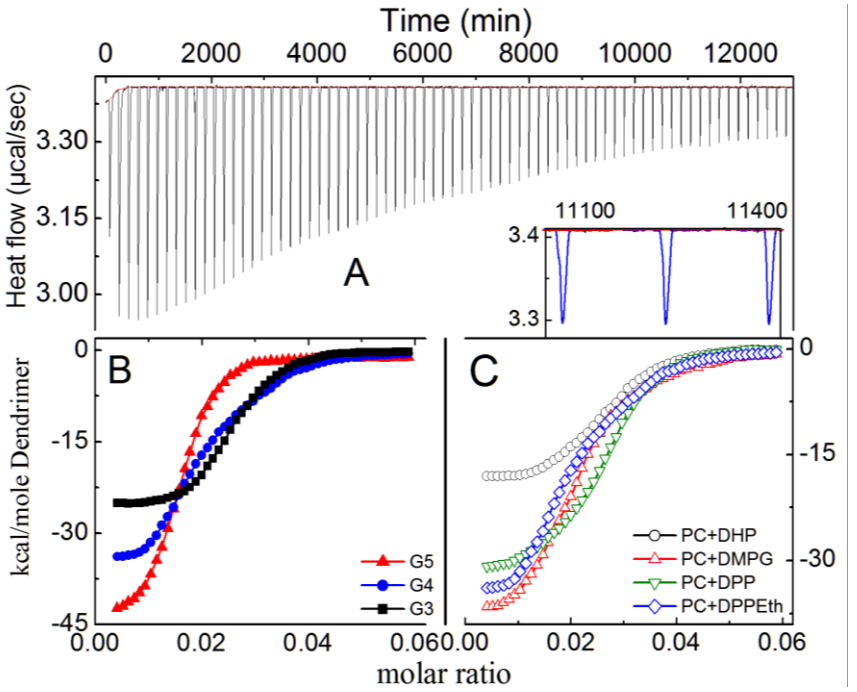

Figure 10. Isothermal titration calorimetric profiles of dendrimer - liposome interaction process. Panel A, the raw ITC data of DPPEth+DPPC liposome; PAMAM G4 dendrimer; Panel B, effect of dendrimer generation on DPPEth+DPPC liposomes and panel C, effect of lipid variation on G4 PAMAM dendrimers.

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

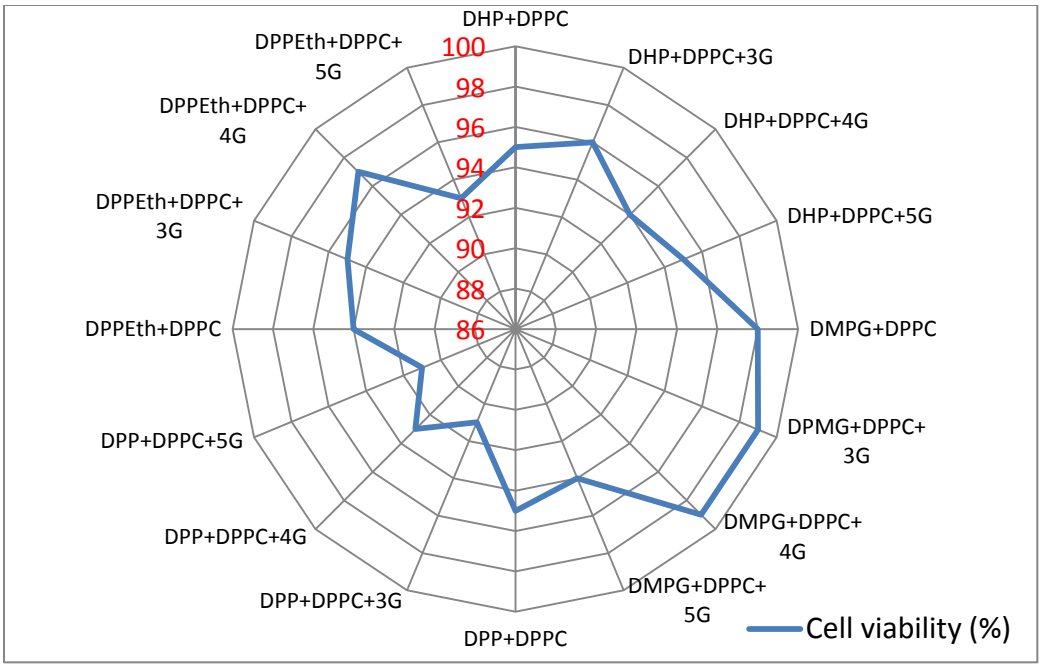

Figure 11. Dose response radar plot of various liposomes and dendrimer-liposome complexes on human blood cell lymphocyte.

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.

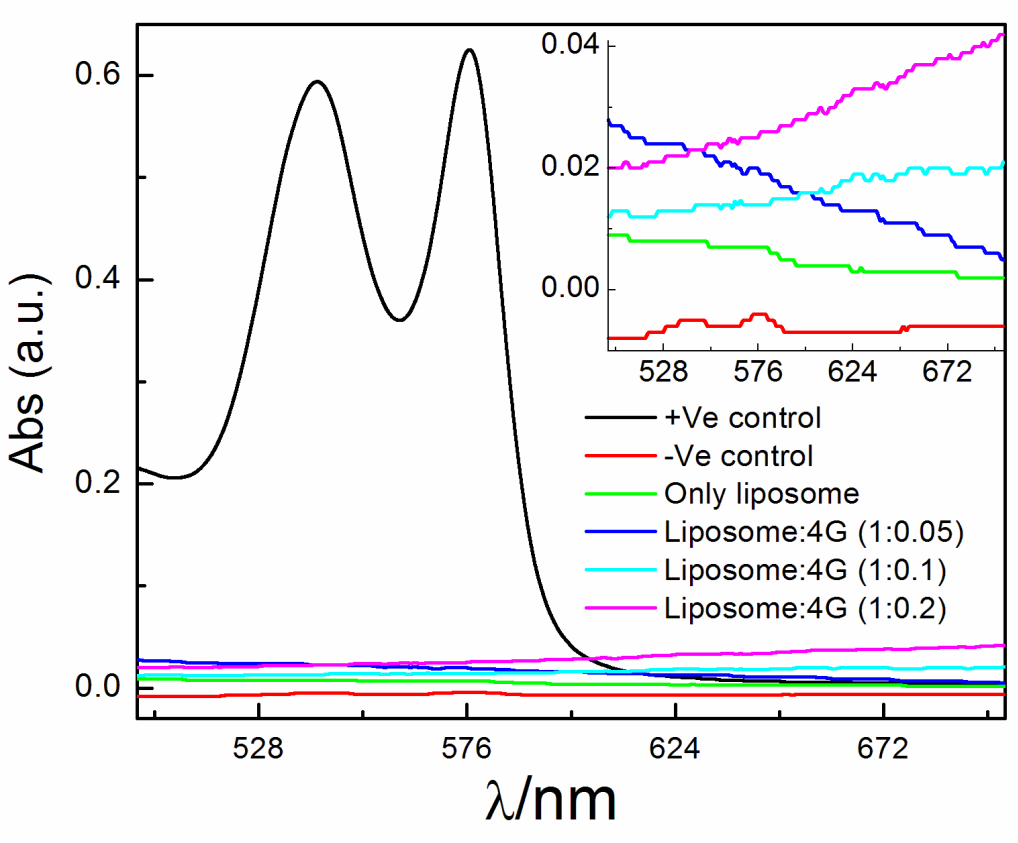

Figure 12. Hymolysis study of DPPEth liposome and dendrimer-liposome aggregates.

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.