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Thermal stability and entrapment properties of hybrid lipid/surfactant vesicles for the incorporation of Ciprofloxacin

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Abstract

Liposomes are popular drug carriers providing several advantages such as an increase in formulation options for the entrapped drug. Ciprofloxacin is an antibiotic used for lung infections that has already been incorporated in liposomal mixtures of HSPC/Cholesterol. The aim of this thesis was to explore and compare already existing liposomes and newly designed hybrid lipid/surfactant combinations for the entrapment of ciprofloxacin HCl with respect to their physicochemical characteristics of size, zeta potential and thermal behaviour through which the stability of the vesicles was assessed. Additionally, the fluidity of the particles was evaluated and compared with previously described so-called Fluidosomes. The motive of this project was to assess optimal lipid/surfactant compositions for potential use in pulmonary antibiotic delivery via nebulisation. The liposomes were prepared by solvating lipid films and thus passively incorporating ciprofloxacin HCl using Dual Centrifugation (DC). The vesicle size measurements were carried out by Dynamic Light Scattering (DLS) whereas the zeta potential was obtained through Electrophoretic Light Scattering (ELS). To gather information about liposome stability and fluidity, Differential Scanning Calorimetry (DSC) measurements were performed. The optimal liposomes chosen regarding this thesis rationale are DPPC/DHDAB 2:1 due to the high amount of antibiotic incorporated as well as DPPG/DPTAP 4:1 for the same reason in addition to a unique thermogram indicating interdigitation. Furthermore, both mixtures of DPPC/DPTAP in the molar ratio of 2:1 and 4:1 showed promising results in terms of their stability as well as an excess positive surface charge which could be useful in terms of mucus penetration in the lung. These successful combinations are comparable to Fluidosome mixtures (DPPC/DMPG) which emerged as being more thermally stable than previously expected, especially in the molar ratio of 9:1, making it a possible candidate to withstand the shear forces of a nebulisation.

Kurzfassung

Liposomen sind gut untersuchte Medikamententräger, die für zahlreiche Vorteile bekannt sind, wie unter anderem die Erweiterung von Formulierungsoptionen für sich in der Entwicklung befindende Arzneimittel. Ciprofloxacin ist ein bekanntes Antibiotikum, das für bakterielle Infektionen in der Lunge eingesetzt wird und bereits in Liposomen aus HSPC/Cholesterol verpackt wurde. Das Ziel dieser Masterarbeit war es, verschiede neu überlegte Lipidkombinationen zur Herstellung von Liposomen, in die Ciprofloxacin HCl geladen werden kann, betreffend ihre physikochemischen Eigenschaften zu untersuchen. Zu diesem Zwecke wurden die Partikelgröße, das Zeta Potential und die thermalen Eigenschaften gemessen und mit bereits bekannten Medikamententrägern und Partikeln verglichen. Durch die erlangten Daten betreffend die thermalen Eigenschaften konnte auf die Stabilität sowohl als auf die Membranbeweglichkeit der Partikel eingegangen werden. Die Membranbeweglichkeit wurde auch bei den bereits bekannten Fluidosomen beschrieben, die in diesem Fall für Vergleiche verwendet wurden. Der Hintergrund dieser Arbeit war es, eine Formulieroption von Liposomen für Ciprofloxacin HCl zu finden, welche man für eine pulmonale Applikation durch Vernebelung verwenden könnte. Die Liposomen wurden hergestellt, indem man zuerst dünne Lipidfilme kreierte. Anschließend formten sich die Partikel durch Duale Zentrifugation (DC) und wurden in diesem Prozess mit Ciprofloxacin HCl beladen. Die Partikelgröße wurde mit Hilfe von Dynamischer Lichtstreuung (DLS) bestimmt und das Zeta Potential durch Elektrophoretische Lichtstreuung (ELS) gemessen. Um Informationen bezüglich der Stabilität und Beweglichkeit zu gewinnen, wurde eine dynamische Differenzkalorimetrie (DSC) durchgeführt und die entstandenen Thermogramme analysiert. Die optimalen Liposomen basierend auf den Kriterien dieser Arbeit sind unter anderem DPPC/DHDAB 2:1 auf Grund des hohen Antibiotikum Gehalts, der eingeschlossen wurde. Desweitern DPPG/DPTAP 4:1, diese Kombination konnte ebenfalls mit der enthaltenen Menge an Ciprofloxacin überzeugen, aber auch aufgrund eines speziellen Thermogramms, das auf eine Verzahnung der Lipidketten hinweist. Die Liposomen aus DPPC/DPTAP 2:1 und 4:1 beeindruckten durch eine gute Stabilität und könnten durch eine positive Oberflächenladung Vorteile in der Reaktion mit Schleim in der Lunge mit sich bringen. Abschließend sind die Fluidosomenkombination aus DPPC/DMPG zu erwähnen, die durch eine unerwartete und untypische Stabilität besonders im Verhältnis 9:1 eine gute Option zur Vernebelung darstellen könnten.

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Abbreviations

CIP	Ciprofloxacin
$ChCl_3$	Chloroform
DC	Dual Centrifugation
DHDAB	Dihexadecyldimethylammoniumbromide
DLS	Dynamic Light Scattering
DMPG	Dimyristoylglycerophosphoglycerol
DPPC	Dipalmitoylglycerophosphocholine
DPPG	Dipalmitoylglycerophosphoglycerol
DPTAP	Dipalmitoyltrimethylammoniumpropane
DSC	Differential Scanning Calorimetry
DSPC	Distearoylglycerophosphocholine
DSPE	Distearoylglycerophopholethanolamine
ELS	Electrophoretic Light Scattering
GUV	Giant Unilamellar Vesicle
LUV	Large Unilamellar Vesicle
MLV	Multilamellar Vesicle
MeOH	Methanol
NaCl	Sodium Chloride
PSPC	Palmitoylstearoylglycerophosphocholine
SUV	Small Unilamellar Vesicle
TRIS	Tris(hydroxymethyl)aminomethane
FTIR	Fourier Transform Infrared Spectroscopy

1. Introduction

Liposomes are spherically-shaped vesicles made primarily from phospholipid bilayers entrapping an aqueous compartment *(Figure 1)* [1]. The formation of liposomes from phospholipids dispersed in water is driven by the hydrophobic effect, whereby Van der Waals interactions between the hydrocarbon chains facilitate assembly of the bilayers, which can be stabilised by interactions between the hydrophilic headgroups [1]. Vesicles consisting of one bilayer are defined as unilamellar vesicles (ULVs) and particles with multiple bilayers are called multilamellar vesicles (MLVs) [2]. Unilamellar vesicles can be divided into further categories, based on their size. Small unilamellar vesicles (SUVs) have diameters ranging from 20 – 100 nm, whereas large unilamellar vesicles (LUVs), are 100 to 1000 nm in diameter [3]. Additionally, there are giant unilamellar vesicles (GUVs), which are larger than 1000 nm [3]. Due to the amphiphilic properties of their component lipids, liposomes have long been considered as ideal carriers for both hydrophilic and lipophilic drugs [4]. Hydrophilic molecules could be entrapped in the aqueous core of a liposome and lipophilic drugs would preferentially localise in the hydrophobic core of the lipid bilayer [4].The incorporation of a drug into liposomes may improve their solubility and stability, depending on its chemical and physical characteristics [4]. In turn, these factors may also improve the in-vivo drug bioavailability [4]. Other benefits of using liposomes as drug carriers include the possibility of a high encapsulation efficiency for hydrophilic as well as lipophilic drugs, broadening their domain as nanocarriers [4]. Moreover, the reduction of unwanted side effects such as tissue irritation can be achieved, due to the fact that liposomes can be used to release drugs site-specifically [4].

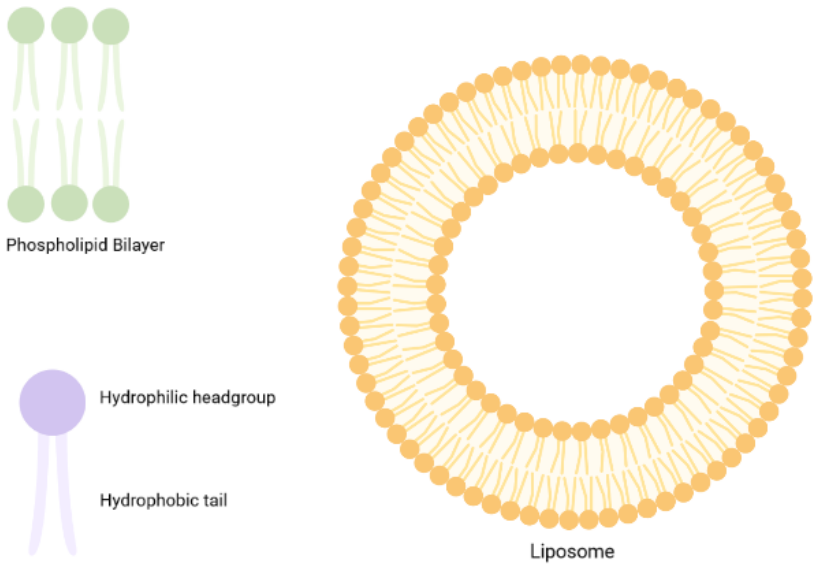

Figure 1: *Structure of unilamellar vesicles (ULVs) made from phospholipid bilayers. The lipophilic tails of the phospholipids point towards each other, while the hydrophilic headgroups point towards aqueous surrounding.*

Antibiotics are often subjects of studies that focus on the effects of liposomes as their drug carriers in order to improve pharmacokinetics, pharmacodynamics, and counter bacterial resistance or toxicity reduction [5] Bacterial resistance to antibiotics is an especially important problem, as is the issue of the systemic side effects created by their administration [5]. Due to the formulation possibilities with liposomes, such problems could be reduced and the efficiency of the drug therapy increased [5].

Of the possible different drug delivery routes for antibiotics, the pulmonary delivery route has been often used for liposomal formulations [6] [7]. Drug delivery to the lung is known to be improved by using liposomes as drug carriers by increasing the concentration of the drug in the pulmonary tissue and reducing unwanted systemic distribution [8]. This can be explained through the influence of particle size on the distribution route [8]. Particles that are inhaled reach alveoli and bronchioles if their sizes range from 500 – 2000 nm, however vesicles with sizes < 500 nm might be exhaled due to their Brownian diffusion [8]. Moreover, the surface charge of inhaled particles plays an important role in the interaction with pulmonary tissueand therefore the absorption process [8]. The mucus that lines areas of the lung such as the bronchioles is negatively charged and the reason why positively charged particles show adhesive behaviour when interacting with it, which is a useful factor to consider for drug application via the pulmonary route [8]. Additionally, only smaller particles with sizes 100 – 200 nm can penetrate the mucus lining [8]. Nebulisers are commonly used for liposomes in order to incorporate them into larger aerosol droplets that then can deposit in the desired location, they transform the applied liquids into a dispersion of aerosol particles [8]. It is important to note that the liposomes are exposed to shear forces when patients use nebulisers [6]. Therefore, liposomes require bilayers of a certain stability to prevent them from being destroyed during the nebulisation process [6]. In addition, unilamellar vesicles with sizes below 100 nm are considered optimal for nebulisation [6]. Due to these different factors playing a vital role for pulmonary drug delivery such as size, surface charge or stability, the physicochemical characterisation of liposomes used for drug delivery is necessary [8]. The main factors that influence liposomal physicochemical properties, are the types and ratios of lipids used [5].

An antibiotic which is used to treat lung infections, but shows known limitations such as developing bacterial resistance as well as systemic side effects is ciprofloxacin (*Figure 2*) [9]. Ciprofloxacin is a broad-spectrum fluoroquinolone, which obstructs bacterial DNA replication, transcription, repair, and recombination through inhibition of topoisomerases II and IV [6]. Due to the fact that ciprofloxacin has already been successfully incorporated in liposomes made from HSPC and cholesterol (Lipoquin) [6], it is possible to compare an already existing formulation with new ones. In this project the fluoroquinolone is to be incorporated in hybrid vesicles made of different lipid/surfactant combinations. The chosen components (DPPC, DPPG, DMPG, DPTAP, DHDAB, PSPC and Cholesterol) are used to create liposomes that vary in their surface charges and membrane stability due to the different lipid/surfactant combinations used. They are a combination of the already studied so-called Fluidosomes made from DPPC/DMPG [10] as well as PSPC and cholesterol, which is a slight variation of the Lipoquin formulation [6] and lipid/surfactant combinations that have not

previously been used for drug incorporation. The liposomal mixtures studied in this project are unique and different from the FDA approved liposomes used in parental drug delivery formulations mostly consisting of DSPC/Cholesterol/Pegylated DSPE [11] as well as liposomes proposed for pulmonary delivery (made from DPPC or DPPC/Cholesterol) [7] [12].

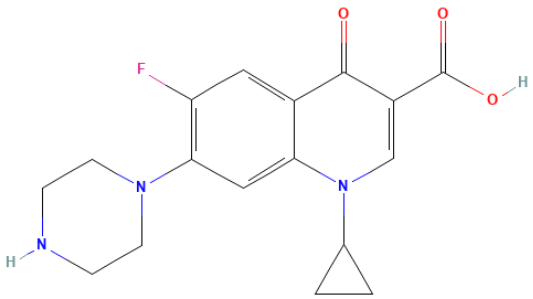

Figure 2: *Structure of ciprofloxacin (derived from PubChem.com)*

The aim of this project is to measure size, zeta potential as well as thermal behaviour of liposomal mixtures for a possible incorporation of Cirpofloxacin. Furthermore to describe physicochemical properties and compare them with the already existing drug carriers [6] [10] in order to propose the optimal liposomes for a possible drug formulation applied by nebulisation. Ciprofloxacin will be entrapped in the chosen liposomal mixtures and the entrapped amount of drug of the different liposomal combinations evaluated and compared. It has previously been speculated that the so-called Fluidosomes made from DPPC/DMPG [10] provide a more fluid lipid mixture than other combinations. This fluidity as well as their negative surface charge is supposed to make them stick more efficiently to Gram-negative bacteria [10]. Nevertheless, this might make their bilayers more dispordered and therefore more prone to destruction under mechanical strain. The membrane fluidity of the hybrid lipid/surfactant combinations will be assessed and compared to the Fluidosomes and the different surface charges examined to possibly assess an advantage in the interaction with Gram-negative bacteria [10]. Another important combination that will be investigated is PSPC/Cholesterol. To recreate the Lipoquin formulation [6] in the following experiments PSPC was used since it is the dominant lipid present in the HSPC mixture. It is expected that this combination will be a particular good drug carrier due to its success in the past [6]. Liposomes made from DPPG/DHDAB are supposed to be more stable due to the formation of an ion pair between the two components [13]. DPPC is also a very successful and well-known liposomal component [14] and is expected to form capable and stable vesicles. It is necessary to understand how the lipids and surfactants work together to build liposomes to design the best possible

drug carrier for ciprofloxacin. Therefore, the size measurement is necessary to see if the constructed particles could be inhaled, withstand the shear forces of a nebulisation process and remain a viable drug carrier [8]. The zeta-potential is important for any drug formulation in the future to predict drug adsorption. In the lung dug adsorption depends amongst other factors on the particle size and on the interaction with the mucus lining [8]. Furthermore, the zeta potential is helpful to avoid interactions regarding stability or particle aggregation within a possible formulation by knowing the zeta potential of particles in a certain bulk solution [15]. The stability, which is additionally necessary to know for withstanding shear forces will be inquired by investigating liposome thermal behaviours by DSC measurements. Through DSC measurements it is possible to obtain information about the different lipid mixture thermotropic transitions and their related enthalpies [16]. Changes observed between phase transitions in pure lipids and specific mixtures can then be an indication of altered bilayer stability at manufacture, storage or administration temperatures [17].

1.1 Preparation methods for liposomes

There are different manufacturing methods for the creation of liposomes, they can be optimized to produce different liposome classes such as SUVs, MUVs, LUVs, GUVs [18]. In this project the liposomes were created by preparing lipid films, adding a buffer/ciprofloxacin mixture together with zirconium beads followed by homogenization using dual centrifugation (DC) (see Materials and Methods). This procedure is a centrifugation process where in contrast to the basic centrifugation the sample is additionally rotated on a second axis [19]. Longitudinal acceleration and sideward acceleration occur through the extra rotation movement [20]. The longitudinal acceleration forces the sample to the top and the bottom of the vial, while the sideward acceleration presses the sample against the wall [20]. As a result of the different accelerations the sample is flowing up and down in the vial rather than being pushed to the top or bottom of its container [20]. Due to these centrifugal forces as well as the high friction created in combination with the zirconium beads, dual centrifugation is eligible for homogenization as well as nanomilling [19] [20].

1.2 Dimensions and DLS

The determination of liposome size is crucial for their optimisation as drug delivery systems. It is important to know the size distribution of the liposomes prepared for drug encapsulation due to the fact that the size of the vesicles influences if and how different barriers can be penetrated (e.g. mucus) or where the particles are deposited in the lung etc. [8] [21].

One of the most common procedures to determine particle sizes is DLS (Dynamic light scattering), which is a non-destructive technique that measures the frequency shift of a laser beam that is scattered by the moving particles in the dispersion [22]. Particles move in their

aqueous surrounding by Brownian motion and the intensity of the scattered light depends on the particle's velocity [22].The moving particles cause constructive and destructive interference, so the intensity of scattered light which is detected by the DLS changes over time [23]. Dynamic light scattering, also referred to as photon correlation spectroscopy, gives us two main results to consider when comparing outcomes of different experiments [24]. One of the most prominent values is the Z-average which is the intensity weighted mean of the particle distribution, whereas the polydispersity index or PDI shows the variance in size, indicating the width of the distribution curve obtained [24]. For a monodisperse distribution the Z-average will be the same as the mean of the size distribution curve [22]. When the distribution is polydisperse, there are more peaks but only one Z-average [22]. In this thesis the obtained Z-average of DLS experiments is given as a diameter in nm.

1.3 Phospholipids and zeta potential

The zeta potential is one of the characteristics of nanoparticles that defines their potential as drug carriers since it can help to understand and sometimes give an estimate how the particles will act in different milieux [15]. It is important to differentiate it from the surface charge which results mostly from ionized functional groups or the adsorption of charges [15]. When particles are dispersed in a bulk solution, they move through Brownian motion [15]. As a result of this movement, a layer of charges builds around each particle called the Stern layer, formed from counter ions existing in the bulk solution [25]. This layer develops around the already existing surface charge layer *(Figure 3*) [25]. The zeta potential is the charge that results on the so-called slipping plane of the Stern layer, which is the part that interfaces with the aqueous surrounding and the diffuse charged layer of the particle [25]. The zeta potential depends on the pH value of the surrounding dispersion [23]. The pH can change the zeta potential due to the fact that there is a higher or lower number of ions present depending on the changes of the pH value [23]. These ions influence the electric double layer consisting of the tight and loose layers of ions around the particles [23] [26]. That is why a dilution of the measured sample might be necessary, depending on the composition of the dispersion [23].The measurement of the zeta potential is possible through the application of an electric field across the dispersion [15] [25]. The particles will move towards the electrode with the opposite charge to their net charge with a certain acceleration that is proportional to the force of the applied electric field on the charges surrounding the particle, this information then is used to calculate the zeta potential [15] [25]. A very common way to determine the zeta potential measurement is Electrophoretic light scattering (ELS), during which the particles moving through an electric field scatter incident laser light [23]. The dispersed beam experiences a Doppler shift (shift in frequency), which is proportional to the speed of the particles, allowing the determination of the zeta potential [23].To gain knowledge about the colloidal stability of dispersed particles, the zeta potentials are the most prominent data to acquire [23]. It is generally considered, that values of > ± 30mV are necessary to form highly stable particle dispersions [23]. These values are to be taken with

caution since the zeta potential only gives information about electrostatic repulsive forces and not the van der Waals forces, which can also impact colloid stability [27].

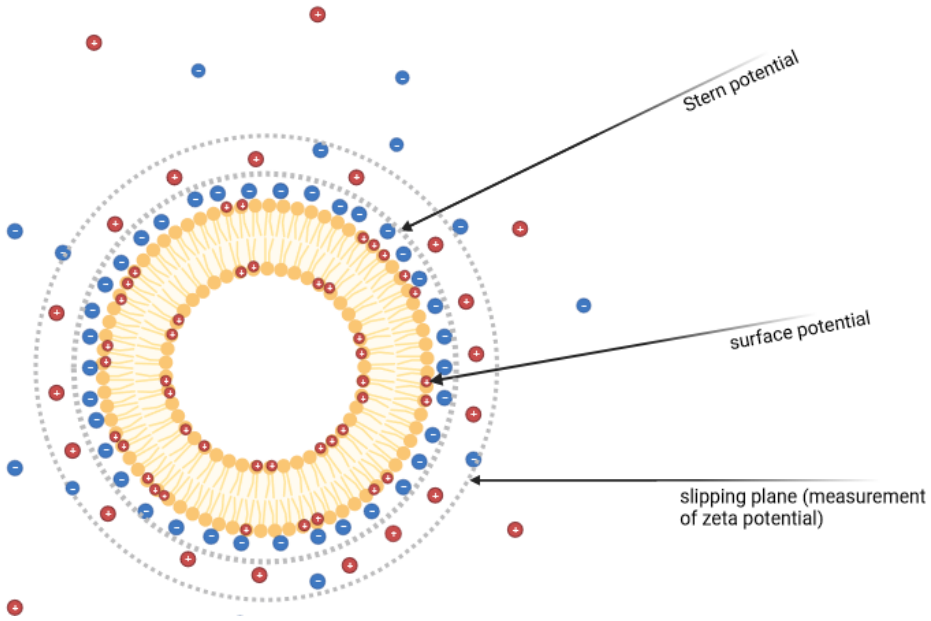

Figure 3: *The different charged layers of a dispersed particle in a bulk solution.*

The zeta potential of lipid vesicles depends on their different phospholipid and/or surfactant components. Different combinations of amphiphiles will give a different zeta potential. The zeta potential is as mentioned an important factor in the adsorption process, it must be considered when working with bacteria/antibiotics. Bacterial membranes containing lipopolysaccharides carry a negative charge, therefore differences in liposome zeta potential will influence the interaction of the particles and their putative targets. Negatively charged vesicles are for example made from mixtures of zwitterionic DPPC and anionic DMPG, which are known to fuse with bacterial envelopes to deliver antibiotic payloads [10]. The Lipoquin-mimicking [6] formulation consists of the zwitterionic PSPC, there is the positive charge of the choline and the negative charge of the phosphate. The added cholesterol is by itself a neutral molecule. These two formulations will be created and used to compare with self-designed hybrid vesicles, containing the lipid/surfactant mixtures mentioned above. Some of the hybrid vesicles contain DPPG, which is a negatively charged phospholipid. The zwitterionic DPPC will be used to create liposomes with DHDAB among others which is a positively charged surfactant. The positively charged DPTAP will also be mixed with DPPC as well, in addition to mixtures containing DPPG. The above-mentioned surface charge properties, conferred by the components used, contribute to the measured zeta potential when the liposomes are formed in their surrounding bulk solution.

1.4 Thermotropic phase transitions and DSC measurements

Phase transitions happen due to the intake or release of energy causing a change in state of a substance, which makes them endothermic or exothermic procedures [28]. During phase transition lipid bilayers undergo structural changes, such as acyl/alkyl chain melting [29]. When liposomes are formed below their chain melting transition temperature (T_m), the phospholipids are in an ordered, almost crystalline state aligning their lipid tails perfectly [29]. When there is heat applied, the ordered state or gel state changes into a more amorphous and fluid state where the lipid tails are more disordered [30]. Sometimes there are additional peaks marking so-called pre-transitions, which are associated with the transition from the gel to a rippled gel phase, which additionally is a marker for impurities [29] *(Figure 4).* Increases of phase transition temperatures of vesicles where different components with known transition temperatures are combined are an indication of a bilayer stability increase [17]. There is more energy needed to bring the lipids to a disordered state, which can be explained through a more stabilized crystalline packing of the lipids [17].

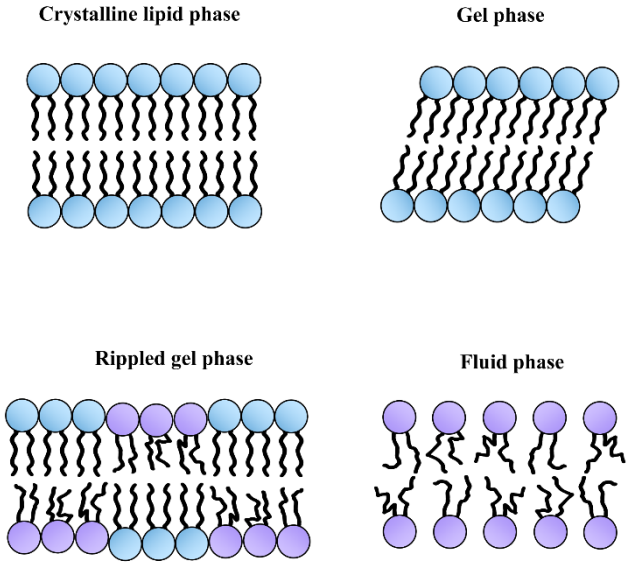

Figure 4: *Illustration of the different phases phospholipids possibly go through during a phase transition.*

The most common method used to determine phase transitions in lipids is Differential Scanning Calorimetry (DSC) [31] *(Figure 5).* DSC measures how lipid physical states are modified due to temperature changes and plots these changes of heat flow against time or temperature [31]. The heat flow difference is always detected through the chosen sample and a reference material and describes the quantity of heat that is released or absorbed [31].

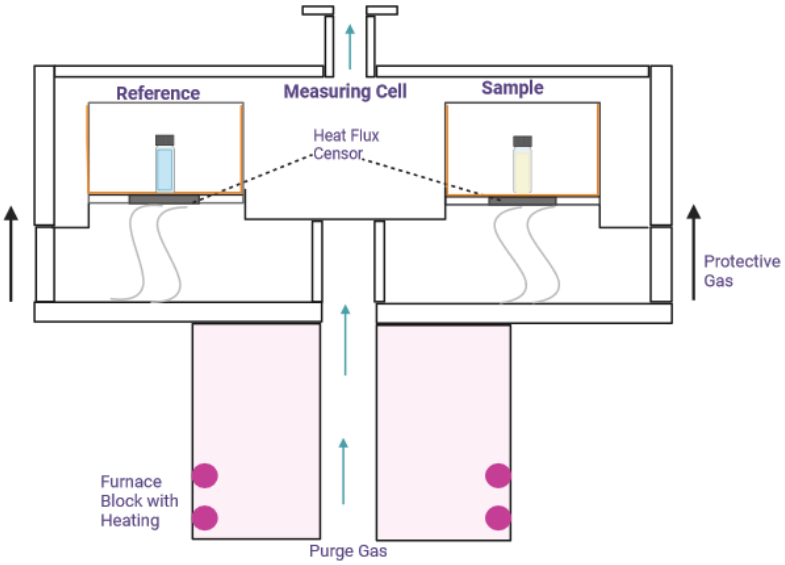

Figure 5: *The principle of the setup of a Differential Scanning Colorimetry (DSC) device.*

Important parameters for the analysis of the DSC thermograms produced by liposomes are T_m (the main chain-melting transition), which is the point where the phase transition happens, and the enthalpy change ΔH of the endothermic or exothermic processes [16] *(Figure 6*). The enthalpy (ΔH) is calculated by integrating the endothermic or exothermic peaks of the thermogram [29]. The enthalpy is the energy that is needed (either release or intake) to achieve a phase transition.

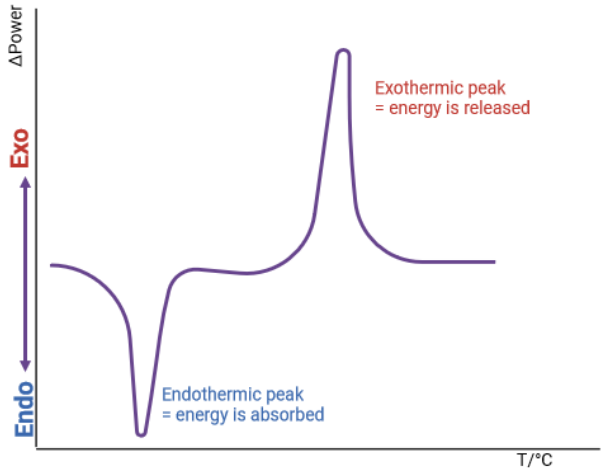

Figure 6: *Basic illustration of a DSC thermogram.*

To make liposomes more fluid at specific temperatures there is the possibility of mixing phospholipids with different transition temperatures together [17]. Liposomes made from components that have relatively high transition temperatures (> 37°C) with respect to the mean body temperature are supposed to be more stable and less permeable for the drugs encapsulated, whereas phospholipids with low transition temperatures (< 37°C) are expected to easier release encapsulated drugs in aqueous surrounding at body temperature [17].

The aim of this project is as mentioned before, is the collection of data (size, zeta potential, transition temperature etc.) on the chosen phospholipid combinations in order to find the most suitable vehicles for the encapsulation of the antibiotic ciprofloxacin possibly used for nebulisation. The suitability will be determined by the incorporation success of the drug in addition to the sizes of the vesicles, as the size distribution of the particles will be determined and signs of aggregation possibly revealed. The zeta potential will be measured in order to determine the particles ionic behaviour in the surrounding bulk solution. It will be revealed, if the zeta potential makes sense in terms of the surface charges of the used components. Moreover, the bilayer stability will be assessed by the thermal analysis through a DSC-measurement. This will show transition temperature increases or not. There might be unplanned pre-transitions or other signs of anomalies which will be investigated and discussed in detail in the following sections of this thesis.

2. Materials and Methods

2.1 Materials

The ultrapure milli-Q water used in all experiments was obtained from a PURELAB flex 3 (ELGA LabWater,UK). Triton^{TM} X-100 (T8787), ciprofloxacin HCl, cholesterol dihexadecyldimethylammonium bromide (DHDAB) and TRIS (Tris(hydroxymethyl)aminomethane) were purchased from Sigma-Aldrich GmbH (Darmstadt, Germany). The lipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'rac-glycerol) (sodium salt) (DPPG), 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine (PSPC), 1,2-dipalmitoyl-3-trimethylammonium-propane (chloride salt) (DPTAP) and 1,2-dimyristoyl-sn-glycero-3-phospho-(1'rac-glycerol) (sodium salt) (DMPG) were all supplied by Avanti Polar Lipids (Alabaster, AL, USA). The solvents chloroform ($CHCl_3$) and methanol (MeOH) were obtained from VWR International (Darmstadt, Germany). Sodium Chloride (NaCl) was supplied by Carl Roth GmbH (Karlsruhe, Germany).

2.2 Manufacturing liposomes using Dual Centrifugation (DC)

Firstly, 40 mg of ciprofloxacin HCl was dissolved in 10 ml of 10 mM tris,150 mM NaCl buffer creating a 10.88 mM solution. Followed by the preparation of 10 mM lipid solutions (DPPC, DHDAB, DPTAP, PSPC, DMPG and cholesterol) in chloroform/methanol 9:1. DPPG was dissolved in chloroform/methanol 1:1. For manufacturing the different liposome formulations, the lipid stock solutions were combined in various ratios *(Table 1)* and a total of 1 ml was transferred to 2 ml volume ZentriMix-vials. Lipid films were created by the removal of solvents under reduced pressure (150 mbar) using a Rotavapor Hei-VAP Expert Control, (Heidolph Instruments, Germany) for approximately 20 minutes, at 40°C. Subsequently, 50µl of a 10.88 mM ciprofloxacin HCl stock solution and 100 mg of Y Beads (made from zirconium oxide) 0.1-0.2 mm were added to the vials. The dual centrifugation was carried out using a ZentriMix 380 R centrifuge (Hettich GmbH, Germany) offset to 2500 rpm, for 20 minutes at 30°C. After this initial centrifugation, 1 ml of 10.88 mM ciprofloxacin HCl was added and another centrifugation cycle was conducted at 30°C for 2 min at 1500 rpm. Prior to storing the liposomes at 4°C overnight, the vials were centrifuged for 10 seconds at 10000 rpm in a Microcentrifuge (Hettich GmbH, Germany) to sediment the beads.

Table 1: *The molar ratios of lipids and surfactants used.*

DPPG/DPTAP 2:1	DPPG/DPTAP 4:1	DPPG/DHDAB 2:1	DPPG/DHDAB 4:1
DPPC/DPTAP 2:1	DPPC/DPTAP 4:1	DPPC/DHDAB 2:1	DPPC/DHDAB 4:1
DPPC/DMPG 9:1	DPPC/DMPG 18:1	PSPC/Cholesterol 1:1	PSPC/Cholesterol 2:1

2.3 Liposome Size and Zeta Potential

To measure the size of the liposomes as well as their zeta potential, the Zetasizer Pro (Malvern Panalytical Ltd, UK) was used. The measurements were taken at room temperature (25°C). For the zeta potential disposable folded capillary cells (DTS1070) filled to the maximum marking and for the size measurements simple disposable plastic cuvettes with 350 µl sample were used. The samples for the measurements were prepared in 10 mM TRIS without sodium chloride, since the high concentration of ions from the salt interfere with the zeta potential measurement. The particle size was measured via Dynamic Light Scattering (DLS), using the instrument general-purpose mode and an equilibration time of 10 seconds. The size and zeta potential measurements were determined from an average of three runs. Prior to starting the measurement, the prepared samples were diluted 1:4 with 10 mM tris buffer and then transferred to the cuvettes. For the analysis the ZS-Xplorer software was used.

2.4 Differential Scanning Calorimetry (DSC)

The DSC measurements were conducted using a Malvern Cal PEAQ-DSC (Malvern Panalytical Ltd, UK). The chosen heating rate was 60 K/h at a temperature range of 2°C to 90°C using medium feedback mode. At least two runs were carried out for each sample, with the data from the second run being selected for the analysis. All prepared samples had a concentration of 2 mM total lipid. Powdered lipids were dispersed in 10 mM tris buffer (pH 7.3) which was also used as the reference. The volume for all samples as well as the reference was 250 µl.

For the analysis, the area under the curve relating to the enthalpy (kJ mol^{-1}) was calculated using the programme Fityk [32].

2.5 Determining the liposomal encapsulation of ciprofloxacin HCl using UV-Vis Spectroscopy

For the purpose of establishing the amount of drug that can be incorporated in the different liposome formulations, a column separation of the passively loaded ciprofloxacin liposomes was conducted using PD-10 size exclusion columns packed with Sephadex™ G-25M resin (Cytiva, Buckinghamshire, UK) to remove any unentrapped antibiotic. Fractions of 0.5 ml were collected after loading 0.5 ml of buffer. The liposomes were detected by eye in the 7^{th} fraction due to its turbidity and 150 µl of this fraction was pipetted on a 96-well plate. Subsequently adding 50 µl of 1% (w/w) Triton x-100 solution (in 10 mM TRIS/150mM NaCl) directly on the plate in order to destroy the liposomes and free the incorporated drug, mixing the samples and carefully removing bubbles that could interfere with the spectroscopic

measurement, the absorption of every sample was determined using an EPOCH 2 NSC microplate spectrophotometer (BioTek Instruments, Germany) reading an absorbance spectrum (200nm to 700nm, read speed: normal, delay: 100 ms, measurement/data point: 8). To compare the acquired results blanks of 150 µl of 10.88 mM ciprofloxacin HCl in 10 mM TRIS buffered saline and 50 µl of 1% (w/w) Triton x-100, 150 µl buffer without ciprofloxacin HCl and 50 µl of 1% (w/w) Triton x-100 and 200 µl of buffer by itself were also measured.

For determination of the drug concentration in the particles, a calibration curve was constructed from absorbances measured with different concentrations of ciprofloxacin HCl (81.6 µM, 40.8 µM, 20.4 µM, 10.2 µM, 5.1 µM and 2.55 µM) in buffer. Each sample consisted of 150 µl of a different drug concentration in 10 mM TRIS/ 150 mM NaCl combined with 50 µl of 1% (w/w) Triton x-100 and the absorbance measured. This absorbance at 320 nm together with the different concentrations was then used to create a calibration *(Figure 12).* The wavelength of 320 nm was chosen because the peak with the highest absorbance of ciprofloxacin HCl was detected at this wavelength, while measuring the absorbance spectrum (200 nm – 700 nm) of the concentrations of ciprofloxacin HCl in buffer mentioned above together with 50 µl of 1% (w/w) Triton x-100.

3. Results

3.1 Liposome characterisation

Inconclusive size measurements can be signs of colloidal instability resulting in aggregation of the particles. For certain lipid or lipid/surfactant combinations (DPPG/DPTAP 2:1, PSPC/Cholesterol 1:1, 2:1, DPPG/DHDAB 2:1 and 4:1) no stable zeta potential measurement could be obtained *(Table 2)*. These liposomal mixtures already showed signs of aggregation during the production of the liposomes, due to the turbid-looking liposomal dispersions. When stable liposomes are manufactured, opalescent dispersions are created. The only combination that seemed to have an acceptable mean particle size of 147.9 nm was PSPC/Colesterol 1:1. The liposomes were still considered to be unstable since the mean size of two populations included one that was beyond 1000 nm, therefore these vesicles would not be suitable for the encapsulation of ciprofloxacin HCl under the chosen conditions.

Table 2: *The results of the DLS measurements of the most unstable liposomes. The size portrayed is the mean of three measurements.*

liposomal combination	**mean size measured in nm**
DPPG/DPTAP 2:1	3741.0
PSPC/Cholesterol 1:1	147.9
PSPC/Cholesterol 2:1	469.6
DPPG/DHDAB 2:1	3208.0
DPPG/DHDAB 4:1	400.6

Additionally, for the formulation development reasons as mentioned in *section 1.2*. the sizes of the liposomes are important to see if the vesicles are comparable amongst the lipid/surfactant combinations currently used. Moreover, it is important that the zeta potential makes sense in terms of the net charges of the lipid combinations used as well as the charges present in the bulk solution, for example it is expected that mixtures of DPPC and DMPG are anionic. When combining DPPC with DPTAP or DHDAB the resulting zeta potential should be cationic. Mixtures of DPPG and surfactants depend on which component makes up the majority of the combination. The zeta potential of the liposomes ended up being in the expected ranges, due to the negatively or positively charged components used. The average particle size *(Figure 7)* for the liposomes made from the combination of DPPC/DHDAB containing more DPPC (4:1) had a larger average size and a lower zeta potential than combinations with DPPC/DHDAB 2:1. One of the few negative zeta potentials was measured for DPPC/DMPG 9:1 whereas the combination with the molar ratio 18:1 was effectively neutral with -11.0 mV, which shows that less DMPG produces less anionic liposomes. DLS measurements showed a size of 85.9 nm for DPPC/DMPG 18:1 and 94.6 nm for the ratio of 9:1. Particles made from DPPC/DPTAP 2:1 resulted in a size of 87.8 nm and a zeta potential of 68.3 mV. The same combination with a ratio of 4:1 had a particle size of 80.8 nm and an average zeta potential of 52.2 mV. Finally, the last negative zeta potential, due to the presence of

DPPG, of -61.6 mV was measured with the combination of DPPG/DPTAP 4:1 with a size of 103.5 nm.

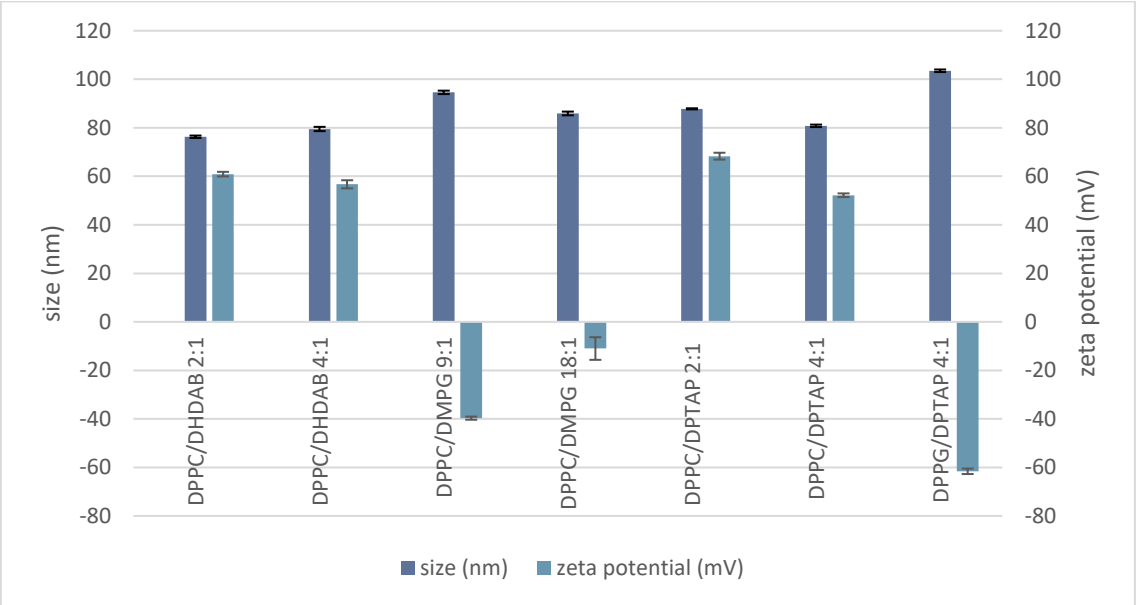

Figure 7: *The mean results of three DLS (size in nm) and zeta potential (mV) measurements of the stable liposome formulations. The error bars represent the standard deviation of the three measurements the means are presented of.*

3.2 Differential Scanning Calorimetry (DSC) measurements

To further investigate the different properties of the lipid/surfactant combinations to formulate the liposomes, the combinations which produced the most stable measurements in terms of size and zeta-potential were chosen to conduct DSC experiments with. The DSC measurements add more information to the stability assessment of the liposomes and allow an evaluation of whether the vesicles are a good option for nebulisation. They might show a tendency for the lipids to phase separate as well as whether the so-called Fluidosomes [10] are as fluid at ambient and body temperatures as their name suggests. For the sake of a more comprehensive overview and the possibility to discuss the different impacts and reasons for the results, liposomes composed of the pure individual components were also measured.

DPPC liposomes showed a pretransition at approximately 35.5 °C and the main chain-melting transition at 41.6°C which is close to previously published values for DSC measurements for DPPC in buffer (pH 7.4) at 41.5°C [33, 34]. The peak onset was measured at ~38.0°C with the end at ~45.9°C. As the area under the curve was integrated, the result was an enthalpy of 45.4 kJ mol^{-1} *(Table 3)* which is close to the previously published enthalpy of 41.0 kJ mol^{-1} [35]. DHDAB vesicles had a main transition temperature (T_m) of 29.1°C which is also close to the previously published value of 28°C [36]. The peak of the thermogram was integrated and

started at ~24.8°C and ended at ~37.9°C with an enthalpy of 32.6 kJ mol^{-1}. For the first combination of DPPC/DHDAB 2:1 a T_m of 49.0°C was measured, which is a definite increase compared to DPPC as well as DHDAB by itself. This is not true for the same combination with the molar ratio of 4:1 (43.6°C) which is a lower enthalpy than for DPPC. DPPC/DHDAB 2:1 did have a very small peak right after the main transition, which was not visible in the measurement of the 4:1 combination *(Figure 8).* The enthalpy of DPPC/DHDAB 2:1 increased by 3.6 kJ mol^{-1} compared to DPPC by itself, whereas the enthalpy of DPPC/DHDAB 4:1 decreased by 1.8 kJ mol^{-1}, indicating that the combination with the molar ratio of 2:1 is more stable than DPPC/DHDAB 4:1 *(Table 3).*

DMPG produced a broad peak with the highest point at 30.4°C with a quoted T_m at 23.3°C [37]. The enthalpy calculated by integrating the area under the curve was 26.2 kJ mol^{-1}. Liposomes made of DPPC/DMPG 18:1 had a main transition at 41.6°C. Liposomes made of the same lipids with the molar ratio of 9:1 produced a much broader peak, indicating disordered bilayers, that was not as narrow as for the 18:1 combination with a main transition temperature at 46.2°C. It is clear that the increased amount of DPPC in the 18:1 ratio has an influence on the transition temperature, which increased compared to DPPC/DMPG 9:1. Both combinations (DPPC/DMPG 18:1 and 9:1) showed higher transition temperatures compared to DPPC and DMPG liposomes by themselves *(Figure 9).* The enthalpies calculated for the mixtures of DPPC/DMPG both increased compared to DPPC. The enthalpy for DPPC/DMPG 9:1 was 67.6 kJ mol^{-1} and for the molar ratio of 18:1 46.1 kJ mol^{-1} whereas for DPPC 45.4 kJ mol^{-1} were calculated *(Table 3).* This indicates that the Fluidosomes [10] are not as fluid as they are claimed to be, due to the indication of a gel phase stabilization due to the higher enthalpies as well as the increase of the transition temperatures. Nevertheless, the onset of the thermogram peaks was lower with ~ 6.7°C for DPPC/DMPG 9:1 and ~ 12.0°C for DPPC/DMPG 18:1 than for DPPC with ~ 38.0°C, which can be an indication for lower stability aligning with the theory of the mixtures being more fluid.

The DPTAP liposomes showed a divided peak with the first transition at ~39.3 °C and the second one at 43.1°C, the main transition of DPTAP in buffer (pH 7.4) was quoted at 52.8°C [38]. Previously published data of divided thermogram peaks suggests interdigitation between the hydrophobic lipid chains [39]. When combined with DPPC at a molar ratio of 2:1 with DPTAP showed a main transition at 53.6°C and in the molar ratio of 4:1 at 52.0°C both combinations resulted in clear single peaks with no sign of phase separation. The DPPC/DPTAP combinations also showed an increase in transition temperatures compared to the pure lipids, which indicates that DPTAP stabilises the liposomes *(Figure 10).* The enthalpies calculated for DPPC/DPTAP 2:1 was 55.8 kJ mol^{-1} and for the molar ratio of 4:1 52.5 kJ mol^{-1}, both being higher than the enthalpy for DPPC (45.4 kJ mol^{-1}) by itself suggesting a gel phase stabilization for the mixtures. The onset temperatures of the integrated peaks for the mixtures were lower than for DPPC but higher than for pure DPTAP *(Table 3).*

Finally, liposomes made from DPPG/DPTAP 4:1 were measured (*Figure 11).* While DPPG liposomes produced a very narrow peak with a transition temperature at 41.4°C quoted at 41.0°C [13] and a very small pretransition ~21.0°C quoted at around 37.0°C [13]. This combination with DPTAP in a molar ratio of 4:1 produced a divided peak with an additional transition before the main transition at ~41.5°C. This could be due to interdigitation taking place

between the hydrophobic tails [39]. This would explain why the range of the measured transition is from ~14.4°C to ~64.1°C, meaning that gel phase is stable to almost 60°C. The enthalpy calculated for DPPG was 47.5 kJ mol^{-1} and 109.6 kJ mol^{-1} for DPTAP. The mixture of DPPG/DPTAP 4:1 showed a lower enthalpy with 44.7 kJ mol^{-1} than both single components. *(Table 3).*

For a better visualisation and comparison of the DSC experiment data, different graphs containing several results were created *(Figure 8 - Figure 11).* The graphs of the single measurements are presented in the Appendix *(Figure 13 - Figure 24*).

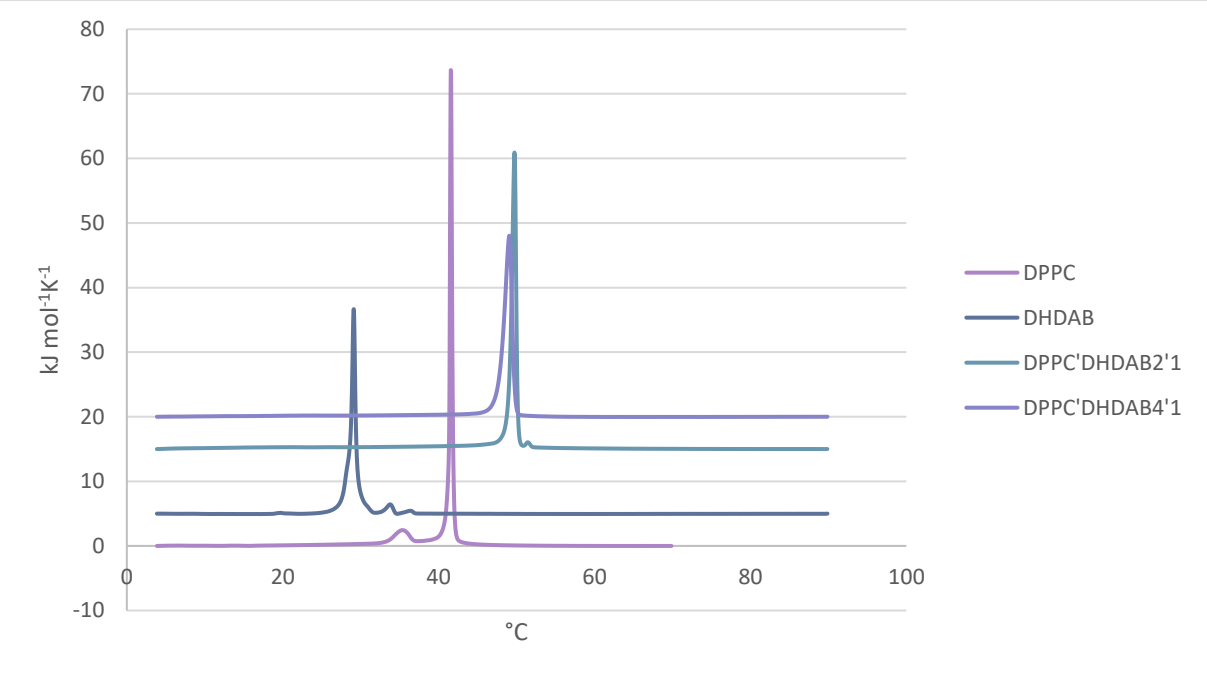

Figure 8: *The measurement results of DPPC, DHDAB, DPPC/DHDAB 2:1 and DPPC/DHDAB 4:1 liposomes. All values on the y-axes have been increased (DHDAB +5, DPPC/DHDAB 2:1 +15 and DPPC/DHDAB 4:1 +20) except for the DPPC results to facilitate the visualisation of the comparison.*

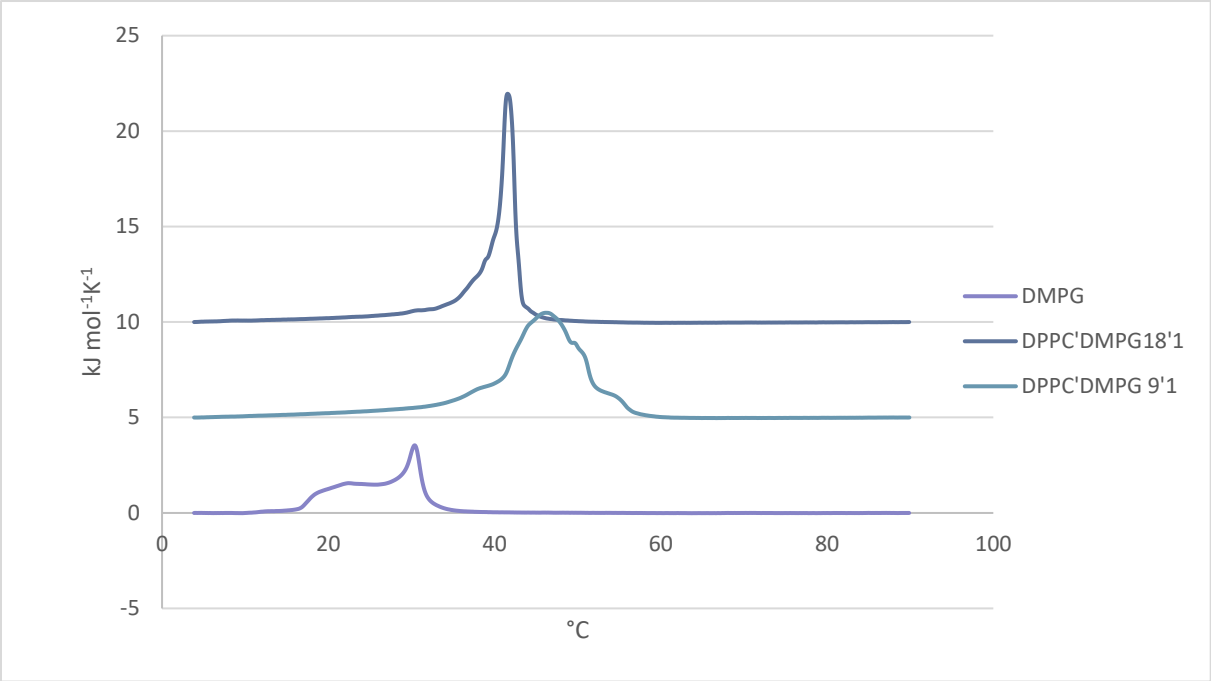

Figure 9: *The DSC measurement results for DMPG, DPPC/DMPG 18:1 and DPPC/DMPG 9:1 particles. The values on the y-axes have been altered in steps of five (DPPC/DMPG 9:1 +5 and DPPC/DMPG 18:1 +10) except for DMPG.*

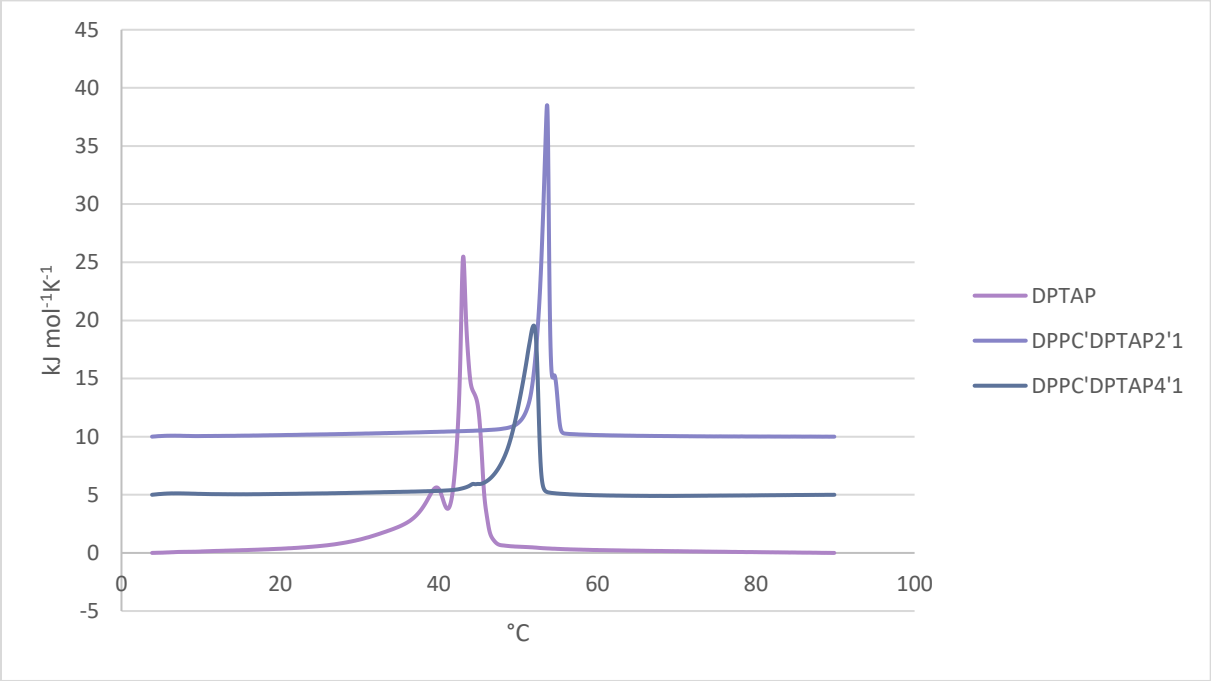

Figure 10: *Visualisation oft the results of the DSC measurements of DPTAP, DPPC/DPTAP 2:1 and DPPC/DPTAP 4:1. The values on the y-axes for DPPC/DPTAP 2:1 and 4:1 have been increased in steps of five.*

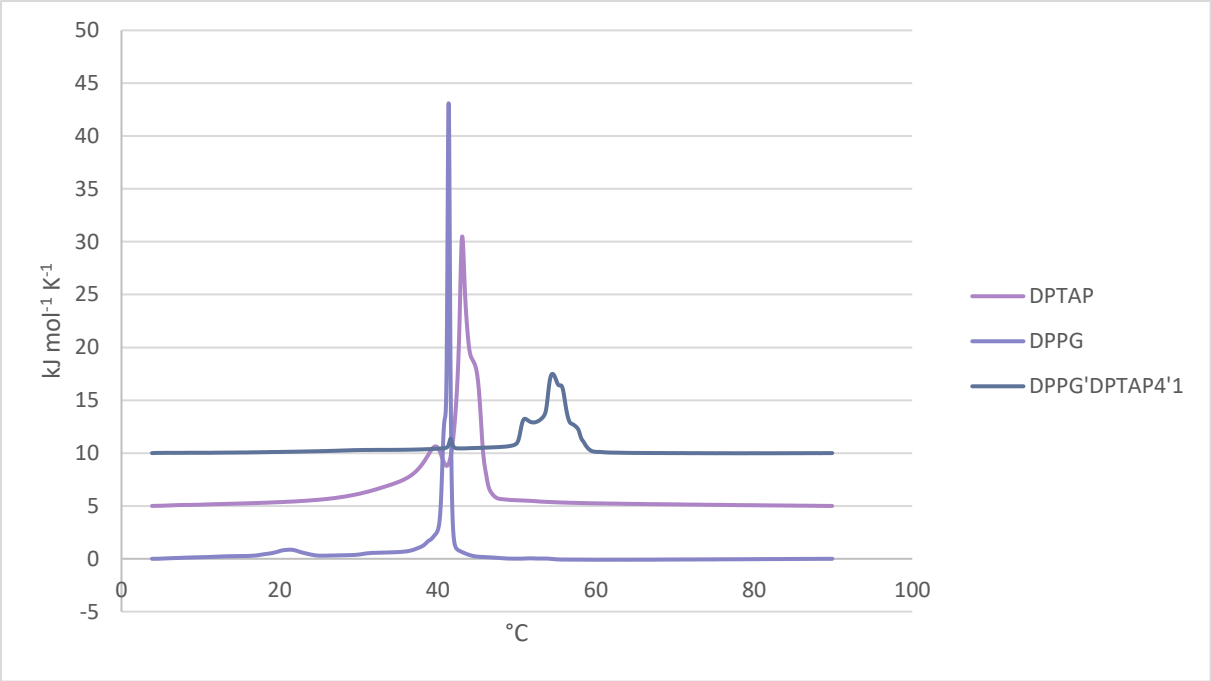

Figure 11: *The measurement results of the DSC experiments of DPTAP, DPPG and DPPG/DPTAP 4:1. The values of DPTAP and DPPG/DPTAP 4:1 on the y-axes have been increased by 5 for DPTAP and 10 for DPPG/DPTAP 4:1.*

The enthalpy of the peaks was determined by calculating the area under the curve (Fityk), which is presented in the table below (*Table 3).*

Table 3: *The calculations of the area under the curve (Fityk) is equal to the enthalpies listed.*

Liposomal combinations	Enthalpy (kJ mol^{-1})	Peak onset (°C)	Peak end (°C)
DPPC	45.4	38.0	45.9
DHDAB	32.6	24.8	37.9
DPPC/DHDAB 2:1	49.0	39.3	54.9
DPPC/DHDAB 4:1	43.6	35.4	53.6
DPTAP	109.6	13.9	68.3
DPPC/DPTAP 2:1	55.8	27.3	61.8
DPPC/DPTAP 4:1	52.5	25.4	57.7
DMPG	26.2	10.0	48.0
DPPC/DMPG 9:1	67.6	6.7	59.1
DPPC/DMPG 18:1	46.1	12.0	50.3
DPPG	47.5	27.3	47.1
DPPG/DPTAP 4:1	44.7	14.4	64.1

3.3 Ciprofloxacin HCl concentration in the liposomes

The calibration curve *(Figure 12)* showing the relationship between ciprofloxacin HCl and the UV absorbance at 320 nm, is linear over the range of concentrations used. The measured concentrations of the incorporated drug in the liposomes measured fell within this chosen range, except for DPPG/DHDAB 2:1 for which no drug incorporation could be evaluated.

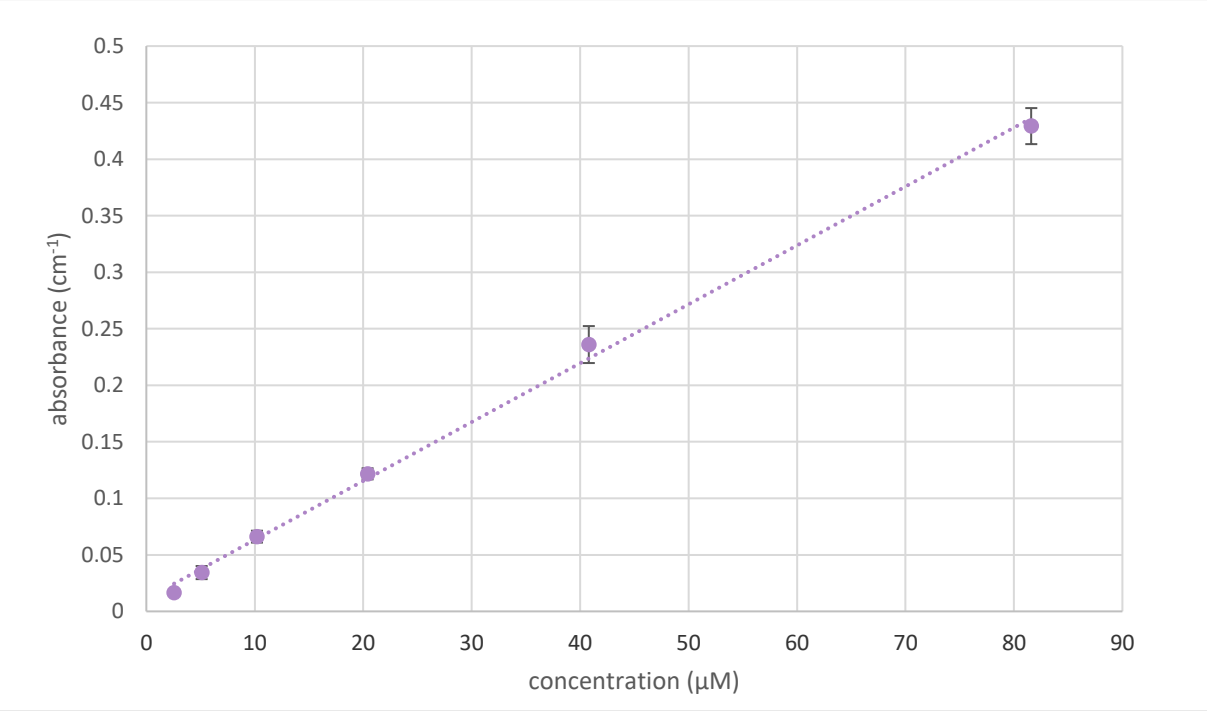

Figure 12: *Calibration curve portraying concentrations of ciprofloxacin HCL on the x-axes and the mean absorbance measured at 320 nm on the y-axes. The error bars show the standard deviations. To set up the calibration curve, the measurements were repeated three times on three different days.*

Another important factor to compare the data on is the encapsulation efficiency, which shows the percentage of the mass of drug that was successfully incorporated in the liposomes.

Table 4: *Results of calculating the concentrations of the drug incorporated in the particles with the help of the linear equation of the calibration curve, as well as the mass of drug before the column separation, the masses of the encapsulated drug and encapsulation efficiency of ciprofloxacin HCl in the liposomes.*

	Absorbance (cm^{-1}) at 320nm	*Concentration (µM)*	*Mass of drug before column separation (µg)*	*Mass of drug after column separation (µg)*	*Encapsulation efficiency (%)*
DPPG/DHDAB 2:1	0.01	- 0.54	2001.0	-	-
DPPC/DHDAB 2:1	0.46	85.62	2001.0	15.75	0.79
DPPC/DHDAB 4:1	0.36	66.96	2001.0	12.32	0.62
DPPG/DPTAP 2:1	0.07	10.42	2001.0	1.92	0.1
DPPG/DPTAP 4:1	0.38	70.81	2001.0	13.02	0.65
DPPC/DPTAP 2:1	0.21	37.35	2001.0	6.87	0.34
DPPC/DPTAP 4:1	0.29	52.92	2001.0	9.73	0.49
PSPC/Cholesterol 1:1 *(Lipoquin variation)*	0.29	53.69	2001.0	9.87	0.49
DPPC/DMPG 9:1 *(Fluidosomes)*	0.14	24.27	2001.0	4.46	0.22
DPPC/DMPG 18:1 *(Fluidosomes)*	0.22	39.85	2001.0	7.34	0.37

The data shown in *Table 4* is the amount of ciprofloxacin HCl that was calculated to be in the sample after the unentrapped drug solution was removed with the size-exclusion column separation and the liposomes were solubilised by Triton x-100, to measure the absorbance of the drug by itself. The combination of DPPG/DHDAB 2:1 had the lowest encapsulation of ciprofloxacin HCl, whereas DPPC/DHDAB 2:1 showed the highest amount of entrapped drug with 15.75 µg. Most particles containing DPPG were less successful in encapsulating ciprofloxacin HCl compared to the liposomes containing DPPC, except for DPPG/DPTAP 4:1 which had the highest amount of drug incorporated with 13.02 µg right after the combination of DPPC/DHDAB 2:1, which is followed by DPPC/DHDAB 4:1. An overall trend was that liposomes made from DPPC/DHDAB held more drug than the particles with DPPC/DPTAP. Particles made of PSPC/Cholesterol 1:1 placed 4th in the ranking of incorporation success, whereas the Fluidosomes [10] made from DPPC/DMPG positioned in the lower half, of the two DPPC/DMPG 18:1 encapsulated more drug with 7.34 µg than the combination with the molar ratio of 9:1 (4.46 µg).

4. Discussion

The aim of this project was to characterize the various liposomes in terms of their size, zeta potential as well as their thermal behaviour in order to evaluate their stability. Furthermore, ciprofloxacin HCl was incorporated in new hybrid lipid/surfactant vesicles, as well as in the previously published liposomal formulations: the PSPC/Chol Lipoquin formulation [6], the DPPC/DMPG Fluidosomes [10] and the combination of DPPG/DHDAB, for which published data already exists [13]. The above-mentioned characteristics are crucial for possible development of an antibiotic formulation for nebulisation in order to be able to assess possible interactions within a formulation itself, to predict the adsorption of the drug at biological interfaces, as well as the interaction with the bacterial target, as well as to obtain the necessary information if the liposomes could potentially withstand the shear forces created through the process of nebulisation.

Prior to discussing the results of the distinct liposomal formulations, it is necessary to state that the encapsulation efficiency of the supposedly good vehicles was low. This result is very likely to be associated with the preparation method of the liposomes. Ciprofloxacin is pH sensitive, and the encapsulation efficiency is influenced by the pH value of the bulk solution [40]. A pH gradient during the preparation in the loading process of the drug could be helpful in order to incorporate more drug into the liposomes [40]. Supposedly the encapsulation efficiency of ciprofloxacin is higher when the vesicles used are negatively charged [40]. It is said that the amine group of ciprofloxacin becomes positively thus facilitating drug/lipid interactions, making it more likely to show increased entrapped in negatively charged vesicles [40] [41]. Additionally, the lipid concentration of the liposomes plays an important role, since a higher lipid concentration is likely to create larger liposomes, therefore increasing the space for the drug to be incorporated. Moreover, the liposome dispersions created in this project with ciprofloxacin HCl entrapped, did not show a high lipid concentration in the first place (10 mM) therefore the density of liposomes in a sample is not high and the amount of drug that can be incorporated is limited.

Although the Lipoquin combination set high hopes for this study due to its documented past as a drug carrier [6], the approximate PSPC/Chol version used in this study did not meet expectations . Their large size indicated a lack of colloidal stability, since the vesicles were expected to have a diameter lower than 100 nm, which is the suitable size for nebulisation [6]. This was not achieved with the performed preparation method used in this study and the size of the liposomes was a lot bigger (*Table 2*). This could be due to aggregation, which is to be expected from neutral liposomes, making them not useful for drug delivery as they have been considered previously [21]. Additionally, the fact that extrusion and remote loading of the Lipoquin liposomes was used [6] [42] , and not passive loading with dual centrifugation, may have been responsible for the results observed in this study. To achieve remote loading, a pH gradient was used [42], which apparently improves the entrapment efficiency of ciprofloxacin [40]. The preparation method has a high impact on the quality of liposomes produced since different preparation methods can lead to different sizes of liposomes and different sizes of vesicles entrap different amounts of drugs. Moreover, the entrapment of

ciprofloxacin can be improved as mentioned by a pH gradient during the loading of the liposomes [40]. Nevertheless, the encapsulation seemed to be more successful than in other mixtures since the liposomes made from PSPC/Cholesterol entrapped more drug than the DPPC/DMPG Fluidosomes [10] among others *(Table 4).*

The second previously published antibiotic-loaded liposomes made from DPPC/DMPG 9:1 and 18:1 [10] performed a lot better than the PSPC/Cholesterol mixtures in terms of size measurement, as they showed no signs of aggregation *(Figure 7).* On the other hand, they did not show a very high drug incorporation *(Table 4).* The DPPC/DMPG 18:1 combination turned out to be more efficient in terms of encapsulating the antibiotic. The 9:1 combination produced a much broader and noisier peak in the DSC measurements which shows that the gel phase was present over a wider range of temperatures, albeit with evidence of overall disorder. The thermogram shows that the 9:1 liposomes have different phase transitions within their system, therefore making the whole composition more flexible. Nevertheless, the enthalpy of DPPC/DMPG 9:1 was higher than the enthalpy of DPPC/DMPG 18:1 as well as DPPC by itself, indicating that the Fluidosomes are not as fluid as they are said to be [10]. This might be due to more efficient packing, leading to stronger Van der Waals forces between the lipid chains, therefore increasing the enthalpy [43] increasing with the reduction of DPPC in this composition.

The DPPG/DHDAB hybrid lipid/surfactant vesicles were expected to be more stable due to the potential for ion pair formation between the components [13]. This could not be proven in the experiments performed, since the combination of DPPG/DHDAB 2:1 did not show sufficient results in the drug encapsulation process *(Table 4)* this is why no DSC measurement and hence stability assessment were conducted. Previously performed DSC measurements showed that when DPPG and DHDAB are added together in different molar ratios (x_{DHDAB}= 0.2-0.7) there is an increase of transition temperatures compared to pure DPPG and DHDAB, indicating a stabilized gel phase [13]. This was proposed to be due to an ion pair forming between the surfactant and the phospholipid [13]. The size measurements of DPPG/DHDAB 2:1 as well as for DPPG/DHDAB 4:1 indicated aggregation of the vesicles *(Table 2)*. This was also visible during the production of the vesicles due to intense cloudiness of the dispersions. Lipoquin and the Fluidosomes [6] [10], on the other hand, were more successful in terms their size and ciprofloxacin incorporation.

The combination of DPPG/DPTAP was much more successful in terms of particle size in a molar ratio of 4:1 than 2:1. The liposomal mixture of DPPG/DPTAP 2:1 showed an anomalous size measurement *(Table 2)* due to the aggregation that was visible with the naked eye at the end of the production of the liposomes. Nevertheless, the same combination with the molar ratio of 4:1 had the second-highest drug encapsulation therefore being more successful than the previously studied drug carriers [6] [10]. This could be explained by more negatively charged phospholipids being present and negatively charged vesicles being more likely to exhibit a good ciprofloxacin encapsulation efficiency [40]. The DSC measurements revealed phase separation due to the presence of an additional peak, which can be explained by excess DPPG that was not included in the binding with DPTAP. Additionally, there was a small shoulder peak visible, indicating interdigitation, which is a phenomenon that has already been observed with PGs in the past [39]. Interdigitation happens due to a lateral repulsion of the headgroups of

the phospholipids, leading to an intertwining of the hydrophobic lipid chains [39]. The range over which the gel phase was stabilized was for DPPG/DPTAP 4:1 from ~ 14.4°C to ~ 64.1°C which a range of ~ 49.7 °C where the gel phase or an interdigitated phase is stabilized. The DSC thermogram for DPTAP revealed an additional shoulder peak as well which in combination with the high enthalpy could also indicate interdigitation taking place.

DPPC/DPTAP 4:1 and 2:1 both showed fewer promising results than the DPPG/DPTAP 4:1 combination in terms of incorporating ciprofloxacin HCl. Nevertheless DPPC/DPTAP 4:1 was equally successful in terms of the incorporated amount of drug as the Lipoquin [6] variation and even entrapped more ciprofloxacin HCl than both Fluidosome mixtures [10]. The liposomes made from DPPC/DPTAP 2:1 and 4:1 showed similar sizes to the previously published drug carrying Fluidosomes [10] *(Figure 7)* making them eligible for nebulisation [6]. A positive factor is that the liposomes made from DPPC/DPTAP showed an increase in transition temperature compared to the pure lipids, which is an indication for increased stability [17]. The cation of DPTAP is likely pairing the anion from the phosphate of DPPC, leaving the choline unpaired and resulting in the particles carrying a positive charge. This charge could be useful for a pulmonary application, causing adhesive behaviour when interacting with the negatively charged mucus [8]. Additionally, the enthalpy calculated for the DPPC/DPTAP liposomes was quite high compared to other combinations, except for DPPC/DMPG 9:1, which might be an indication for tighter lipid packing [43] in this ratio compared to the others. This is another point to prove why the Fluidosomes [10] are not as fluid as they are claimed to be, since they cannot show such a high enthalpy compared to this relatively stable combination of DPPC/DPTAP when they are supposed to be more disordered in terms of their phospholipid membrane and are therefore probably more likely to rupture under shear forces.

The combination that seems to be the most promising in terms of incorporating the antibiotic and showed decent stability results is DPPC/DHDAB. In the molar ratio of 2:1 this mixture had the highest concentration of ciprofloxacin HCl entrapped and in the ratio of 4:1 it placed third after DPPG/DPTAP 4:1. All of these three combinations contained more drug than the drug carrier variation of Lipoquin [6] and the Fluidosomes [10]. The DPPC/DHDAB liposomes are most likely more stable in a nebulisation process than the pure DPPC and DHDAB liposomes because the transition temperatures are higher [17]. In terms of enthalpy only the combination with DPPC/DHDAB 2:1 is higher than both pure phospholipids, making it more likely that the Van der Waals forces were stronger [43], therefore more stable in the molar ratio of 2:1 than in DPPC/DHDAB 4:1. The bilayer stability is an advantage for the a possible nebulisation, due to the particles being better able to withstand the shear forces created in the process. Additionally, the sizes measured of both liposomal mixtures of DPPC/DHDAB were in a range that is useful for nebulisation as well [6] complementing the other optimal results. The DSC thermogram peaks are narrow showing that the bilayers are well ordered, and no phase separation is taking place.

4.1 Extension experiments

To consolidate the information gathered it would be useful to conduct further experiments to see if the liposomes as vehicles for ciprofloxacin HCl have the ability to increase the potency of the drug and conduct microbiological experiments for testing the liposomal combinations on bacteria. Moreover, different preparation methods should be studied to determine if the combinations such as PSPC/Cholesterol would be more successful and then possibly qualify for further experiments as well.

5. Conclusion

To conclude this study on liposome characteristics it can be said that a very good liposomal combination for possibly developing a drug formulation qualifying for nebulisation taken all the different results in account is DPPC/DHDAB 2:1. This combination outmatched the other liposomal mixtures regarding the entrapped amount of drug. Furthermore, the stability assessment proved to be successful due to the relatively high enthalpy calculated. The hybrid lipid/surfactant combination of DPPG/DPTAP 4:1 had a surprisingly high drug incorporation and the DSC thermogram suggests increased stability at the temperature range of the peak due to the long range of stabilized gel phase as well as possible interdigitation taking place [44]. Additionally, the liposomes made from DPPC/DPTAP proved themselves to be quite stable relative to the DPPC/DHDAB combination when compared with DPPC by itself. An additional positive factor that DPPC/DPTAP shows, but DPPG/DHDAB could not provide is an excess positive surface charge. This would facilitate adhesion to the mucus lining of the lung, which is particularly helpful for nebulised drug formulations for pulmonary delivery [8]. The Fluidosomes [10] are more likely to be permeable to their drug payload, especially under the shear forces created by nebulisation, due to possible phase separation causing formation of disordered domains. Nevertheless, they do contain a stabilised gel phase and a high enthalpy was calculated for DPPC/DMPG 9:1. This could be advantageous for a drug formulation as well as their decent encapsulation of the antibiotic (DPPC/DMPG 18:1) and their success as drug carriers in the past [10].

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Appendix

Thermograms of the single DSC measurements of the different liposomal combinations.

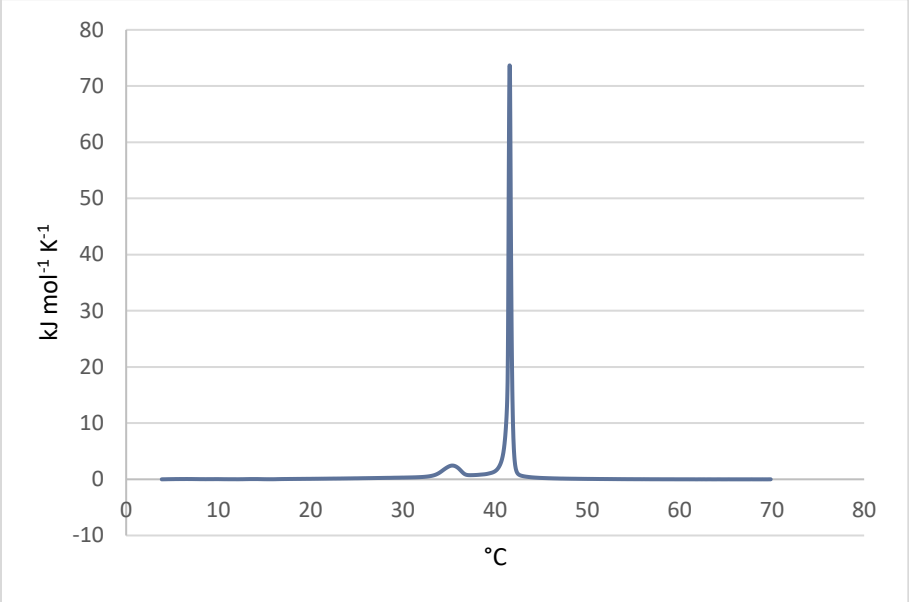

***Figure 13**: DPPC*

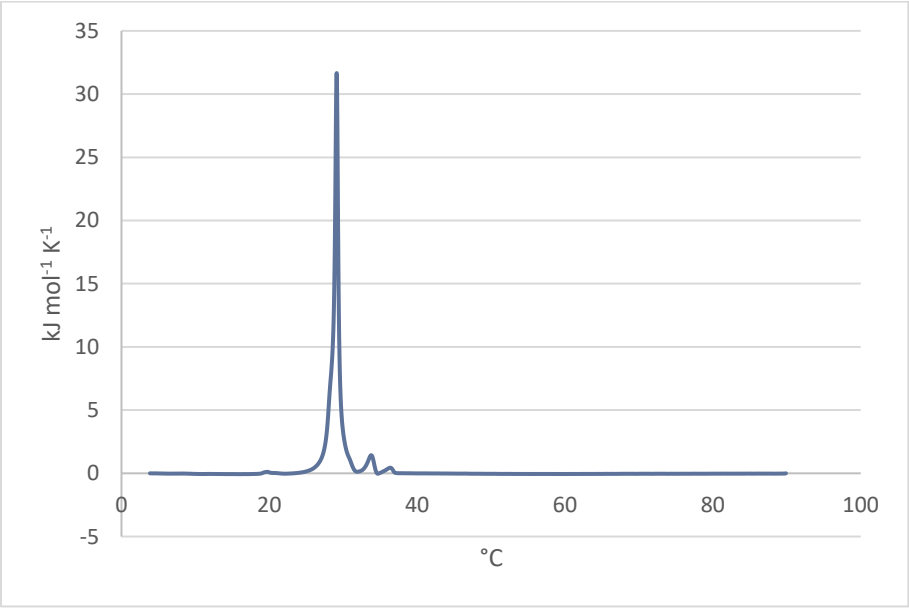

***Figure 14:** DHDAB*

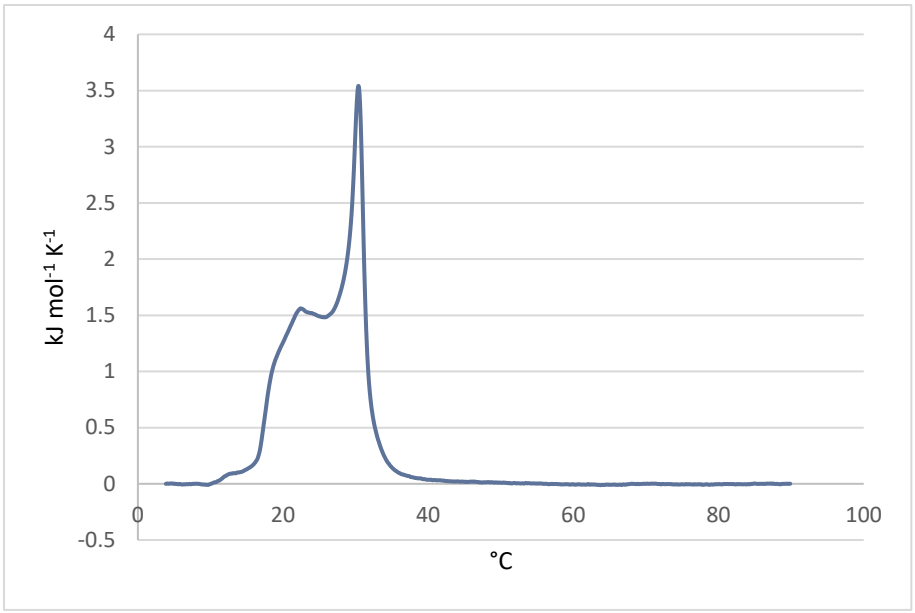

Figure 15: *DMPG*

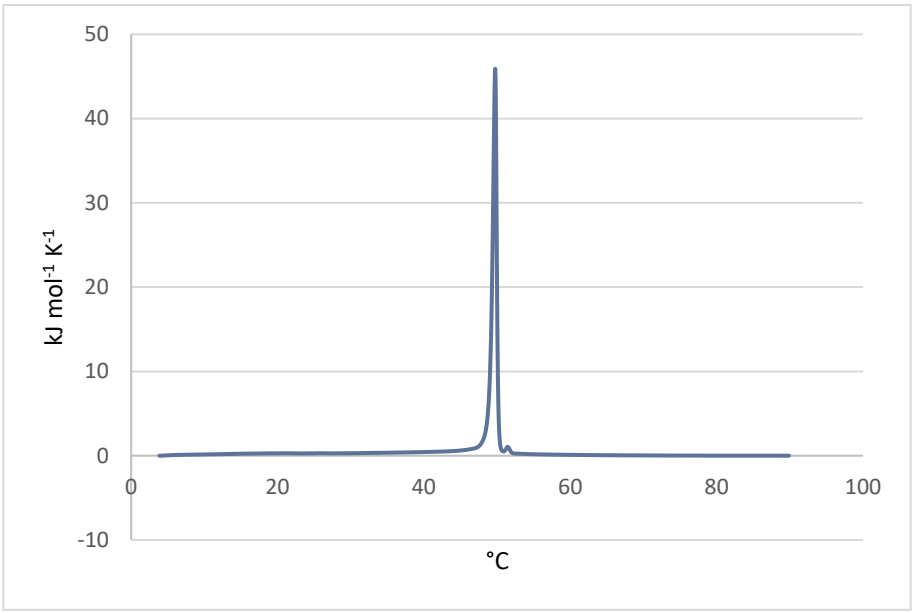

Figure 16: *DPPC/DHDAB 2:1*

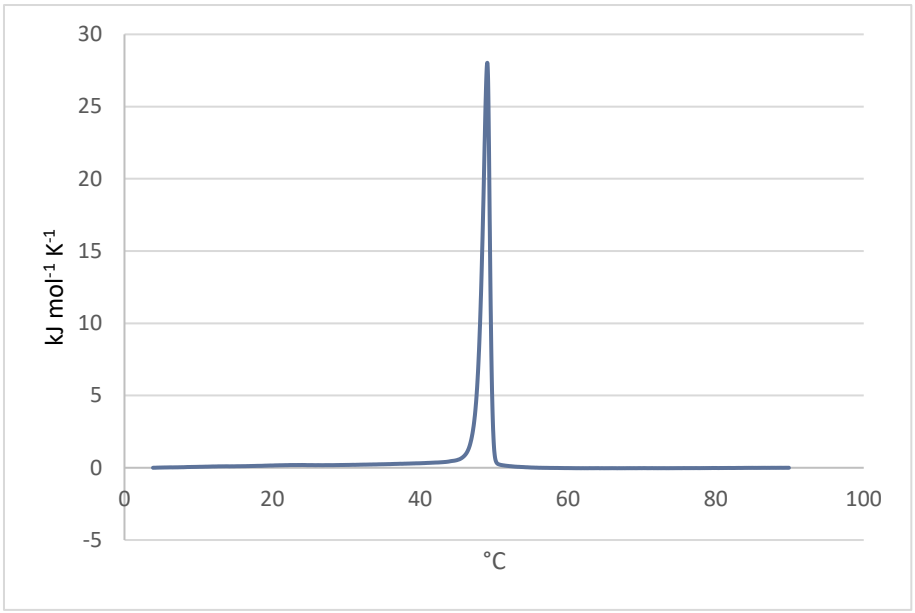

Figure 17: *DPPC/DHDAB 4:1*

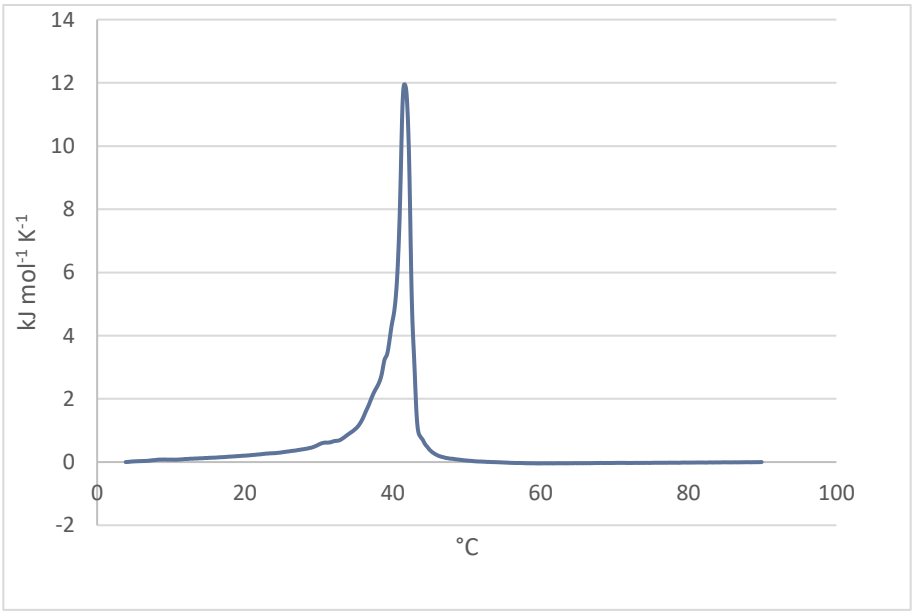

Figure 18: *DPPC/DMPG 18:1*

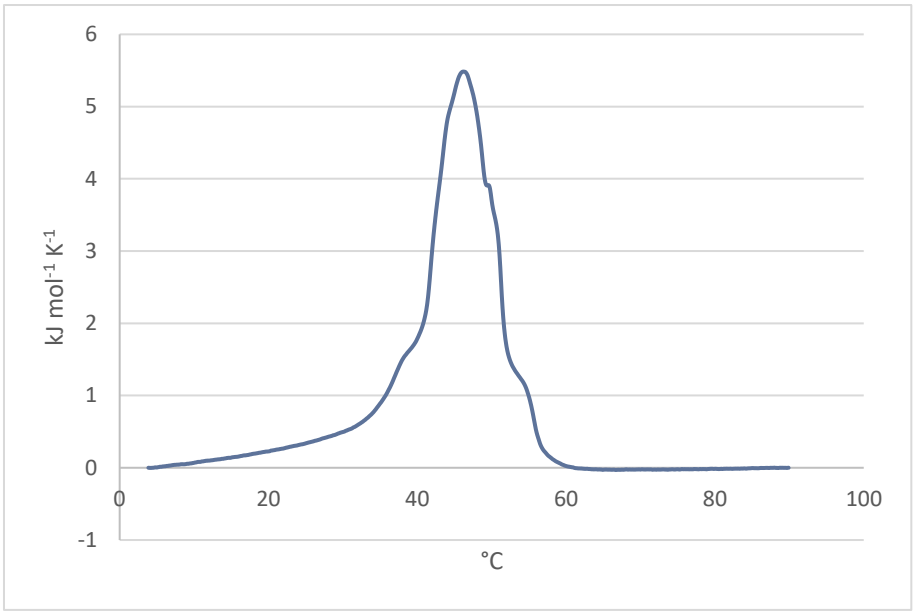

Figure 19: *DPPC/DMPG 9:1*

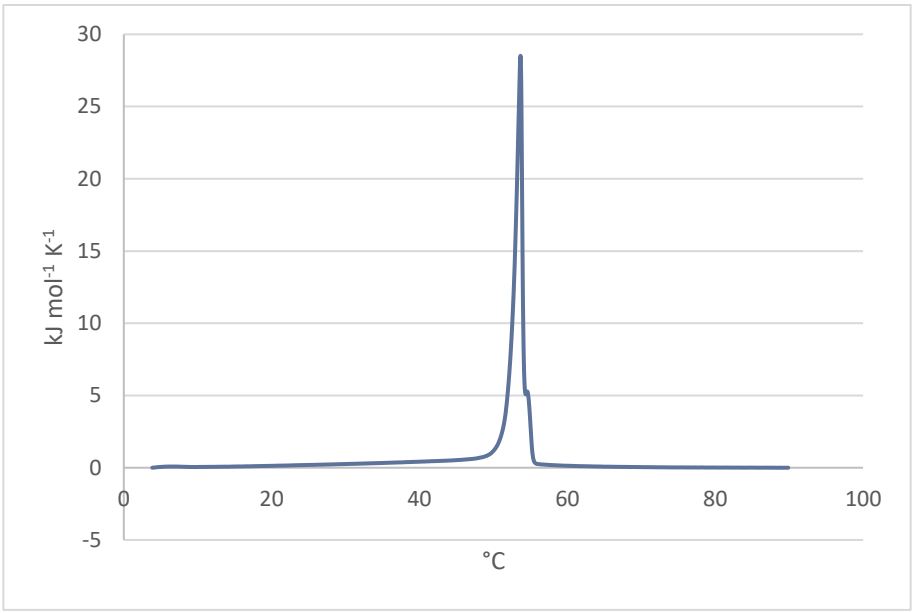

Figure 20: *DPPC/DPTAP 2:1*

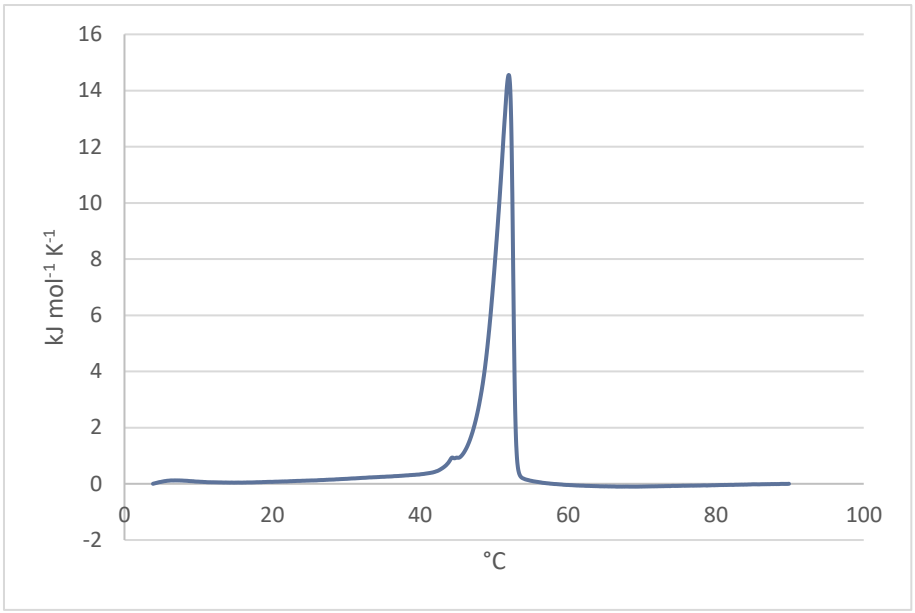

Figure 21: *DPPC/DPTAP 4:1*

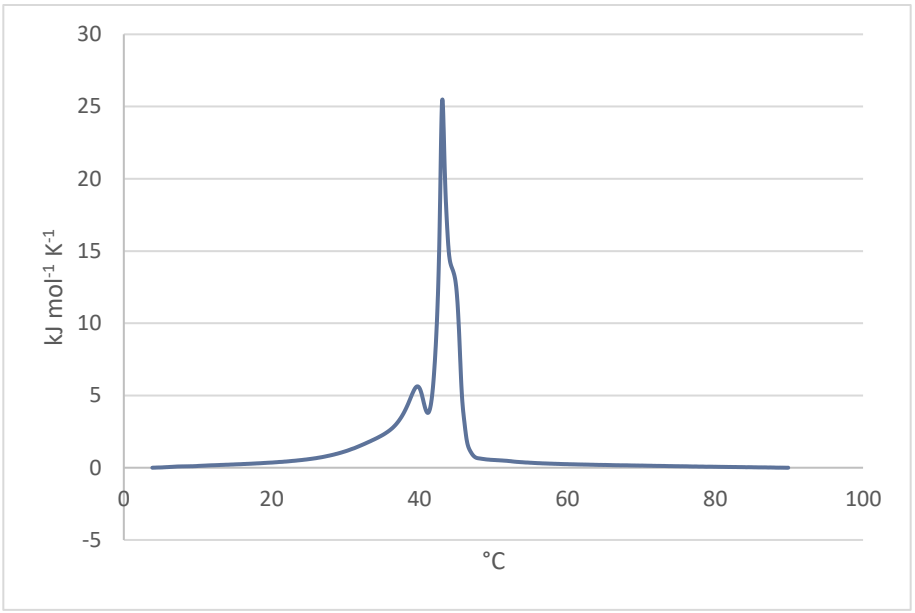

Figure 22: *DPTAP*

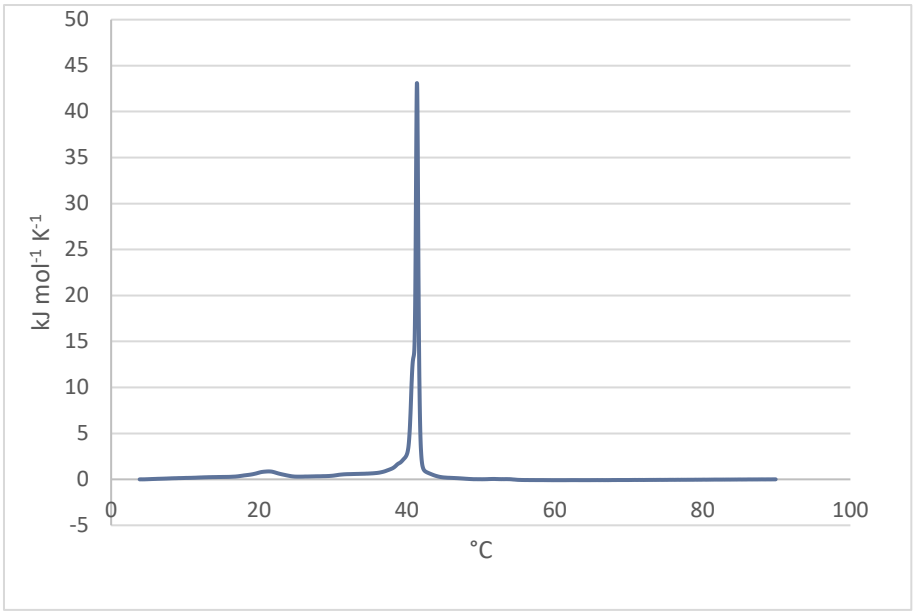

Figure 23: *DPPG*

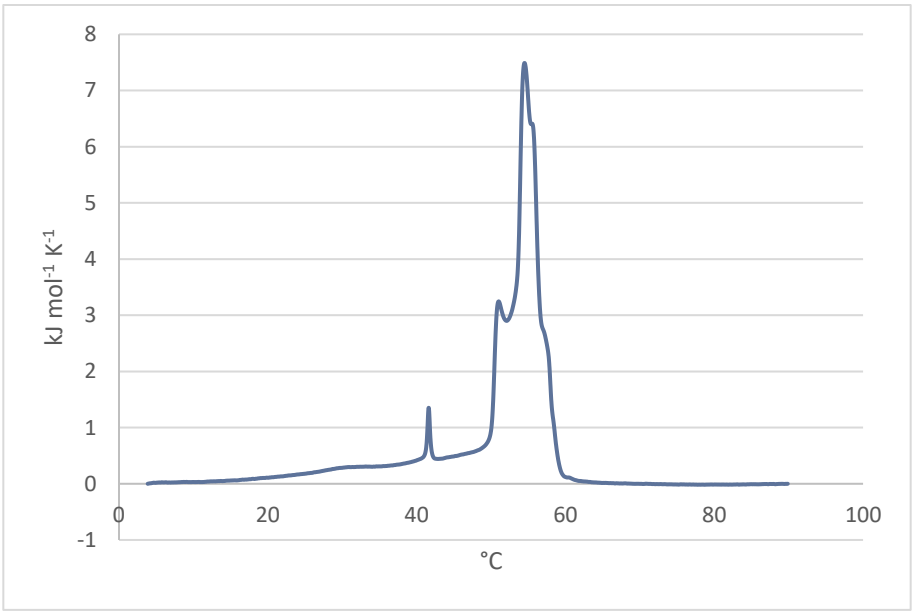

Figure 24: *DPPG/DPTAP 4:1*