Physiochemical Properties Associated with Transfection of Cationic Liposome-Based Gene Delivery Vectors in the Absence of DOPE

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Abstract
In this article we report on novel cationic lipids and their physiochemical characteristics. Intrinsic pKa of the cationic lipids were studied utilizing the surface charge active fluorescent probe TNS.TNS constitutes the aminonaphthaline-6-sulfonate based fluorescent dyes identified to benefit from a polarity-dependent fluorescence and have been used for long to probe structural modification in membranes. Langmuir monolayer of the novel lipids were analyzed on air/buffer interface by the film balance technique and the differential scanning calorimeter (DSC) was also explored to study the level of purity and the phase transition behavior of the lipid derivatives. The thermodynamic figures determined from π—A isotherm gave information regarding the critical temperature (T_c), which was in approximate conformity with the lipid phase transition temperature from gel-liquid state (T_m) found by both fluorescence polarization studies and differential scanning calorimetry.

1. Introduction

In recent years, the theory and principles of gene delivery for therapeutic function have been well documented. Nonetheless, numerous limitations in the development of strategies employed for effective delivery have perturbed ground breaking advancement in this area [1]. Even though viral vectors, weighed against non-viral vectors such as lipoplexes and polyp lexes, are far more competent in bringing about cellular transfection, inherent immunity problem compromise their protective use in vivo [2]. Although vulnerabilities consist of mutational insertion hazard and exposure, potentially leading to oncogenicity[3,4], large scale production of viral vectors as such might also represent an impediment. In addition to being less efficient, particularly in vivo [5], both lipoplexes consisting of a complex of nucleic acids and cationic lipids, and polyp lexes composed of cationic polymers and nucleic acids, are considered to be immunologically inert, and importantly safer than viral vectors for in vivo use. In view of the fact that they are also comparatively easy to produce and to amend chemically for enhancement of transfection efficiency, research efforts in this potential area have been significantly augmented over

the last decade [6]. To this end, the accomplishment of non-viral gene therapy will depend on a vital approach on novel efficient lipids. Thus novel cationic lipid derivatives have been synthesized and for rational development physical and chemical parameters were investigated in a systematic manner.

2. Materials and Methods

2.1. Materials

The synthetic methods of the cationic lipid derivatives are described elsewhere (Donkor et al., 2014). Monolayer experiments were performed in 40 mM tris buffer, pH 7.2, Tris HCl (99.8 + %), 2-(p-toluidino)-6-naphthalenesulfonic acid (TNS) was used for pKa studies, Water for buffer preparation was obtained from a Barnstead NANO pure ultrapure water system (Barnstead, Dubuque, IA). The calorimetric measurements were performed on a DSC Q100 Thermal Analyzer Control Software equipped with cell base 2 Gottingen Model CP225D Electrobalance (Sartorius, AG Gottingen) was used for weighing. Hermatic aluminum pans of the DSC were purchased from TA Instruments.

2.2. ¹H NMR Spectral Data of the Lipid Derivatives

N,N′−dilauroyl-1,3-diaminopropyl-2-[bis-(2-aminoethyl)]carbamate (LDVAD). $C_{32}H_{65}N_5O_4$ (MW 583.5): MS (Positive/Negative ES) m/z 590.3 [M+Li], ¹H NMR (400 MHz, CDCl$_3$, 20°C, TMS) δ 0.874 (t, 6H, CH$_3$), 1.21-1.26 (coherent peak, 32H, 8(CH$_2$)$_2$,), 1.66 (m, 4H, CH$_2$CH$_2$CO,), 2.18 (m, 8H, CH$_2$CO, *CH$_2$*NH$_2$), 3.24-3.74 (m, 8H, *CH$_2$*NHCO, OCON(CH$_2$)$_2$), 4.68 (m, 1H, (CH$_2$)$_2$*CH*OCO), 6.26 (t, 2H, HNCO).

N,N′−dimyristoyl-1,3-diaminopropyl-2-[bis-(2-aminoethyl)]carbamate MDVAD). $C_{36}H_{73}N_5O_4$ (MW 639): MS (Positive/Negative ES) m/z 646.4 [M+Li] , ¹H NMR (400 MHz, CDCl$_3$, 20°C, TMS) δ 0.92 (t, 6H, CH$_3$), 1.21-1.25 (coherent peak, 40H, 10(CH$_2$)$_2$), 1.55 (m, 4H, CH$_2$CH$_2$CO), 2.18 (m, 8H, CH$_2$CO, *CH$_2$*NH$_2$), 3.24-3.69 (m, 8H, *CH$_2$*NHCO, OCON(CH$_2$)$_2$), 4.76 (m, 1H, (CH$_2$)$_2$*CH*OCO), 6.36 (m, 2H, HNCO).

N,N′−dipalmitoyl-1,3-diaminopropyl-2-[bis-(2-aminoethyl)]carbamate (PDVAD). $C_{40}H_{81}N_5O_4$ (MW 695): MS (Positive/Negative ES) m/z 702.4 [M+Li]. ¹H NMR (400 MHz, CDCl$_3$, 20°C, TMS) δ 0.87-0.90 (t, 6H, CH$_3$), 1.26-1.30 (coherent peak, 48H, 12(CH$_2$)$_2$,), 1.60 (m, 4H, CH$_2$CH$_2$CO), 2.18 (m, 8H, CH$_2$CO, *CH$_2$*NH$_2$), 3.30-3.70 (m, 8H, *CH$_2$*NHCO, OCON(CH$_2$)$_2$), 4.74 (m, 1H, (CH$_2$)$_2$*CH*OCO), 6.42 (m, 2H, HNCO).

N,N′−distearoyl-1,3-diaminopropyl-2-[bis-(aminoethyl)]carbamate (SDVAD). $C_{44}H_{89}N_5O_4$ (MW 751): MS (Positive/Negative ES) m/z 758.5[M+Li]. ¹H NMR (400 MHz, CDCl$_3$, 20°C, TMS) δ 0.84 (t, 6H, CH$_3$), 1.21-1.25 (coherent peak, 56H, 14(CH$_2$)$_2$,), 1.56 (m, 4H, CH$_2$CH$_2$CO), 2.19 (m, 8H, CH$_2$CO, *CH$_2$*NH$_2$), 3.40-3.74 (m, 8H,

*CH$_2$*NHCO, OCON(CH$_2$)$_2$), 4.75 (m, 1H, (CH$_2$)$_2$*CH*OCO), 6.22 (m, 2H, HNCO).

N,N′−dioleoyl-1,3-diaminopropyl-2-[bis-(2-aminoethyl)] carbamate (OVDAD). $C_{44}H_{85}N_5O_4$ (MW 747), MS (Positive/Negative ES) m/z 754.177 [M+Li]⁻; ¹H NMR (400 MHz, CDCl$_3$, 20 °C, TMS) δ 0.86 (t, 6H, CH$_3$), 1.24-1.28 (coherent peak, 40H, 10(CH$_2$)$_2$), 1.62 (m, 4H, *CH$_2$*CH$_2$CO,), 1.96-2.25 (m, 16H, *CH$_2$*CH=CH*CH$_2$*; CH$_2$CO, *CH$_2$*NH$_2$), 3.20-3.54 (m, 8H, *CH$_2$*NHCO, OCON(CH$_2$)$_2$), 4.75 (m, 1H, (CH$_2$)$_2$*CH*OCO), 5.30 (m, 4H, CH=CH), 6.24 (m, 2H, HNCO).

2.3. pKa Studies

Liposomes composed of DOPC/Cholesterol/cationic lipid (50:45:5 molar ratios) at 2 mM total lipid concentration in 10 mM tris, 10 mM MES and 10 mM ammonium acetate, pH 7.5 were prepared. Each liposomal preparation was diluted to 0.1 mM total lipid concentration in 2 ml of the same buffer and 1 μM TNS in deionized water was added to give a total lipid-probe ratio of 50: 0.5. After stirring each sample for 30 minutes, TNS fluorescence was recorded with a carry eclipse fluorescence spectrophotometer at an excitation wavelength of 321 nm and emission wavelength of 445. The pH of each sample was measured immediately after fluorescence measurement.

The pH titration curves were fitted with PSIPlot software (Poly Software International, version 3, New York, USA) to the following equation,

$$F = A + \frac{B}{1 + 10^{C(pH - pKa)}} \qquad (1)$$

Where, A is the minimum TNS fluorescence, B indicates the difference between the maximum and minimum emission intensities, and C is a correction factor for the slope of the curve.

2.4. Monolayer Studies

The interfacial properties of the novel cationic lipid derivatives in isolation were investigated using the Langmuir film balance technique. A computer controlled KSV Minitrough film balance (KSV instruments LTD, Finland) equipped with two hydrophilic polyacetal made barriers and a platinum Wilhelmy plate was used to construct the Π-A isotherms of monolayers of the different cationic lipid derivatives at the air/water interface. In this study, a trough made of solid PTFE with dimensions of 1364 × 75 mm was used. The trough was filled with 145 ml of 40 mM tris buffer, pH 7.2, as the sub-phase. Π-A isotherms of the cationic lipid derivatives were constructed at different sub-phase temperatures maintained by the aid of an external water bath circulator. Cationic lipid solutions at 0.4 mg/mL concentration were prepared in chloroform, stored at –20°C and used within 3 hours of preparation. Each experiment was repeated 3-4 times in an open-air vibration-free environment to ensure isotherms reproducibility. Carefully, 25 μL aliquots

of cationic lipid solutions were applied drop by drop to the surface of the aqueous sub-phase with the aid of a Hamilton glass micro syringe. After an early delay time of 15 minutes, to ensure chloroform evaporation, the barriers were compressed at a constant speed of 10.0 mm/minute. Analysis of the constructed Π-A isotherms, phase transitions, monolayer collapse, and compressibility moduli of each compression isotherm were determined from the first derivative of the monolayer Π-A isotherms applying the equation below;

$$K = -A \left(\frac{\partial \pi}{\partial A} \right)_T \qquad (2)$$

Where K is the isothermal compressibility modulus of the monolayer, A is the area per molecule at the corresponding surface pressure (π). Values of the compressibility moduli at the monolayer collapse pressure were calculated from the plots of the compressibility modulus as a function of the mean molecular area.

Regarding the important functional role(s) that have been attributed to cationic lipids in delivering DNA-based biopharmaceuticals to damaged cells and tissues, understanding the structural basis for their biophysical behavior takes on added significance. Here, the influence of the changes in the acyl chain length on the interfacial behavior of the novel cationic lipid derivatives at various temperatures using Langmuir film balance is explored. This approach provides a means to investigate the influence of acyl chain length in the lipid derivatives over a range of molecular cross-sectional areas known to occur in membrane systems, while avoiding the changes in lipid mesomorphic behavior and aggregation state that often occur in model bilayer systems as temperatures are varied across the liquid-crystalline to gel phase transition temperature. The results not only supply insights into the in-plane elastic interaction of different lipid derivatives but also provide a foundation for understanding the physical environment produced when cationic lipid mix with other membrane lipids (Li et al., 2000).

The dependence of the transition enthalpy ΔH of the liquid-expanded to liquid condensed phase on the subphase temperature of the lipid derivatives was studied, applying a modified Clausius-Clapeyron equation [7]:

$$\frac{d\pi}{dT} = \frac{\Delta H}{T(A_E - A_C)} \qquad (3)$$

where T denotes the sub phase temperature, A_E represents the molecular area in the liquid expanded phase at the plateau onset, A_C is the molecular area at the liquid condensed phase extrapolated to the transition pressure, and $d\pi/dT$ refers to the temperature coefficient of the transition pressure.

2.5. Differential Scanning Calorimetry

The calorimetric measurements were performed on a DSC Q100 Thermal Analyzer Control Software equipped with cell base 2. The experiments were performed in a temperature range of −50ºC to 150ºC at a scan rate of 1º/min. In all the experiments the lipids were hydrated by heating constantly at 60ºC for 1 hour except for the purity test which was conducted in the absence of water. Appropriate amount of each of the lipids was weighed on Gottingen Model CP225D Electrobalance (Sartorius, AG Gottingen) directly into Hermatic aluminum pans of the DSC instrument after the samples were thoroughly dried under vacuum. Appropriate amounts of water were added and the pans were sealed and reweighed. The sealed pans were votexed, reweighed again to ensure no loss of water.

3. Results and Discussion

3.1. pKa Determination

The intrinsic pKa of the cationic lipid derivatives were determined utilizing the surface charge active fluorescent probe TNS [8, 9]. TNS constitutes the aminonaphthaline-6-sulfonate based fluorescent dyes identified to benefit from a polarity-dependent fluorescence and have been used for long to probe structural changes in membranes and proteins [10]. This class of fluorescent dyes exhibits an increased quantum yield upon transfer from a polar, example, aqueous solution, to a nonpolar environment, example phospholipid membranes. In the system described here, the fluorescence intensity of TNS is further enhanced in a pH-dependent fashion upon incorporating bivalent amine cationic lipid derivative in the DOPC/Cholesterol vesicles. This enhancement can be attributed to two mechanisms, (1) an increased quantum yield due to reduction in polarity of the interfacial region of DOPC/Cholesterol upon inclusion of protonated cationic lipids and (2) stoichiometric and affinity reasons as more of the anionic probe is expected to associate with the vesicle in the presence of positively charged cationic lipids.

Titration curves of DOPC/Cholesterol liposomes containing 5% of each of the five cationic lipid derivatives are presented in Figure 1. Non-linear curve fitting of the cationic lipid titration curves gave an intrinsic pKa values ranging from 6.5 to 7.5. In principle, all the derivatives were expected to have the same intrinsic pKa value due to the fact that all the five lipid derivatives share the same head group and linker region. When these lipids are diluted within the phospholipid bilayer, their acyl chains will be embedded in the hydrophobic domain of the bilayer and their fairly separated bivalent primary amine head groups could experience the same degree of pH-dependent protonation and deprotonation. This in fact should be the case for the five cationic lipid derivatives and therefore, any variation of the intrinsic pKa values for these series of lipids bearing the same head group is actually an indication of a lipid mixing problem, that is, phase separation and accumulation of different lipids in certain domains. In other words, the lipids of interest were not diluted any more within the phospholipid bilayer and one would end up

determining a pKa value in the range within the apparent and not intrinsic pKa.

However, the intrinsic pKa values of the cationic lipid derivatives diluted in pH insensitive DOPE/Cholesterol vesicles were expected to differ from their apparent pKa values in vesicles formulated with these lipids alone. In addition, the intrinsic pKa values obtained did not explain the different lipid–DNA interactions and transfection profiles of the cationic lipid derivatives[11]. Therefore, an experiment to estimate the intrinsic pKa of the cationic lipids pure vesicles was considered based on monitoring variation of particle size distribution of the cationic lipids as a function of pH. All the cationic lipid derivatives, LDVAD, MDVAD, PDVAD and SDVAD, resulted in aggregates at pH 8 (Data not shown), that is, the pH at which the hydrated liposomal lipid derivatives began to be deprotonated. This implies that their intrinsic pKa values are higher than that of the apparent pKa. Small unilamelar vesicles (SUV) prepared with the unsaturated oleoyl derivative aggregated at approximatelypH 9.5, resulting in higher fraction of the molecules deprotonated just above pH 9. The intrinsic pKa value of the ionizable dioleoyl derivative correlates with its capacity to mediate considerable in vitro transfection activity as well as its ability to complex plasmid DNA compared with the other lipid derivatives.
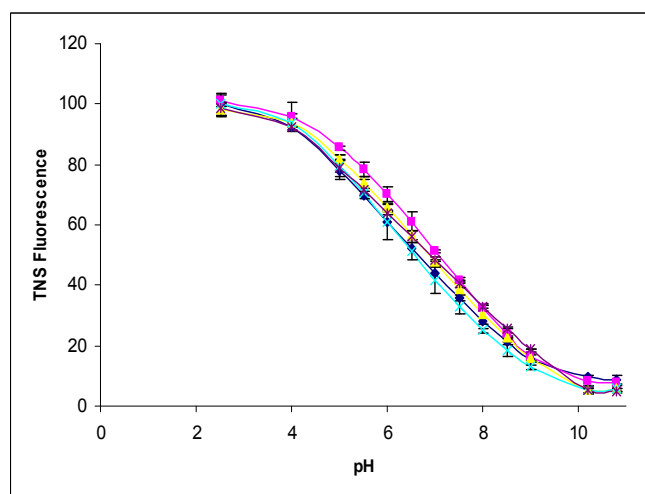

Fig. 1. *pH titration curve of DOPE/Cholesterol (55:45) vesicles containing 5 % mole of cationic lipid derivatives. Data points indicated are the mean and standard deviations obtained from duplicate experiments. Error bars are smaller than data points unless presented otherwise. Continuous line is the fit curve obtained by non-linear curve fitting of the experimental data points. (◂) LDVAD, (♦) MDVAD, (■) PDVAD, (▾) SDVAD, (•) ODVAD. Goodness of fit statistics was assessed with 95% confidence interval.*

3.2. Monolayer Studies

The interfacial properties of the novel cationic lipid derivatives in isolation, at different temperatures were investigated using the Langmuir film balance technique. Aliquots of cationic lipids in chloroform were spread on the surface of the same buffer system used to prepare the aqueous dispersions for transfection studies [11] and for the biophysical characterization studies, that is, 40 mM Tris buffer, pH 7.2. Monolayers of the saturated derivatives either exhibited an all liquid-condensed state or a two-dimensional phase transition toward a liquid-condensed state in their π-A isotherms at most temperatures studied except for the LDVAD, and ODVAD derivatives. More specifically, the compression isotherm of the shortest carbon chain derivative, LDVAD and the unsaturated ODVAD with a double bond between 9 and 10 carbon atoms exhibited an all liquid-expanded phase transition observed at temperatures between 15 ^{o}C and 37 ^{o}C. The surface pressure π was measured as a function of the molecular area A (π-A isotherms) at different temperatures between 5°C and 45°C for all the lipid derivatives. Figure 2, 3 & 4 show the temperature-dependent behavior of the lipid derivatives of monolayers on subphase with pH 7.2. Two dimensional phase transition of a liquid-expanded (chain-disordered) to liquid condensed (chain-ordered) nature were observed at all the temperatures used. The sharpness of the two-dimensional transitions evidently depicted the homogeneity of the acyl chain composition in the cationic lipid derivatives [12]. Only liquid expanded behavior, that is., chain-disordered, was observed (between 15°C and 37°C) for both LDVAD and ODVAD, indicating highly fluid nature of the two cationic lipid derivatives. At lower temperatures (< 10°C), the π-A isotherms for LDVAD showed pronounced change of slope, manifesting a second order transition from a condensed phase with tilted molecules to a condensed phase with non-tilted molecules [13]. The phase transition pressures shifted to higher pressures with increasing temperature and at temperatures above 5°C, the kink in the isotherm of LDVAD was replaced by a small plateau, which is attributed to a first order phase transition. The results evidently show that, the length of the saturated acyl chain 12 or 14 carbons or introducing a double bond in the acyl chain length (18:1), significantly alters the two dimensional phase behavior of the cationic lipid derivatives.
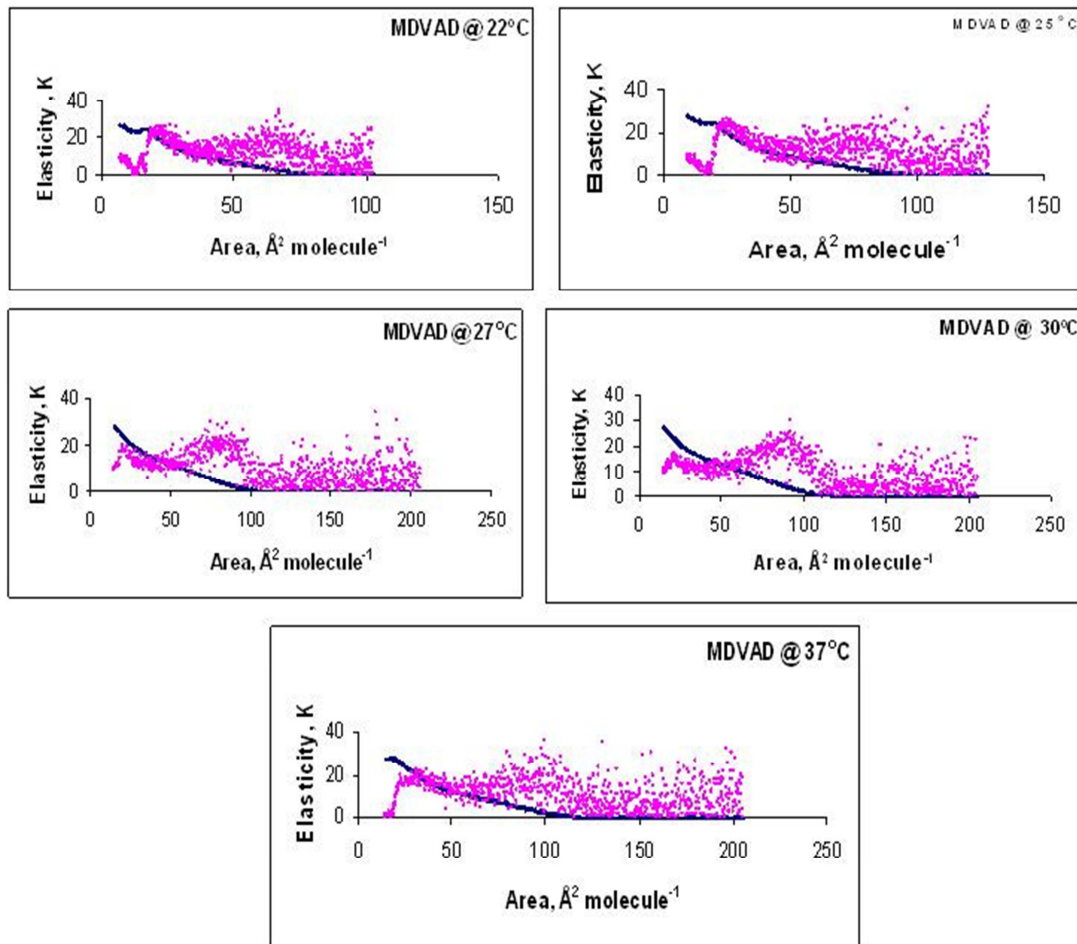
Fig. 2. *Plot of compressibility modulus (K) versus mean molecular area. The plots were used to determine surface transition pressure (Π_t) of main transition (liquid expanded – liquid condensed), and the liquid expanded area and liquid condensed area A_E and A_C respectively of the MDVAD cationic lipid monolayers on tris buffer, pH 7.2.*
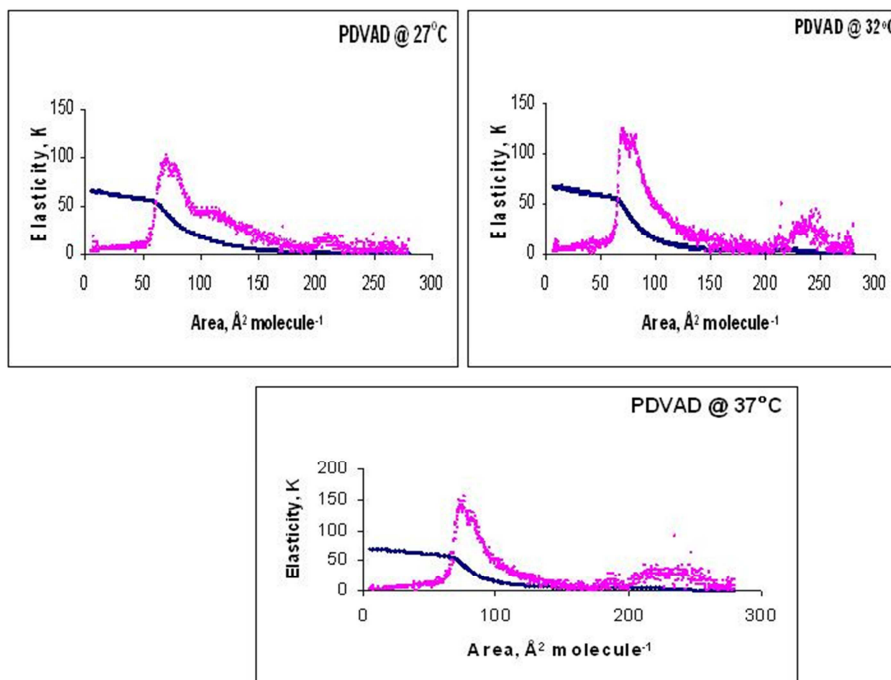

Fig. 3. *Plot of compressibility modulus (K) versus mean molecular area, to determine surface transition pressure (Π_t) of main transition (liquid expanded – liquid condensed), and also A_E and A_C of the PDVAD cationic lipid monolayers on tris buffer, pH 7.2.*
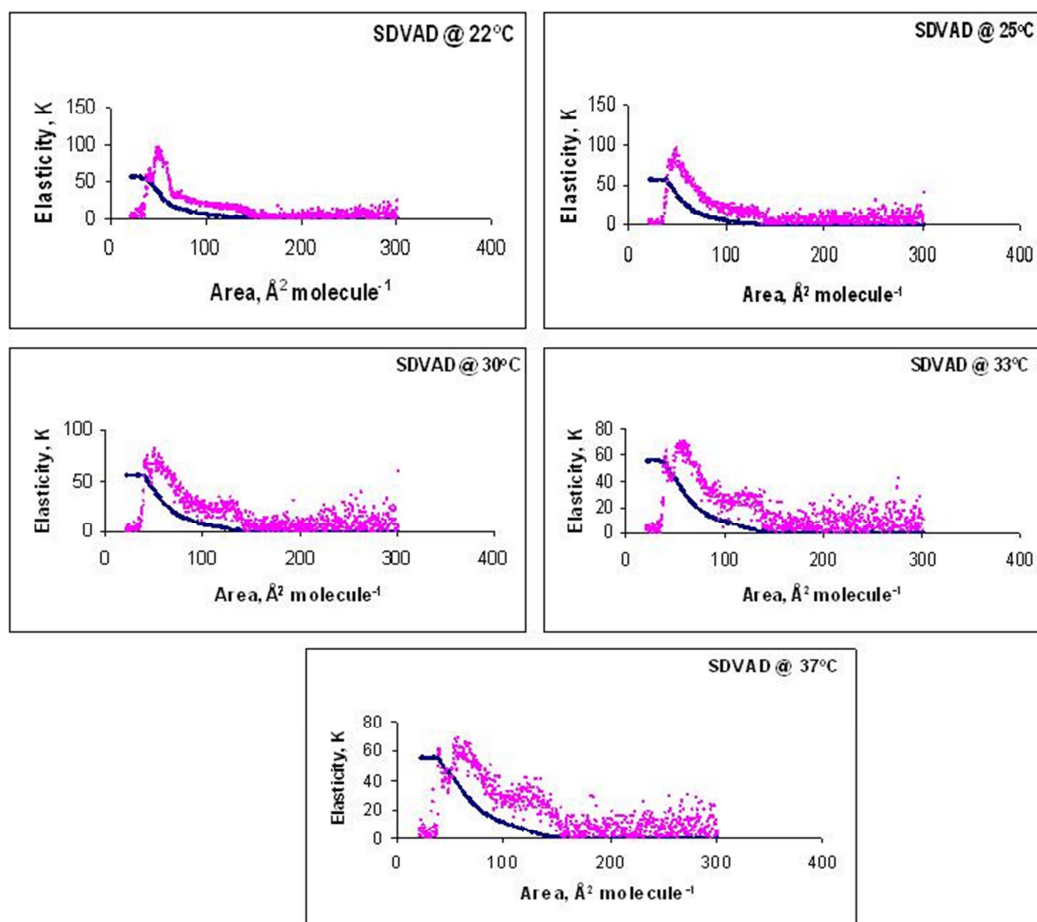
Fig. 4. Plot of compressibility modulus (K) versus mean molecular area, to determine surface transition pressure (π_t) of main transition (liquid expanded – liquid condensed), and also A_E and A_C of the SDVAD cationic lipid monolayers on tris buffer, pH 7.2.

Upon reaching 16 carbons in length, additional increase had minor impact on the two dimensional phase transition behavior with respect to transition onset pressure. This is illustrated quantitatively by comparing the lowest temperature (T_o) at which the lipids exhibit a two-dimensional phase transition, which were determined from plots of surface phase transition onset pressures versus temperature. Extrapolation to the x-intercept provided the distinctive T_o value for each of the cationic lipid derivatives (Figure. 5). Generally, the lower the T_o value, the weaker the intermolecular forces that stabilize the monolayers [14]. Examination of T_o values (Table 2) indicates that, the saturated acyl chain of 12, 14 and 16 carbons have similar T_o values at temperatures (between 15 ^{o}C and 37 ^{o}C). Comparison does reveal differences of monolayer T_o and bilayer T_m values. The bilayer T_m of the cationic lipid derivatives which were determined from anisotropy analysis do show a definite upward trend with increasing acyl chain length that is not seen in the monolayer T_o values for carbon chain of 14:0, 16:0, and 18:0 of the cationic lipid derivatives. This difference most likely reflects the net effect that chain interdigitation had on producing increasingly thicker, more stable bilayer gel phases for the cationic lipid derivatives; whereas, in the monolayers, no such interdigitation occurred.
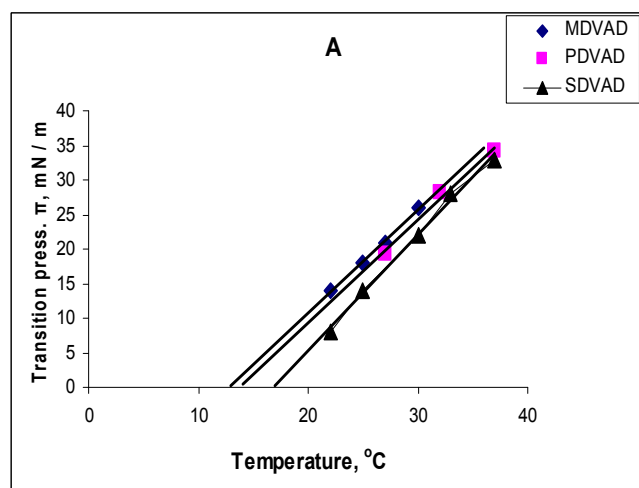

Fig. 5. Surface pressure (π_t) of main transition (liquid expanded – liquid condensed) of cationic lipid monolayers on tris buffer, pH 7.2 versus temperature. (■) MDVAD, (♦) PDVAD, (◄) SDVAD.

The dependence of the transition enthalpy of the liquid expanded (chain-disordered) to liquid condensed (chain-ordered) phase transition on the subphase temperature was studied by applying the modified Clausius-Clapeyron equation:

$$\frac{d\pi}{dT} = \frac{\Delta H}{T\left(A_E - A_C\right)} \qquad (4)$$

where T denotes the subphase temperature, A_E represents the molecular area in the liquid expanded phase at the plateau onset, A_C is the molecular area at the liquid condensed phase extrapolated to the transition pressure, π and $d\pi / dT$ refers to the temperature coefficient of the transition pressure. The plot of absolute enthalpy (ΔH) versus temperature (Figure 6) yielded the critical temperature (T_C) by linear extrapolation toward |ΔH| = 0. The critical temperature values found for the cationic lipid derivatives (Table 1) are the temperatures above which no liquid condensed phases exist. The Tc values were found to roughly correlate with the phase transition temperature values determined from differential scanning calorimetric experiments.
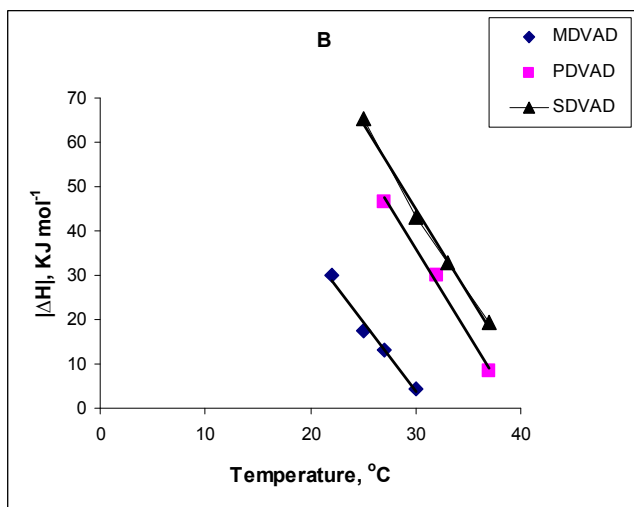

Fig. 6. *Transition enthalpy (|ΔH|) as a function of temperature of the cationic lipid derivatives on tris buffer, pH 7.2. ΔH was calculated by use of a modified Clausius-Clapeyron equation (■) MDVAD, (♦) PDVAD, (◄) SDVAD*

Representative compression isotherms characteristics of the cationic lipid derivatives are shown in (Table 2). At 22 and 37°C the isotherms of four of the analogues LDVAD, MDVAD, PDVAD, and ODVAD formed monolayers that existed in an all liquid-expanded state (Figure not shown) with molecular collapse area and pressure shown by the first derivative of dπ/dA at 65.94 Å^2, 34.91 mN/m; 20.89 Å^2, 27.84 mN/m; 49.49 Å^2, 58.8 mN/m; 64.62 Å^2 , 36.24 mN/m in that order, with the exception of the SDVAD analogue which exhibited an all liquid condensed state at a collapse area and pressure at 49.97 Å^2, 40.8 mN/m. Interfacial compressibility modulus (K) values of the monolayer prepared from the different cationic lipid derivatives, calculated at molecular collapse area are represented in Table 2.

Drawing conclusion relating to the compressibility of lipids with different molecular dimensions using compressibility modulus K has been indicated to be uncertain [5]. Therefore, for evaluation purpose between monolayers with changeable collapse areas, the rate of change of surface pressure versus the change of mean molecular area, referred

to as dπ/dA of the monolayer could be used as a pointer of monolayer state and compressibility. In this light, taking into account both the compressibility modulus and the corresponding first derivative of π versus area values, the five cationic lipid derivatives showed similar and low compressibility and high elasticity, with LDVAD and ODVAD having the lowest slope, dπ/dA of approximately. 1.0 mN.m^{-1}Å^{-2}, indicative of high fluidity and elasticity.

3.3. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry is especially suitable for the determination of purity and hydration properties of lipid bilayers. From DSC experiments several parameters could be obtained such as: (1) midpoint melting temperature of the lipid bilayers, (2) enthalpy of fusion, (3) the number of bound water molecules (unfreezable water) per molecule of lipid in the gel state if the melting temperature of the water is below that of the lipid investigated or in the liquid crystalline state if it is above the melting temperature of the lipid [15], (4) the melting temperature of the lipid as a function of hydration, (5) gel to liquid phase transition of the lipid bilayer (Figure 7). Here, we have used differential scanning calorimeter (DSC) for the investigationof thermodynamic parameters of a novel class of cationic lipid derivatives, LDVAD, MDVAD, PDVAD, SDVAD and ODVAD respectively as indicated (Table 3). We further investigated the compositional aspect of the lipid hydration (Manuscript in preparation). It has been previously shown that the number of unfreezable water molecules generally increases with the size and charge of the polar headgroup of the lipid molecule and with the degree of unsaturation of the hydrocarbon chains, but inter and intramolecular hydrogen bonding may also compete with the hydration. Here, we investigated the lipid derivatives, LDVAD, MDVAD, PDVAD, SDVAD and ODVAD, all having the bis[(aminoethyl)]-amine polar headgroup withvariable acyl chain length.

The membrane fluidity of the cationic liposomes prepared from the bis[aminoethyl]-amine derivatives was scrutinized through investigating the gel to liquid crystalline phase transition of these bilayers by means of differential scanning calorimetry. In this transition, the highly ordered hydrocarbon chains in all-trans conformation in the low temperature gel phase undergo a transition to a disordered, mixed cis-trans conformation in the high temperature liquid crystalline phase. The importance of acyl chain state in the bilayer arises from the fact that numerous phenomenon during the lipofection chain can efficiently advance when the temperature is above the phase transition temperature of the lipid bilayer. Examples of these processes include preparation of cationic liposomes, formation of lipoplexes and escape from endosomal degradation. All the presently used cationic lipids, with a few exceptions, entail the presence of the neutral phospholipid DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) for high transfection activity [16, 17]. Homogeneous integration of this lipid requires that the

cationic lipid acyl chains exist in a disordered fluid state. Rigidity of the cationic lipid will result in phase separation of the different lipid components upon storage. Moreover, membrane fluidity of cationic liposomes, along with other properties, governs the fusogencity of cationic liposomes with each other as well as with biological membranes. It has been verified that significant restructuring of cationic lipid and fusion of liposomes occurs during the interaction of cationic liposomes with plasmid DNA [18]. Therefore, only cationic lipids with phase transition temperatures below the experimental temperature will promote efficient complexation and condensation of plasmid DNA during the lipoplex formation process. In addition, interaction and fusion of lipoplexes with biological membrane, such as endosomal membrane, and the subsequent delivery of DNA cargo into the cytoplasm is essential for efficient *in vitro* transfection of plasmid DNA. Rigidity of the lipid bilayer is believed to suppress the fusogenic events involved during this process thereby, resulting in degradation of lipoplexes in the endosome and failure to induce significant *in vitro* transfection.

In Fig. 8 is presented the DSC heating scans of hydrated bilayers of the five cationic lipid derivatives in 40 mM tris buffer, pH 7.2. The thermodynamic parameters extracted from these scans are summarized in Table 3. The shortest acyl chain derivative, carbon 12 (LDVAD), and the monounsaturated derivative, ODVAD exhibited all fluid state. These phase transition temperatures appear to increase steadily as the length of the acyl chain is increased from 14 to 18. As shown in Table 3, the degree of temperature for the phase transition increases with increasing carbon chain length. The difference in the main phase transition temperature was approximately 10 degrees between MDVAD and PDVAD. Increasing the chain length by an additional 2 carbons resulted in only 4 degree increase in the phase transition temperature. Unlike the main phase transition temperature, the transition enthalpy (ΔH) of bilayers prepared with the saturated lipids increased in a linear fashion as the length of the hydrocarbon acyl chain length increased. On average, increasing the acyl chain length of the saturated derivatives of carbon 14 to 16 was accompanied by 400.03 ± 0.89 kJ/mole. Addition of 2 more carbons to 18 resulted in an increase of the enthalpy to about 1500 ± 0.02 kJ/mole.
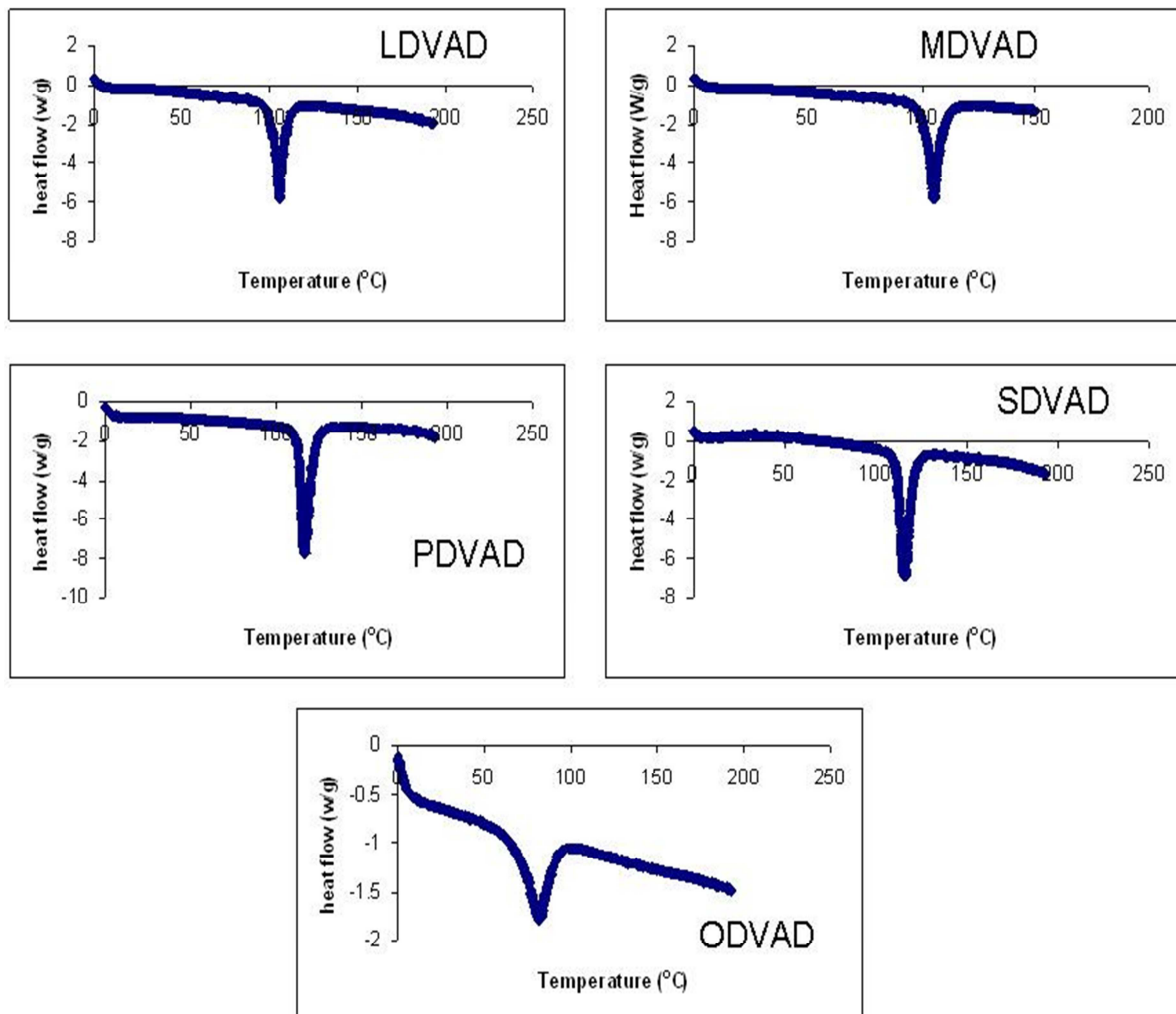

Fig. 7. *Representative heat of fusion thermograms (purity experiment) of the cationic lipid derivatives by differential scanning calorimetry (DSC)*
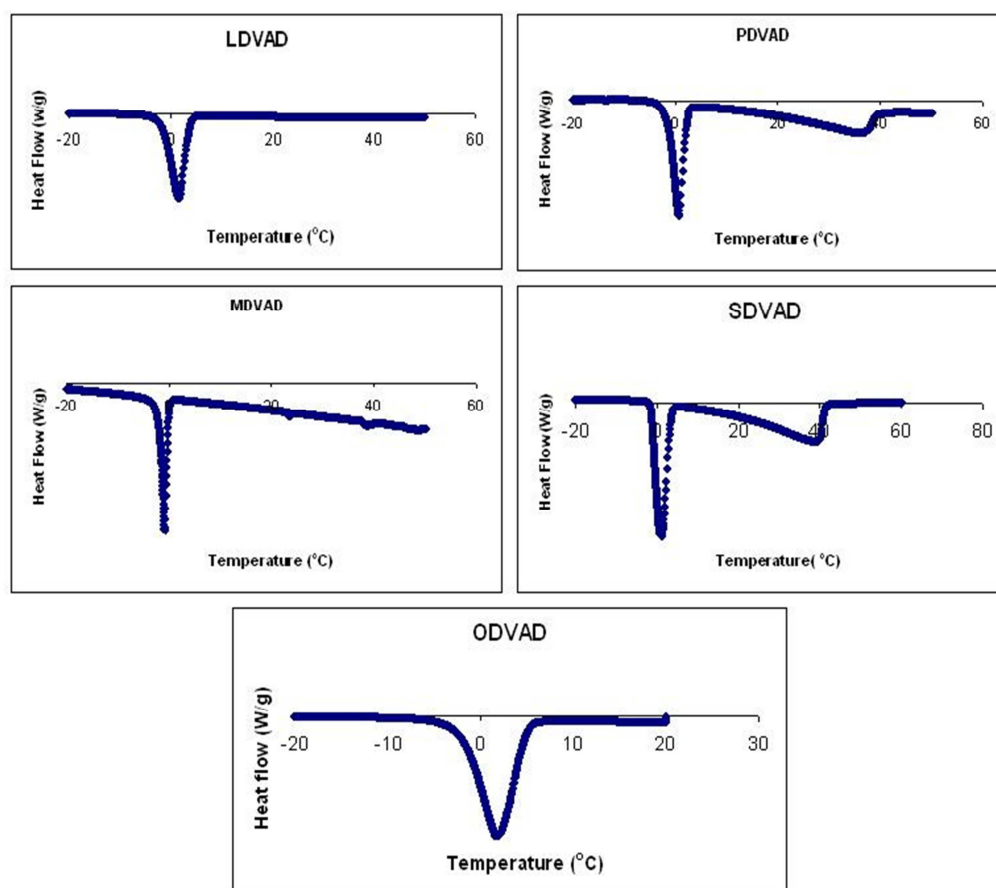
Fig. 8. *Phase transition thermograms of the cationic lipid derivatives determined by differential scanning calorimetry (DSC).*

Table 1. *Thermodynamic phase characteristics of the cationic lipid derivatives*

Cationic Lipid	T_o (°C)	T_c (°C)	$d\pi/dT$
LDVAD (12:0)	--------	--------	-------
MDVAD (14:0)	12.8	31.1	1.50
PDVAD (16:0)	13.7	39.6	1.49
SDVAD (18:0)	16.9	42.4	1.68
OVDAD (18:1)	-------	-------	-------

Values for $d\pi/dT$, and T_o (°C) were calculated from the slopes and x-intercepts respectively, of the best fit lines to the phase transition onset surface pressure versus temperature plots. Values for T_c (°C) were calculated from the x-intercept of the transition enthalpy ($|\Delta H|$) versus temperature plot.

Table 2. *Monolayer parameters of the bis[(aminoethyl)]-amine cationic lipid derivativesa*

Cationic Lipids	MmA (Å²)		Π^c (mN/m)		K (mN/m)		$d\Pi/dA^b$		Phase state	
	22 °C	37 °C	22 °C	37 °C	22 °C	37 °C	22 °C	37 °C	22 °C	37 °C
LDVAD	65.32 ± 1.59	65.94 ± 1.95	34.13 ± 0.64	35.51 ± 0.98	65.09 ± 4.20	68.75 ± 1.29	0.86 ± 0.10	0.81 ± 0.02	L_1	L_1
MDVAD	20.27 ± 1.78	22.89 ± 1.25	27.05 ± 0.93	27.84 ± 0.77	25.78 ± 3.02	33.09 ± 0.69	2.62 ± 0.09	2.3 ± 0.19	L_1	L_1
PDVAD	49.49 ± 1.95	52.88 ± 0.23	58.86 ± 0.95	61.06 ± 1.59	63.7 ± 2.71	72.54 ± 5.29	2.15 ± 0.10	2.17 ± 0.14	L_1	L_1
SDVAD	49.97 ± 0.26	54.4 ± 1.51	40.86 ± 1.15	40.94 ± 1.45	49.24 ± 13.56	58.83 ± 8.10	2.4 ± 0.06	3.01 ± 0.10	L_2	L_2
ODVAD	64.6 ± 1.95	65.53 ± 1.85	36.24 ± 1.52	36.45 ± 1.22	75.5 ± 1.29	80.9 ± 1.54	1.03 ± 0.04	1.05 ± 0.08	L_1	L_1

L_1 denotes liquid-expanded phase state.
L_2 denotes liquid-condensed phase state.
MmA denotes mean molecular area expressed in Å² per molecule.
Derivatives [a]measured in 40 m M tris buffer pH 7.2. All parameters are 'onset' transition values.
$d\Pi/dA^b$ values are at the collapse pressure or other two dimensional phase transition.
Π^c phase transition denoted by a maximum in the $d\pi/dA$ plot as a function of mean molecular area.

Table 3. *DSC Main Phase Transition Parameters of the Cationic Lipids.*

Cationic Lipid	Phase Transition Tempt, T_m(°C)	ΔH (KJ mol⁻)	ΔH (J/g)
LDVAD	< 5	-------	------
MDVAD	23.37	1.592	2.492
PDVAD	35.3	401.054	572.2
SDVAD	39.15	1500.05	3663
OVDAD	< 5	-------	-------

4. Conclusion

The observation that several lipid mixing and fusogenic actions take place during the processes of liposome preparation, lipoplex formation and endosomal escape point to the consideration toward the importance of investigating the physicochemical properties of cationic lipids in isolation such as, phase state, interfacial elasticity and extent of protonation.

In the current study, the assessment of membrane fluidity by means of Langmuir monolayer techniquesand differential scanning calorimetry studies indicated that the twotransfection efficient lipid derivativessynthesized, exhibited a phase transition below physiological temperature. The current findings imply that the bilayers of MDVAD and ODVAD exhibited an all liquid expanded state below physiological temperature.

In conclusion, in a novel approach, a relationship between in vitro transfection activity of cationic lipids and their interfacial elasticity has been established by means of Langmuir film balance technique and differential scanning calorimetric analysis. Monolayers of the transfection active compounds, MDVAD and ODVAD analogs exhibited highly elastic behavior accompanied by low compressibility modulus expressed in mN/m and low dπ/dA values expressed in mNm⁻¹.A⁻². Similar conclusion has been deduced from transfection active agents and interfacial properties of other dialkylamidopropane-based cationic lipids [19, 20]. Even though, the cationic lipid analog, LDVAD exhibited an elastic behavior and has its phase transition temperature below physiological temperature, its transfection studies fell below expectation and it is a subject of future studies in our laboratory.

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Abbreviations

DOPE, dioleoylphosphatidylethanolamine; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; LDVAD, N,N'-dilauroyl-1,3-diaminopropyl-2-carbamoyl-bis[(aminoethyl)]-amine; other cationic lipids follow the same nomenclature as LDVAD but with different acyl chains. MDVAD, dimyristoyl derivative; PDVAD, dipalmitoyl derivative; SDVAD, distearoyl derivative; ODVAD, dioleoyl derivative; N/P, Nitrogen/Phosphate; T_m, maximum transition temperature; TNS (6-(p-toluidinyl)naphthalene-2-sulfonic acid).

References

[1] Akao, T., Nakayama, T., Takeshia, K., Ito, A. 1994. Design of a new cationic amphiphiles with efficient transfection ability. *Biochem. Mol. Biol. Int.* 34, 915-920.

[2] Aljaberi, A., Chen, P., Savva, M. 2005. Synthesis, in vitro transfection activity and physicochemical characterization of novel N,N'-diacyl-1,2-diaminopropyl-3-carbamoyl-(dimethylaminoethane) amphiphilic derivatives. Chemistry and Physics of Lipids. 133, 135-149.

[3] Anderson, W. F. 1992. Science. 256, p. 808

[4] Benatti, C. R., Ruysschaert, J., Lamy, M. T. 2004. Structural characterization of diC₁₄-amidine, a pH-sensitive cationic lipid used for transfection. *Chem. Phys. Lipids*. 131, 197-204.

[5] Bennett, M. J., Aberle, A. M., Balasubramaniam, R. P., Malone, J. G., Malone, R. W., Nantz, M. H. 1997b. Cationic lipid-mediated gene delivery to murine lung: correlation of lipid hydration with in vivo transfection activity. *J. Med. Chem.* 40, 4069-4078.

[6] Bhattacharya, S., Dileep, P. V. 2004. Cationic oxyethylene lipids. Synthesis, aggregation, and transfection Properties. *Bioconjug. Chem.* 15, 508-519.

[7] Maria N. Antipina, Ingo Schulze, Bodo Dobner, Andreas Langner, and Geral Brezesinski (2007). Physicochemical Investigation of lipid with a new core structure for gene transfection. Langmuir 23, 3919 – 3926

[8] Hafez, I. M., Cullis, P. R. 2000. Cholesteryl hemisuccinate exhibits pH sensitive polymorphic phase behavior. *Biochim. Biophys. Acta*. 1463, 107-114.

[9] Chen, F. J., Asokan, A., Cho, M. J. 2003. Cytosolic delivery of macromolecules: I. Synthesis and characterization of pH-sensitive acyloxyalkylimidazoles. *Biochim. Biophys. Acta*. 1611, 140-50.

[10] Brand, L., and Gohlke, J. R. 1972. Fluorescence Probes for Structure. Annual Review of Biochemistry, 41: 843 – 868

[11] Donkor, A-M, Spelios, M., Savva, M. 2014. Synthesis, biophysical characterization and *in vitro* transfection activity of novel bivalent amine cationic lipids in the absence of dioleoylphosphatidylethanolamine (DOPE). Pharmacology & Pharmacy, 5, 1015-1028

[12] Smaby, J. M., Momsen, M., Kulkarni, V. S., and Brown, R. E. 1996. Cholesterol-Induced Interfacial Area Condensations of Galactosylceramides and Sphingomyelinswith Identical Acyl Chains. Biochemistry,*35* (18): 5696–5704

[13] Kaganer, V. M., Mohwald, H., and Dutta. P.1999. Structure and phase transitions in Langmuir monolayers. *Rev. Mod. Phys.* 71:779–819.

[14] Li, X-M., Smaby, J. M., Momsen, M. M., Brockman, H. L., and Brown, R. E. 2000. Sphingomyelin Interfacial Behavior: The Impact of Changing Acyl Chain Composition. Biophysical Journal, 78 , 1921–1931

[15] Bach, D., and Miller, I. R. 1998. Hydration of phospholipid bilayers in thepresence and absence of cholesterol. Biochimica et Biophysica Acta (BBA) - Biomembranes1368 (2): 216-224

[16] Farhood, H., Serbina, N., & Huang, L. 1995. The role of dioleoylphosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim. Biophys.Acta 1235, 289-295.

[17] Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Taai, Y. J., Border, R., Ramsey, P., Martin, M., Felgner, P. L. 1994. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Bio. Chem.* 269, 2550-2561.

[18] Huebner, S., Battersby, B. J., Grimm, R., Cevc, G. 1999. Lipid-DNA complex formation: reorganization and rupture of lipid vesicles in the presence of DNA as observed by cryoelectron microscopy. *Biophys J.* 76, 3158-3166.

[19] Savva, M., Aljaberi, A., Feig, J., Stolz, D. B. 2005a. Correlation of the physicochemical properties of symmetric 1,3-dialkoylamidopropane-based cationic lipids containing single primary and tertiary amine polar head groups with in vitro transfection activity. *Colloids Surf. B*. 43, 43-56.

[20] Kearns, M., Donkor, A-M., Savva, M. (2008). Structure-transfection activity studies of novel cholesterol based amphiphiles. Mol. Pharmaceutics, 5(1): 128-139.