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

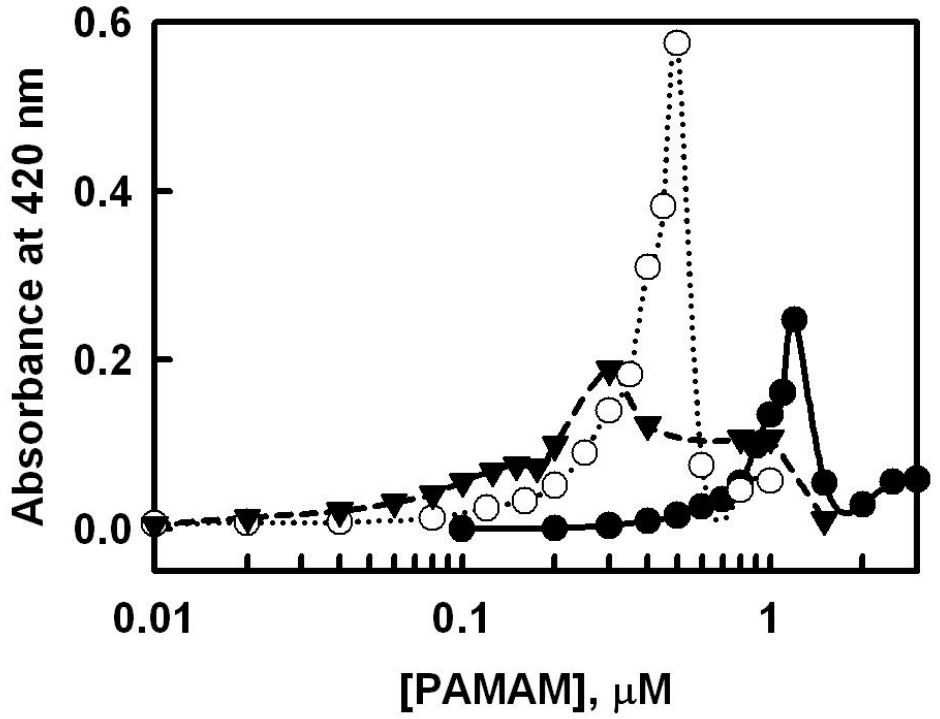

Figure 1. Effect of dendrimers on the abosorbance of the 0.1 m mol dm^{-3} (SLC+CHOL) liposome solutions (with respect to phospholipid). ●,2G PAMAM, O,4G PAMAM, ▲,6G PAMAM.

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

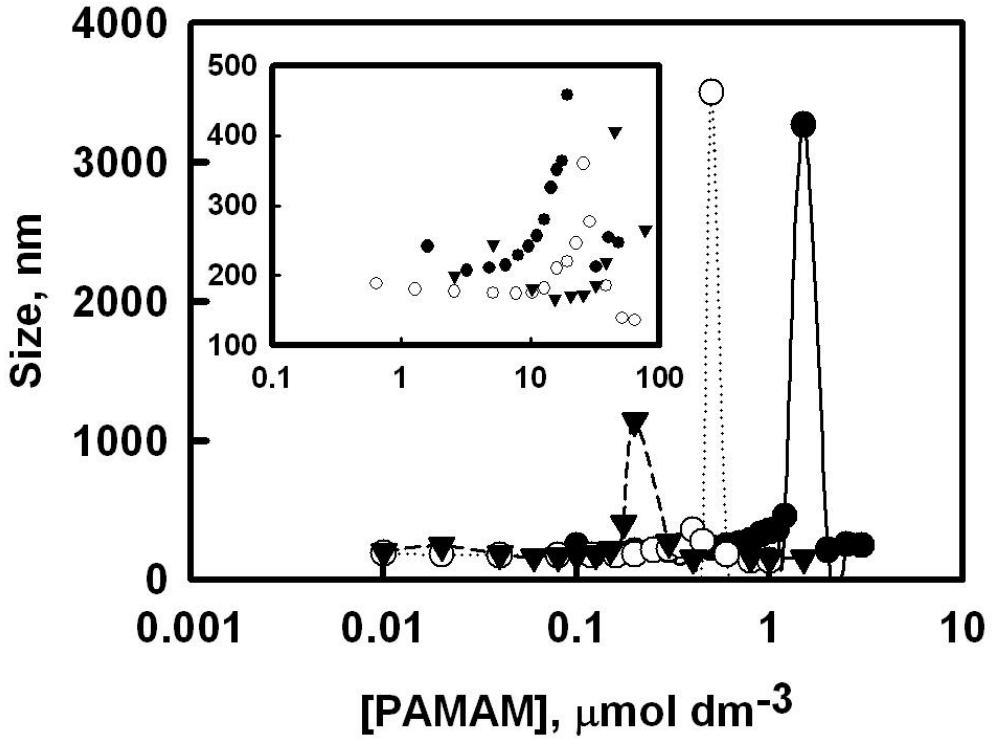

Figure 2. Effect of dendrimers on the size of 0.1 m mol dm^{-3} (SLC+CHOL) liposome solutions (with respect to phospholipid). ●,2G PAMAM, O, 4G PAMAM, ▲,6G PAMAM. Inset: Similar plots in terms of end group concentration of dendrimers.

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

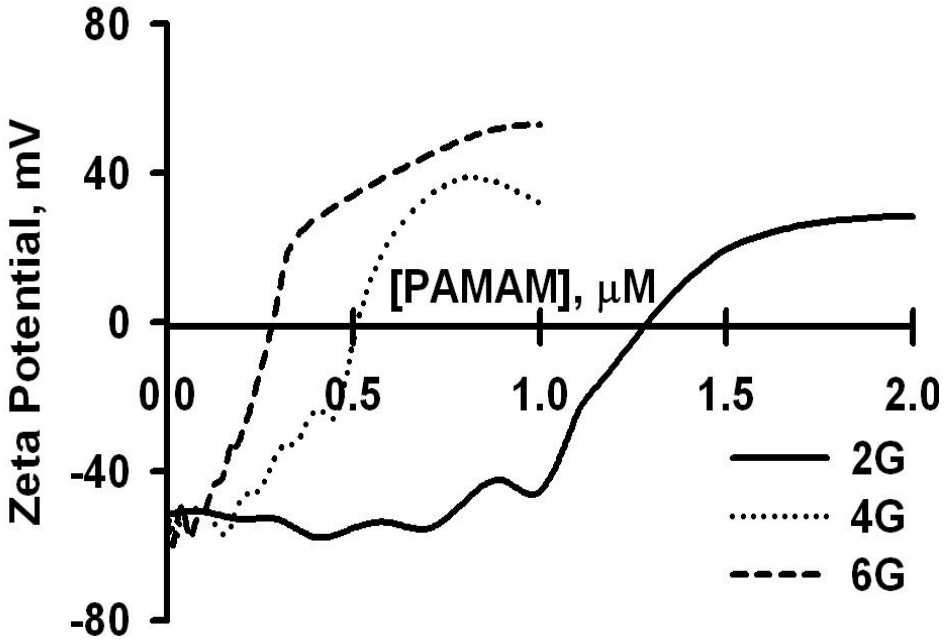

Figure 3. Variation of zeta potential upon the progressive addition of different generation dendrimers on a 0.1 m mol dm^{-3} (SLC+CHOL) liposome. Dendrimer generations are indicated in the graph.

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

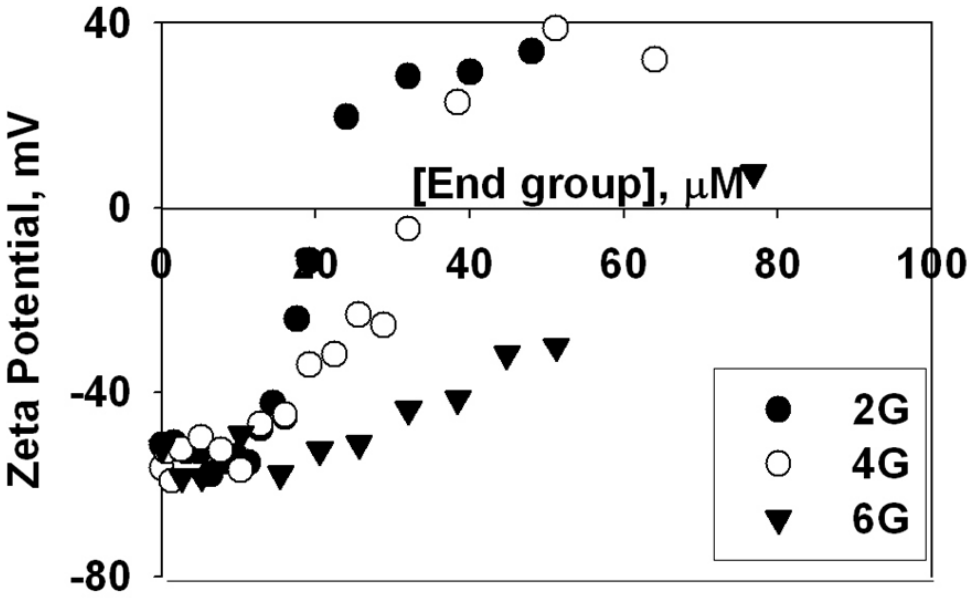

Figure 4. Effect of different generation PAMAM dendrimers on a 0.1 m mol dm^{-3} (SLC+CHOL) liposome. Dendrimer generations are indicated in the graph. Concentrations are expressed in terms of total end group concentration.

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.

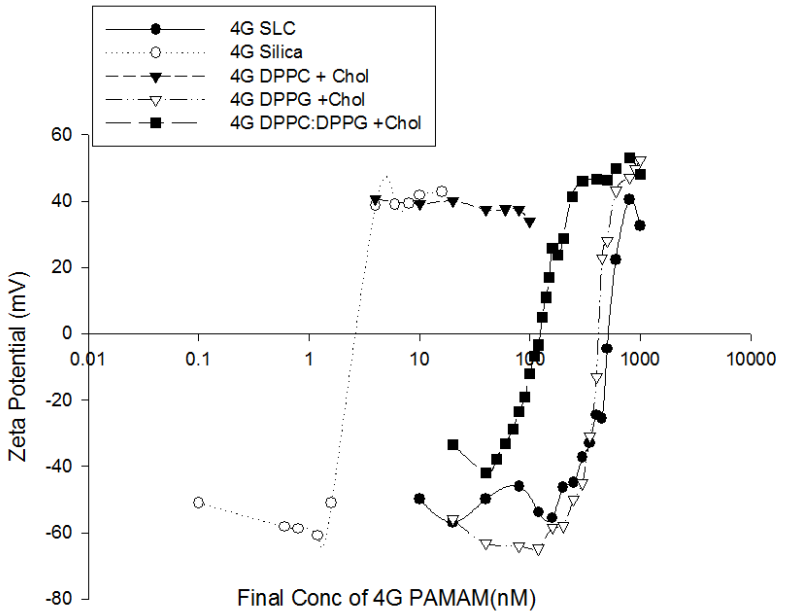

Figure 5. Comparative studies on the interaction of different substrates with 4G PAMAM by zeta potential measurements. Liposome concentration = 0.1 m mol dm^{-3} (with respect to the phospholipid). Similar strength (in terms of weight) of silica was used for a comparison.

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.

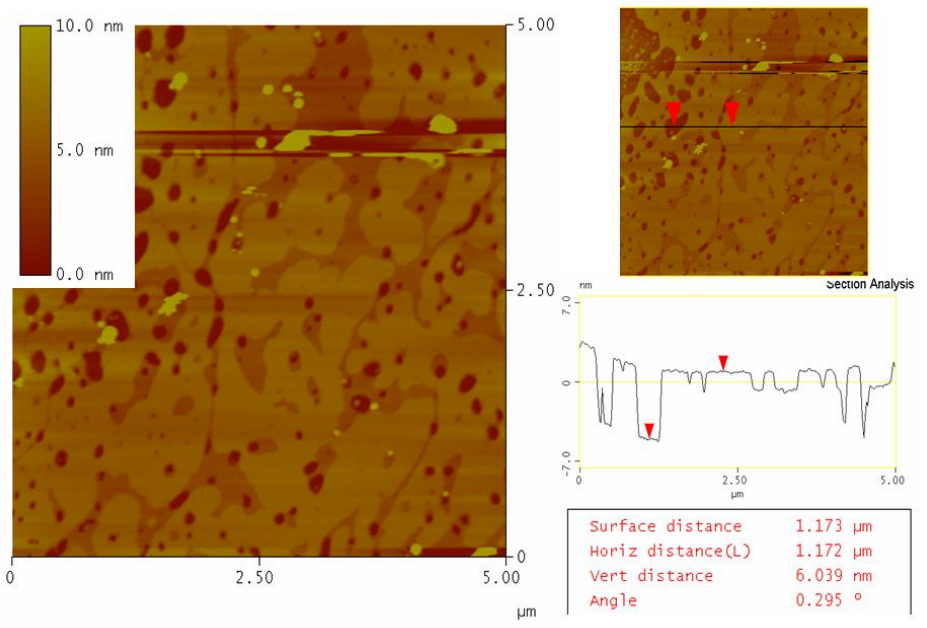

Figure 6. AFM image of a (DPPC+DPPG+CHOL) vesicle, fused on freshly cleave mica. Height analysis on the right reveals the bilayer formation.

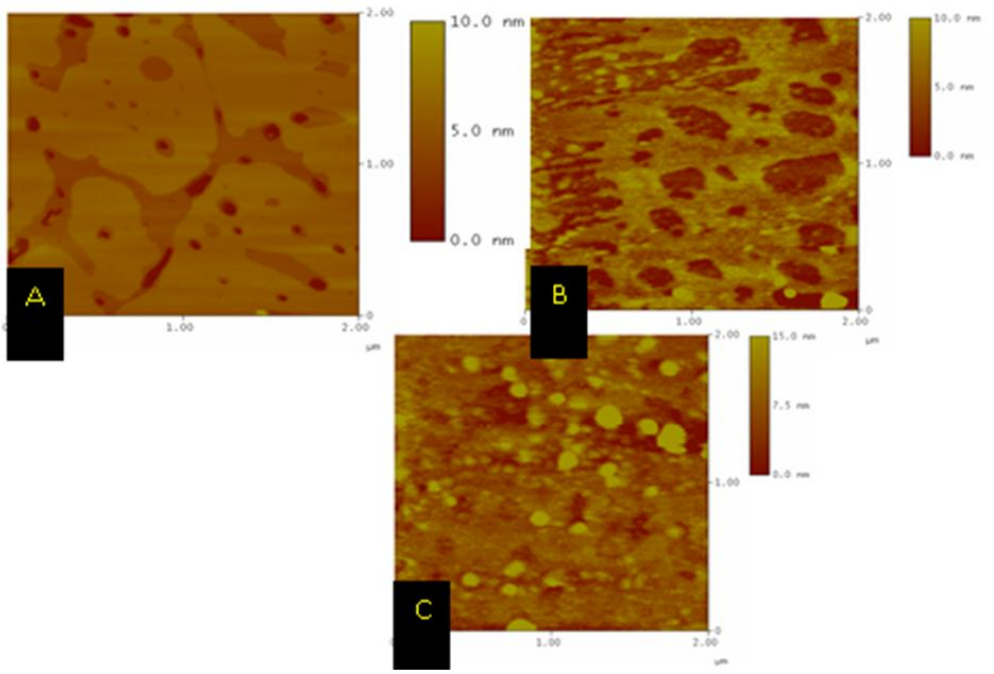

Figure 7. Effect of 6G PAMAM on the (DPPC+DPPG+CHOL) bilayer, fused on mica substrate. A. No dendrimer. B. 100 n mol dm^{-3} 6G PAMAM, C. 500 n mol dm^{-3} 6G PAMAM.

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.