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Research Paper

High Sensitivity Differential Scanning Calorimetry Study of DNA-Cationic Liposome Complexes

Mark Saunders,[1] Kevin M. G. Taylor,[1,4] Duncan Q. M. Craig,[2] Karen Palin,[3] and Hazel Robson[3]

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Purpose. To investigate plasmid DNA interactions with liposomes prepared from dimyristoylglycer-oethylphosphocholine (EDMPC) and DOPE using high sensitivity differential scanning calorimetry (HSDSC).

Materials and Methods. Large unilamellar liposomes of EDMPC with DOPE (mol ratio 0–50%) were prepared. Plasmid DNA was added to give a final DNA/lipid (−/+) charge ratio of 0.5. Samples were placed into an HSDSC and cooled to 3°C, held isothermally for 30 min and then the temperature was ramped to 120°C at a rate of 1°C/min.

Results. On heating EDMPC liposomes, the main phase transition occurred at 21.2°C, with a low temperature shoulder on the endothermic peak. At low DOPE concentrations the main phase transition temperatures and enthalpies of transition were lower than for pure EDMPC, with a peak corresponding to a pure EDMPC phase occurring at DOPE concentrations of 12–17 mol%. At 50 mol%, no main transition endotherm was observed. DNA solution produced two endothermic peaks with numerous 'satellite' peaks indicating thermal denaturation. DNA binding to EDMPC changed the shape of the thermogram, indicating alteration in lipid packing within the bilayer. DNA induced demixing in the bilayers of DOPE-containing liposomes.

Conclusion. HSDSC provided information for characterizing liposome formulations and DNA interactions with such vesicles.

KEY WORDS: calorimetry; cationic; DNA; liposome; phospholipid.

INTRODUCTION

Liposomes have been widely studied as non-viral vectors for gene delivery. Their versatility with respect to size, composition, surface charge, bilayer fluidity and ability to incorporate a wide variety of molecules, allows liposomes to be tailored to produce optimal vectors for clinical use (1). Due to the high negative charge density of the DNA molecule brought about by the phosphate backbone, it is advantageous to use cationic lipids to prepare liposomes, as this increases association of the DNA with the liposomes due to electrostatic effects, and also enables liposomes to adhere to cell surface, which have a net negative charge due to their sialic acid residues (2). The addition of DNA to pre-formed cationic liposomes triggers structural changes in the liposomes as well as the DNA, resulting in a liposomal aggregation and formation of heterogeneous structures. Originally, it was thought that the binding of plasmid DNA to cationic liposomes followed a dynamic equilibrium and that the vesicles bound DNA at the interface whilst maintaining their original size and shape (3,4). This, however, is inconsistent with reports suggesting DNA induced fusion of the cationic vesicles into elongated bilayers, encapsulating the DNA molecules (5). The fusion of the membrane is thought to be accompanied by a liposome-induced DNA collapse, which plays an important role in facilitating and enhancing the encapsulation process. A variety of other complexes have also been visualized, including fibrallar structures (spaghetti–meatball complexes) (6), map–pin (7,8) and fusion and aggregation products (9). It is possible that a range of structures coexist, with different experimental procedures necessary to observe them (10). It is thought that complex formation is dependent on both the ratio of cationic to helper lipid present in the liposome formulation (11) and on the charge ratio of anionic DNA to cationic lipid.

In order to develop efficient liposome carriers for DNA, the characterization of the overall structure of these complexes needs to be established. Despite their extensive use as transfection agents, the actual mechanism by which the DNA is bound to the liposome and the resulting morphology of the complex is poorly understood. Much work has been carried out using such methods as freeze-fracture (6) and cryo-electron microscopy (12), but the results obtained seem to be inconsistent with other findings. In this study we have used thermal analysis to explore the interaction between cationic liposomes and plasmid DNA.

[1] School of Pharmacy, University of London, 29-39 Brunswick Square, London, UK.
[2] The School of Pharmacy, University of East Anglia, Norwich, UK.
[3] BioPharmaceutical Formulation Sciences, GlaxoSmithKline Research and Development, Ware, Hertfordshire, UK.
[4] To whom correspondence should be addressed. (e-mail: kevin.taylor@pharmacy.ac.uk

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Dispersed in aqueous media, hydrated lipids may exist in one or more intermediate liquid-crystalline or mesomorphic forms. Thermal analysis of pure single component lipids has shown that transitions occur over a very narrow temperature range and are well defined. Mixtures of different phospholipids containing different hydrocarbon chain types melt over a broader temperature range and hence exhibit a broader transition. The temperature at which the transition occurs (T_c), the onset temperature and the shape of the transition all depend on the type and ratio of the lipid composition (13).

Within the liposome bilayer, the thermotropic behaviour is extremely sensitive to alterations in lipid packing, such that if the packing is interrupted by the penetration of a material into the bilayer the order parameter of the transition is reduced, with the entropy of the lipid increasing and the phase transition occurring at a lower temperature. Inclusion of material into a phospholipid bilayer generally has a more pronounced effect on the pre-transition than the main transition. Changes in the thermogram of the main transition are dependent on the localisation of the drug within the bilayer, with the biggest changes occurring when the drug is located in positions C_1–C_{10} of the hydrophobic chain region (14).

Until recently, with the advent of differential scanning calorimetry (DSC) instrumentation with increased sensitivity, the study of such interactions between DNA and cationic liposomes was not possible. Conventional DSC has an operating sensitivity of about ±10 μW. High Sensitivity DSC (HSDSC) as employed in this study is capable of detecting signals of ±0.5 μW, and larger sample volumes, typically up to 1 ml may be analysed. This increased sensitivity permits accurate and reliable data to be obtained, particularly for small thermal events, such as pre-transitions which are hard to detect and measure quantitatively using conventional DSC. Subramanian *et al.* (15) studied the interaction of plasmid DNA using DSC with a series of MLV formulations containing the cationic lipids bis[2-(11-phenoxyundecanoate)-ethyl]dimethylammonium bromide BPDAB), bis[2-(11-butyloxyundecanoate)ethyl]-dimethylammonium bromide (BBDAB) and N-hexadecyl-N-{10-[O-(4-acetoxy) phenylundecanoate]ethyl} di-methylammonium bromide (HADAB) mixed with various mole fractions of the neutral lipid DMPC. Although their studies provided a valuable insight into such interactions, indicating a partial phase separation induced upon binding DNA, the formulation used (comprising BPDAB/DMPC 5:95 molar ratio) does not represent a viable formulation for delivery of DNA into eukaryotic cells.

Barreleiro *et al.* (16) used DSC, isothermal titrimetric calorimetry and turbidity measurements to study the interactions between DNA and cationic liposomes. They showed that addition of 1,2 dioleoylphosphatidylethanolamine (DOPE) into a cationic liposome formulation containing the lipid dioctadecyldimethylammonium bromide (DODAB) resulted in a phase separation into two domains with a heterogeneous distribution of DOPE and DODAB. Increasing the concentration of DOPE in the formulation had a significant effect on the binding of plasmid DNA by decreasing the enthalpy associated with such binding, indicating that the amount of cationic lipid present in the vesicles determines the reaction enthalpy. However, a similar study (17) indicated that an increased presence of DOPE in the formulation did not significantly change the shape or energy associated with the binding isotherm from that resulting from titration of pure cationic lipid with DNA. These results may reflect the different bilayer ordering of DOPE, which in turn may be dependent on the type of cationic lipid used.

In this study, HSDSC was used to explore the interaction of plasmid DNA with cationic liposomes prepared from 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (EDMPC) with and without DOPE. EDMPC was chosen as a model cationic lipid as it offered an under-researched and interesting alternative to the more established cationic lipids such as dioleoyltrimethylammoniumpropane (DOTAP).

MATERIALS AND METHODS

Materials

Chloroform, acetone, tris(hydroxymethyl)aminomethane (Trizma base), tris(hydroxy-methyl)amino-methane hydrochloride (Trizma hydrochloride), ethylene-diaminetetraacetic acid (EDTA) and hydrogen fluoride (HF) were purchased from Sigma Chemical Company, Poole, UK and were of AnalaR grade. 1,2 dioleoylphosphatidylethanolamine (DOPE) (99.9%) was purchased from Sigma Chemicals (UK). 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (EDMPC) and a model supercoiled plasmid DNA (9,067 bp) were gifts from GlaxoSmithKline, Ware, Hertfordshire, UK.

Liposome Preparation

Multilamellar vesicles (MLVs) were prepared using the classical film method (18). The cationic lipid EDMPC (30 mg) was dissolved in chloroform (5–10 ml) and the neutral helper lipid DOPE added to give a final mol/mol ratio ranging from 0–50% in a 50 ml round-bottomed spherical Quick-fit flask and the solvent evaporated under vacuum in a Buchi rotary evaporator (Buchi, Switzerland) at approximately 40°C. This resultant thin lipid film was flushed with oxygen free nitrogen for 2 min to remove residual solvent. Buffer was added (10 mM Tris, pH 8) to produce a lipid concentration of 5 mg/ml and the flask shaken vigorously to produce the liposome dispersion. The flask was left at 40°C for 30 min, after which the shaking process was repeated. Subsequently, the flask was flushed with nitrogen, sealed and stored at 4°C until further use. MLVs were placed in a Liposofast-50 extruder (Avestin, Canada) and extruded 20 times through a single-polycarbonate membrane of defined pore size (100 nm, Poretic Products, Osmonics, USA) at ambient temperature.

Preparation of Liposomal/DNA Complexes

One and a half milliliter of the required liposome suspension was placed into a small cuvette and placed onto a RotaMixer (Hook and Tucker, U.K) and gently vortexed whilst plasmid DNA was slowly added using a Hamilton microsyringe to give a final DNA/lipid (−/+) charge ratio of 0.5. The preparation was gently vortexed at 25°C for 5 min to

aid in the mixing process and then left to stand at 25°C (>T_c) for 2 h prior to thermal analysis.

Sizing of Vesicles by Photon Correlation Spectroscopy

Liposomes were sized by photon correlation spectroscopy (PCS) on a Malvern Autosizer 2c (Malvern Instruments, U.K.) at 25°C by dispersing approximately 30 µl of the liposome suspension into 3 ml of buffer (10 mM Tris, pH 8) in a suitable cuvette. Results are expressed as z-average median diameter.

Cryo-transmission Electron Microscopy

A single drop of liposome dispersion was deposited onto a 700-mesh carbon grid and mounted on a guillotine. Residual water was removed by the double blotting technique to leave a thin film. As the filter paper detached from the grid, the guillotine was released and the sampled plunged into a bath of liquid ethane. After sample vitrification, the grid was rapidly removed from the bath using nitrogen-cooled forceps and transferred to a liquid nitrogen bath, then to the transmission electron microscope (Philips FEG 200, Philips, USA) using a dedicated liquid-nitrogen cooled specimen stage. Once transferred, the sample and stage were maintained at a temperature between −160 and −185°C. After the sample and cryo-blades reached thermal equilibrium, micrographs were obtained.

High Sensitivity Differential Scanning Calorimetry (HSDSC)

Liposome Preparations

Liposome dispersion (0.8 ml) was placed into an HSDSC sample cell and sealed using a cell cap. A rubber o-ring was used to seal the cap to ensure an airtight fit. Buffer (10 mM Tris, pH 8) was then added to the reference cell until the weight difference with the matched sample cell was less than 0.001 g, and sealed. The sample and reference cells were loaded into the furnace chambers of a Seteram Micro DSC III (Seteram, France) held at 25°C. The samples were left until the heat flow between the sample and reference remained constant (approximately 20 min). After thermal equilibration had been attained, the samples were cooled to 3°C, held isothermally for 30 min and then the temperature was ramped to 120°C. All scanning was undertaken at a rate of 1°C/min.

After completion of the run, the enthalpy of any transitions, the onset temperature and temperature at the peak of the transitions were calculated using the instrument software. Prior to sample analysis, a buffer versus buffer run was obtained to create a "blank" for subsequent data analysis. All DSC data are presented as mean ± s.d for three experiments.

Plasmid DNA

Plasmid DNA solution (0.8 ml, 4.2 mg/ml) in 10 mM Tris buffer, pH 8, was placed into the sample cell, with the reference cell containing 10 mM Tris buffer. The samples were then loaded into the HSDSC and scanned over a temperature range of 3–120°C at a scan rate of 1°C/min. The cooling signal was also monitored to investigate the reversibility of any denaturation.

RESULTS AND DISCUSSION

DSC Thermogram of Pure EDMPC LUV Liposomes

Extruding all liposome formulations 20 times resulted in a homogeneous population (polydispersity <0.08) of large unilamellar vesicles (LUVs) having a median diameter approximating the pore diameter (100–105 nm) of the polycarbonate membrane filter, as measured by PCS, with a single bilayer as determined by electron microscopy

On heating pure EDMPC liposomes, the main gel (L_β) to liquid crystalline (Lα) phase transition was seen at 21.2±0.1°C (Fig. 1).

A low temperature shoulder on the endothermic peak occurred at a temperature approximately 1.2°C below the peak of the main transition. The overall enthalpy of the main phase transition was 17.4±0.4 kJ/mol, with a half-height width of 2.6±0.3°C. No pretransition was seen within the temperature range scanned.

EDMPC exhibited a relatively broader phase transition endotherm, compared to most single lipid systems (19) with a low temperature shoulder. The lack of symmetry in the endotherm is indicative that within the liposomal bilayer, there may be two distinct types of lipid packing, each undergoing the cooperative main phase transition at slightly different temperatures. Compared to saturated phosphatidylcholines, esterification of the phosphate moiety of the PC molecule in EDMPC increases the steric bulk of the polar headgroup and converts the zwitterionic lipid into a positively charged species. Small angle X-ray scattering of EDMPC MLVs (20) showed that there was an increase in the lamellar *d*-spacing at the main phase transition temperature. Such an increase is atypical of most hydrated lipid bilayers, and has been attributed to a change in the lipid packing of the bilayer and the coexistance of two lamellar lattices at temperatures near the main transition. Below the main phase transition, the thickness of the EDMPC bilayer is less than that of the unesterifed DMPC in the gel state, resulting in the formation of a gel phase wherein the acyl chains of the lipid molecules are thought to interdigitate, resulting in a somewhat thinner

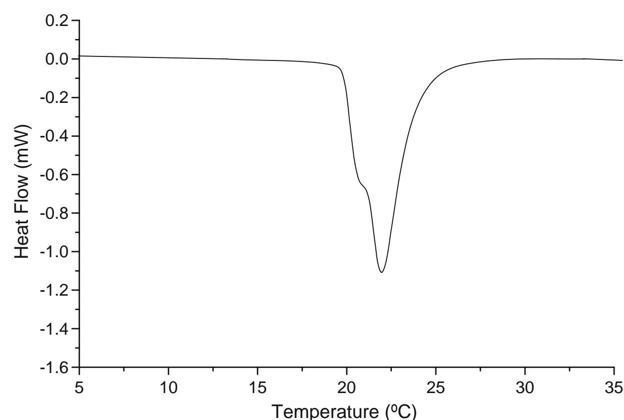

Fig. 1. DSC thermogram of EDMPC LUVs.

bilayer (21). This interdigitation may be facilitated by the relief of stress arising from charged headgroup repulsion and steric crowding of the polar headgroups (22) brought about by the ethyl group on the phosphate moiety.

Thermal Behaviour of DOPE Containing EDMPC Liposomes

The transfection ability of DNA-liposome complexes is greatly improved when a 'helper' lipid is added to the bilayer. The presence of DOPE within the bilayer enhances lipid bilayer fluidity and promotes cell liposome fusion and membrane destabilization (23). Inclusion of DOPE over the concentration range 12–33 mol% ratios was investigated (Fig. 2, Table I). DOPE is a zwitterionic phospholipid which does not usually pack into a bilayer structure but forms an inverted hexagonal phase at high concentrations (23).This results from the small headgroup of phosphatidylethanolamines compared to phosphatidylcholines, such that the overall shape of the lipid molecule is conical, rather than cylindrical. However, due to the electrostatic repulsion between the headgroups of the EDMPC molecules brought about by the positive headgroup charge and the steric crowding by the ethyl moiety, the overall size of the space occupied by the headgroup is significantly larger than its neutral counterpart. This results in sufficient space between the EDMPC molecules for DOPE molecules to insert and partake in a bilayer structure when mixed with such a lipid. At low DOPE concentrations, the main phase transition temperatures and enthalpies are significantly lower ($p<0.05$) than for pure EDMPC.

The increase in HHW of the transition with increasing DOPE concentration suggests that the fluidity of the liposome bilayer is increased with a reduced order of packing within the bilayer, resulting in a reduction in the overall transition cooperativity, leading to enhanced water penetration into the bilayer during the transition at lower temperatures. The low temperature shoulder on the main phase transition of EDMPC was not seen on DOPE incorporation, suggesting DOPE alters the packing of the EDMPC molecules sufficiently to abolish the interdigitated region of the bilayer.

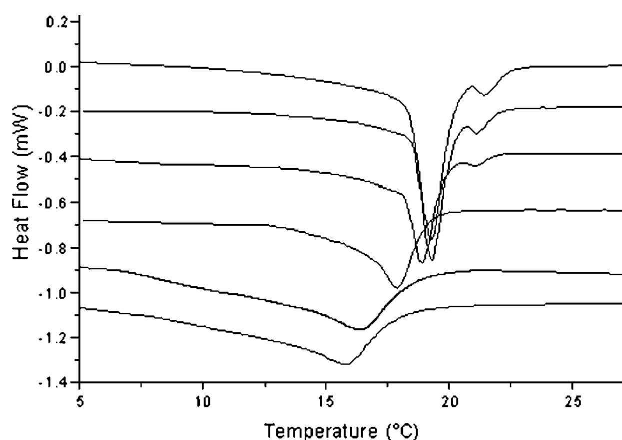

Fig. 2. DSC thermograms of EDMPC liposome containing 12 (*top line*), 15, 17, 20, 25 and 33 (*bottom line*) mol% DOPE.

At 12 mol%, DOPE lowered the main phase transition of the bilayer with a decrease in the overall enthalpy of the transition and a narrowing of the half height width (Table I). A smaller transition, corresponding to a pure EDMPC phase, was also seen at 21.9°C (Table I). Inclusion of DOPE in the liposomal bilayer at between 12 and 17 mol% resulted in further decreases in the peak temperature of the main transition, accompanied by decreases in both the enthalpy of the main phase and higher temperature transition, with an increase in HHWs.

The transition at higher temperature, occurring at approximately 21.2°C was abolished at DOPE concentrations higher than 20% mol. At 20 mol% a single broad endotherm occurred at 18.2°C. Single endothermic peaks with decreasing peak temperatures and enthalpies occurred with increasing DOPE concentrations (25 and 33 mol% DOPE, Fig. 2).

The small phase transition seen at low DOPE concentrations (less than 17% mol DOPE), was seen at temperatures indicative of pure EDMPC bilayers. This was possibly due to EDMPC molecules being in great excess compared to the DOPE, such that there were regions within the bilayer structure where only EDMPC molecules are present, resulting in a bilayer transition at temperatures corresponding to a pure EDMPC phase. This transition, seen at all DOPE concentrations from 12–17% mol, decreased in enthalpy as DOPE concentration increased, suggesting that the amount of EDMPC molecules partaking in the transition decreased due to better mixing of the two lipids. At concentrations greater than 17% mol DOPE, this small transition was abolished and only one broad main phase transition was seen, due to ideal mixing of the two lipids. Although the EDMPC cationic lipid was still in excess, the presence of DOPE at these concentrations was sufficient to alter the packing such that there were no significant regions of pure EDMPC phospholipid molecules within the bilayer.

When DOPE was incorporated at 1:1 mol/mol ratio, no endothermic peak corresponding to the gel- to liquid crystalline phase transition was observed over the temperature range investigated, indicating that such liposomes exist in the fluid state at all temperatures. The conical shape of DOPE molecules hinders the close packing of the neighbouring phospholipid molecules such that no orientation of the molecules occurs so that an ordered domain required for the gel state can be formed.

Thermal Denaturation of Plasmid DNA

In order to understand the effects of plasmid DNA binding to a liposome vector, it was first necessary to understand the effect of changing temperature on the plasmid DNA itself. On heating the plasmid DNA solution, two distinct endothermic peaks were seen (Fig. 3). There was one main predominant broad endothermic melting peak at 89.3±0.5°C, suggesting that this was the main form of plasmid structure. However, there was another prominent endothermic peak at 68.9±0.4°C, with numerous 'satellite' peaks occurring at close temperatures, indicating the presence of numerous fragments of DNA within the sample, corresponding to the thermal denaturation of the double strand helix of the DNA molecule (Fig. 3).

Table I. Transition Temperatures, Enthalpies and Half Height Widths of EDMPC LUV Liposomes Containing Various mol Fractions of DOPE (n=3±s.d)

Percentage of DOPE Concentration	Transition Temperatures (°C)		Transition Enthalpy (KJ/mol)		Half Height Width (°C)	
0	21.2±0.1		17.40 ±0.35		2.55±0.3	
12	19.3±0.2	21.9±0.2	13.01+1.28	0.55+0.12	1.04±0.24	0.59±0.05
15	19.2±0.1	21.1±0.1	12.22±0.4	0.51±0.04	1.15±0.14	0.68±0.06
17	18.9±0.1	21.2±0.0	11.13+4.45	0.39±0.06	1.28±0.12	0.72±0.09
20	18.2±0.2		9.32±0.3		2.04±0.24	
25	16.7±0.3		6.82±0.39		4.28±0.35	
33	15.4±0.4		5.74±0.29		5.17±0.32	

At temperatures higher than the melting point of all the GC pairs, the DNA strands will dissociate and become single stranded, which will be present in coiled and unstructured forms. The presence of multiple peaks in Fig. 3 suggests denaturation to several DNA fragments of varying base compositions and size. The dissociation of the strands takes place in a stepwise fashion, with domains dissociating in a cooperative process by one of five subtransitions which depend on where the domain occurs (24). Gel electrophoresis experiments carried out on the plasmid sample prior to analysis (GlaxoSmithKline in-house data) showed the presence of the DNA plasmid in its supercoiled form, with trace amounts of linear DNA fragments with lower molecular weights. It is suggested that the main endothermic peak in Fig. 3 results from the denaturation of the higher concentrated supercoiled plasmid form, with the linear fragments denaturing at a lower temperature.

From the equation:[1]

$$T_m = 69 + 4.4(\%G + C)$$

Which describes the relationship between base pairing ratios and the temperature at which thermal denaturation occurs, it was calculated that within the bulk of the species present within the sample, the % GC content was approximately 48.8±1.3%.

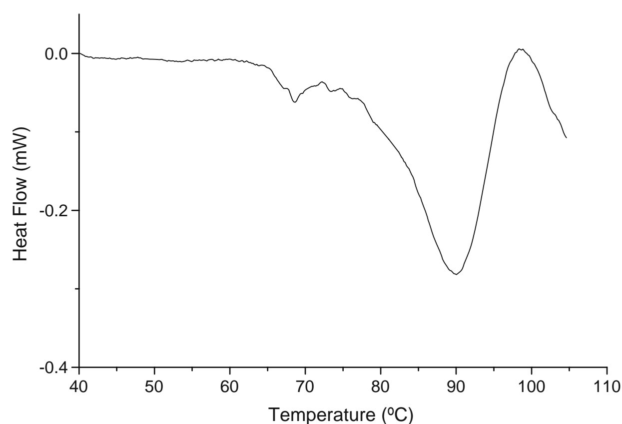

Fig. 3. DSC thermogram on heating plasmid DNA in buffer.

[1] http://www.chemistry.nmsu.edu/studntres/chem435/Lab4/. (Accessed 4th January, 2007).

From the results in Fig. 3, in conjunction with the gel electrophoresis experiments, it is possible to distinguish between the two main types of DNA present (i.e. supercoiled and linear fragments) due to the large differences in the enthalpy of transition. However, due to the nature of the dissociation and the endotherms produced, it is difficult to assign specific melting profiles witnessed in the thermogram and relate them to the fine structure of the DNA in the sample.

From the DSC thermogram obtained (Fig. 3), it can be seen that an exothermic process occurred, with an onset temperature of approximately 98.5°C. This was due to the air space (0.1–0.2 $cm^{3)}$ between the surface of the sample and the lid of the cells of the HSDSC containing the sample and reference (buffer) under study, being sufficient to allow some evaporation to occur from the surface. Since this transition is witnessed within the thermogram and not cancelled out by evaporation from the reference cell, it suggests that the presence of dissolved DNA within the sample alters the vaporization profile of the sample, resulting in significantly different evaporation temperatures between the sample and reference. Such effects have previously been described for biological samples in closed (sealed) containers analysed using the HSDSC instrumentation employed in these studies (25).

After the heating process was completed and thermal denaturation of the DNA had occurred, the sample was cooled back through the transition temperatures to room temperature. Cooling the dissociated DNA strands back to room temperature resulted in no exothermic peak (data not shown). Reassociation of the strands back into their helical form is dependent on the formation of hydrogen bonds between the base pairs of the corresponding strands of DNA, which is an exothermic process. Since no transition was seen which deviated from the baseline, this indicates that little or no reassociation of the single stranded coiled or unstructured chains back into their ordered helical conformation occurred during the time-course of this experiment.

Interaction of Plasmid DNA with EDMPC LUVs

Electrostatic binding of plasmid DNA to pure EDMPC liposomes produced a change in the overall shape of the thermogram, indicating that the binding process alters the lipid packing within the bilayer (Fig. 4). A single main phase transition was seen at 21.9±0.1°C (Fig. 4), which was not significantly different ($p<0.05$) to that of the non-complexed liposomes (Table I).

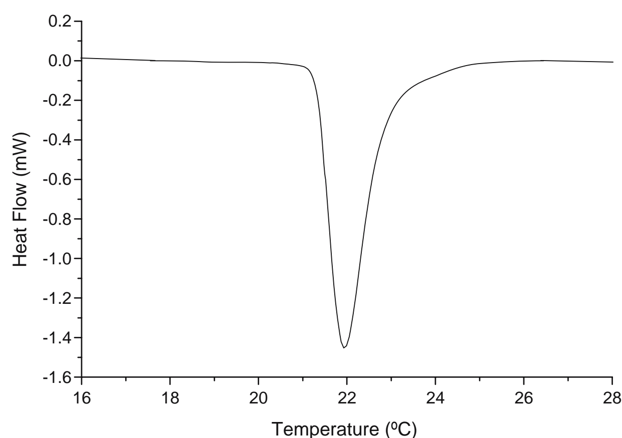

Fig. 4. DSC trace for EDMPC liposomes complexed with plasmid DNA.

Penetration of drug molecules into the hydrophobic acyl region of the bilayer is usually accompanied by a decrease in transition enthalpy and temperature, since the order of both the lipid packing and cooperativity of the phase transition are reduced. Since addition of plasmid DNA to pure EDMPC liposomes had no effect on the peak temperature of the transition, this suggests that the electrostatic binding process between the DNA molecules and the cationic headgroups of the lipid molecules occurs only at the bilayer surface, with no penetration of the plasmid DNA into the bilayer.

The low temperature shoulder on the endotherm of non-complexed liposomes (Fig. 1) was abolished on complexation with plasmid DNA, producing a symmetrical endothermic transition with a narrow temperature range. The HHW of the main phase transition was decreased from 2.6±0.3°C to 1.1±0.2°C, indicating that binding of plasmid DNA to the liposome increased the order parameter of the lipid packing within the bilayer, producing a sharper endothermic transition. The resultant endotherm is more symmetrical, suggesting that complexation of the liposome with plasmid DNA leads to some lipid reorganization, eliminating the interdigitation that occurs between the acyl chains of the EDMPC molecules. This process was also accompanied by a significant decrease ($p<0.05$) in the total enthalpy of the transition from 17.40±0.35 to 16.21±0.19 kJ/mol.

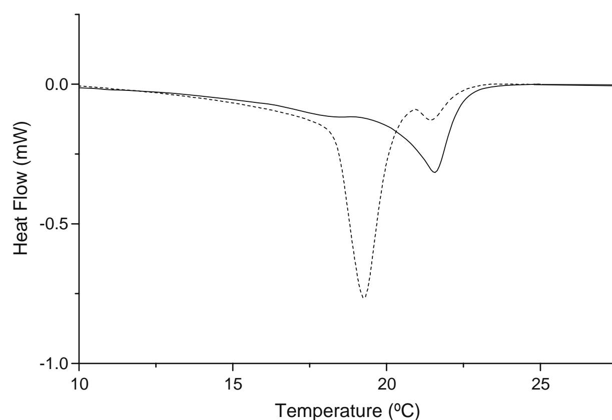

Fig. 6. DSC thermogram for EDMPC/DOPE (15 mol% DOPE) liposomes non-complexed (*dotted line*) and complexed (*solid line*) with plasmid DNA.

Interaction of Plasmid DNA with EDMPC:DOPE Liposomes

Figures 5, 6, 7, 8 and 9 show the thermograms obtained upon heating plasmid DNA complexed with EDMPC LUVs containing varying mole percentages of DOPE.

Complexation of plasmid DNA with EDMPC liposomes containing DOPE at 12 mol% had a pronounced effect on the DSC thermogram (Fig. 5). Non-complexed EDMPC: DOPE liposomes (12 mol% DOPE) showed two endothermic peaks corresponding to the main gel- to liquid crystalline phase transition of an EDMPC enriched (lower transition peak) domain and a pure EDMPC domain within the bilayer. On complexation of plasmid DNA with the liposome surface, there was an increase in the enthalpy of the higher temperature transition from 0.553±0.112 to 8.45±0.233 kJ/mol, accompanied by a decrease in the enthalpy of the lower temperature transition from 13.01±1.28 to 0.305±0.024 kJ/mol. There was no significant difference ($p<0.05$) between the peak temperatures of the two higher transitions, however, the lower peak transition temperature significantly decreased ($p<0.05$) from 19.3±0.04 to 18.2±0.2°C.

Increasing the DOPE concentration from 12 to 17 mol% lowered the overall transition enthalpy of the melting endotherms (Fig. 2). Addition of plasmid DNA to EDMPC

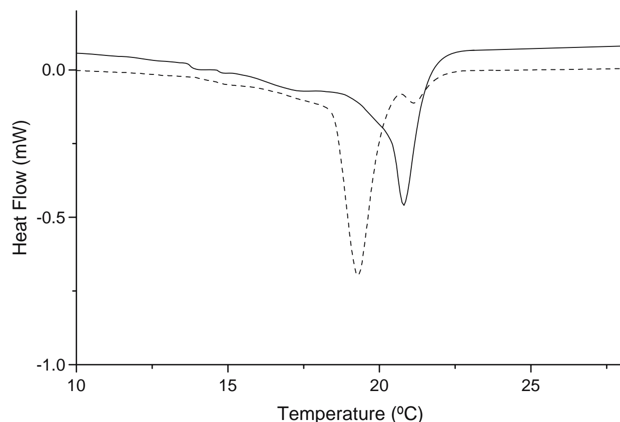

Fig. 5. DSC thermogram for EDMPC/DOPE (12 mol% DOPE) liposomes non-complexed (*dashed line*) and complexed (*solid line*) with plasmid DNA.

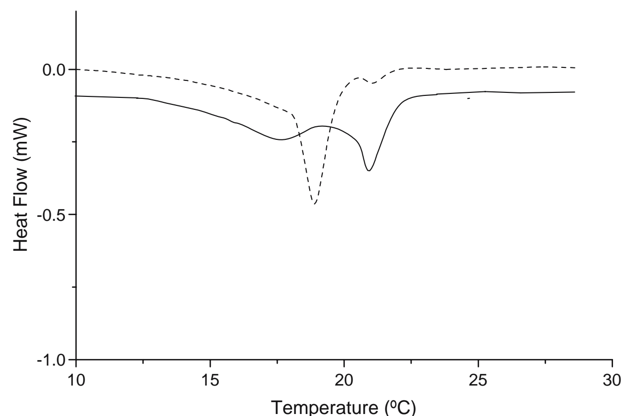

Fig. 7. DSC thermogram for heating EDMPC/DOPE (17 mol% DOPE) liposomes non-complexed (*dashed line*) and complexed (*solid line*) with plasmid DNA.

liposomes with 17 mol% DOPE reduced the temperature of the lower transition from 18.9±0.1 to 17.6±0.2°C (Fig. 7), and the enthalpy of transition decreased from 11.13±1.05 to 1.53±0.05 kJ/mol. There was no significant change in the temperature of the higher transition process; however, a significant increase in the enthalpy from 0.394±0.06 to 5.01±0.11 kJ/mol occurred.

At DOPE concentrations of 12–17% without plasmid DNA, a small peak corresponding to a pure EDMPC phase was seen, indicative of non-ideal mixing in the presence of excess EDMPC molecules. Addition of plasmid DNA to these liposomes increased the enthalpy of the main phase transition (at approximately 21–22°C) in all cases, implying that a greater proportion of EDMPC molecules are present within the domains responsible for this transition. This suggests that the binding of the plasmid DNA to the surface of the liposome resulted in lateral separation of the two bilayer components; producing liposomes with DNA bound EDMPC phases along with EDMPC/DOPE mixed phases. This is confirmed by the change in the temperature and enthalpy of the lower temperature transition, since due to the separation process, less EDMPC molecules will be present in this phase, increasing the ratio of DOPE to EDMPC molecules within these domains, increasing bilayer fluidity. Lipid demixing and the formation of domains within bilayers, resulting from the interaction of lipid membranes with DNA (26) and charged proteins (27,28) has been previously described and discussed.

At 25 mol% DOPE with plasmid DNA, the thermogram indicated the presence of two separate domains within the bilayer, compared to the uniform packing seen in the non-complexed liposomes (Fig. 8). Complexation produced significant reductions in the temperature of the main transition from 16.7±0.3 to 15.7±0.3°C and in the enthalpy from 6.82±0.39 to 5.23±0.26 kJ/mol. A small endothermic peak, with an enthalpy of transition of 1.01±0.13 kJ/mol, was produced at higher temperatures (20.9±0.1°C), corresponding to the generation of an EDMPC enriched phase. The temperature at which this transition occurs is significantly different ($p<0.05$) to pure EDMPC liposomes complexed with plasmid DNA (Fig. 4). At 50% mol fraction DOPE, no

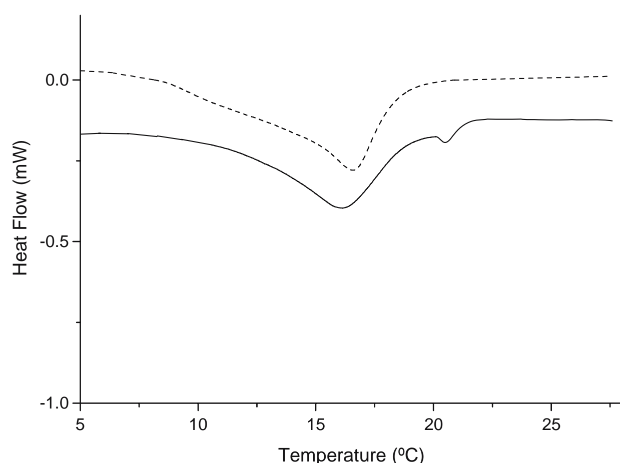

Fig. 8. DSC thermogram for EDMPC/DOPE (25 mol% DOPE) liposomes non-complexed (*dashed line*) and complexed (*solid line*) with plasmid DNA.

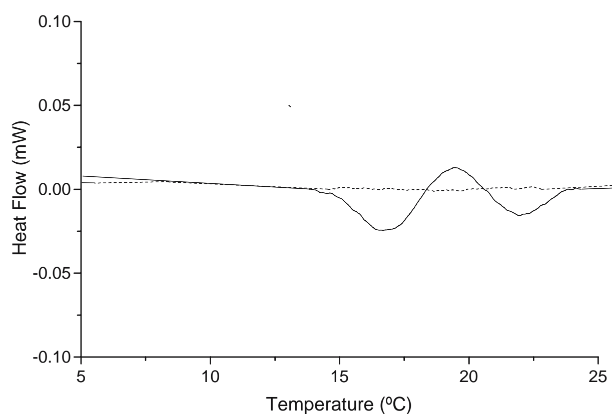

Fig. 9. DSC thermogram for EDMPC/DOPE (50 mol% DOPE) liposomes non-complexed (*dotted line*) and complexed (*solid line*) with plasmid DNA.

endothermic transitions were observed for non-complexed liposomes (Fig. 9). Addition of plasmid DNA resulted in two discrete peaks, occurring at 16.7±0.5 and 21.4±0.3°C, with enthalpies of 0.698 and 0.495 kJ/mol, respectively. Equimolar mixtures of cationic lipid and DOPE are commonly used for optimal transfection, so it was interesting to observe the different thermal profile for the equimolar mix (Fig. 9) in this study. Further work is required to the relationship between the thermal behaviour of these membranes and transfection potential.

At DOPE mol fractions of 25 and 50%, the interaction of plasmid DNA results in a demixing process, with an EDMPC enriched, rather than a pure EDMPC domain produced. Since the enthalpies of the higher temperature transition decreased as the DOPE concentration within the bilayer increased, this suggests that the extent to which the liposome bilayer will demix is dependent on the amount of EDMPC molecules present within the bilayer. As the DOPE concentration is increased, the charge ratio between the positive EDMPC molecules and the negative phosphate backbone of the plasmid DNA molecules is reduced. Not only does this result in a reduced amount of EDMPC molecules available for binding per DNA molecule, it also alters the surface charge of the liposome, changing the overall affinity of the bilayer surface for the plasmid DNA.

CONCLUSIONS

These experiments have established that HSDSC can provide valuable information for characterizing both the initial formulation of liposomes to be used for gene delivery and the interactions of plasmid DNA with such vesicles. When choosing a liposome formulation for use as a gene delivery vector, careful consideration should be taken, since changing the ratio of the components within the bilayer has a pronounced effect on the binding process taking place. DNA binding to mixed cationic liposomes results in a structural change of the bilayer. Therefore, care must be taken in understanding and predicting the effects of these structural changes if a viable formulation is to be developed.

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