

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

INTERACTIONS OF BILE SALTS WITH MODEL LIPID MEMBRANES

M.Sc. THESIS

Gökçe ENGÜDAR

Department of Food Engineering

Food Engineering Programme

Thesis Advisor: Prof. Dr. Beraat ÖZÇELİK

OCTOBER 2012

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SAFRA TUZLARININ MODEL LİPİD MEMBRANLAR İLE ETKİLEŞİMİ

YÜKSEK LİSANS TEZİ

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To my mother and father,

FOREWORD

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ABBREVIATIONS

CDCA	: Chenodeoxycholic acid
CMC	: Critical Micelle Concentration
DCA	: Deoxycholic Acid
DLS	: Dynamic Light Scattering
DPPC	: 1, 2-palmitoyl- <i>sn</i> -glycero-3-phosphatidylcholine
DSC	: Differential Scanning Calorimetry
ITC	: Isothermal Titration Calorimetry
LUV	: Large Unilamellar Vesicles
MLV	: Multilamellar Vesicles
NBD-PE	: 1, 2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt)
PC	: Phosphatidylcholine
POPC	: 1-palmitoyl- 2-oleoyl- <i>sn</i> -glycero-3-phosphatidylcholine

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INTERACTIONS OF BILE SALTS WITH MODEL LIPID MEMBRANES

SUMMARY

Bile salts are biological surfactants that play an important role in fat digestion and absorption through intestinal wall by forming mixed micelles with lipids, fats and cholesterol. Liposomes are lipid vesicles which are spherical self-assembled aggregates, consisted of amphiphilic molecules such as phospholipids. Because liposomes have structural and compositional similarities with biological membranes, they can be used as model lipid membranes to achieve the challenges of working with biological membranes. The interactions of bile salts with model lipid membranes include partitioning of bile salts into membranes and solubilization of membranes by bile salts. Bile salts can form micelles differently from phospholipids which are constituents of lipid membranes. Bile salts can partition into lipid membranes below the critical micelle concentrations (CMC) and they can solubilize the lipid vesicles into mixed micelles above CMC.

Isothermal titration calorimetry (ITC) was used to determine complete thermodynamic profile of interactions including, enthalpy change, Gibbs free energy, entropy change, and partition coefficients. Partitioning of bile salts between water and lipid membranes was investigated by determination of partition coefficient, considering the electrostatic interactions of negatively charged bile salt monomers and vesicles in the model. Solubilization effect of bile salts on lipid membranes was investigated by establishing the vesicle-to-micelle phase diagrams and some critical parameters such as surfactant concentrations to saturate (D_t^{sat}) and solubilize (D_t^{sol}) the membranes and critical surfactant/lipid ratios for saturation and solubilization (R_e^{sat} and R_e^{sol} , respectively) were determined from phase boundaries to quantify membrane solubilization.

In this study, chenodeoxycholic acid (CDCA) as primary bile acid and deoxycholic acid (DCA) as a secondary bile acid, which are known as dihydroxybile acids, were used. Their structures, consisted of two hydroxyl groups bound to hydrophobic steroidal framework and carboxylic acid affect their interaction activities. 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylcholine (POPC) and 1,2-dipalmitoyl-sn-glycerol-3-phosphatidylcholine (DPPC) were used to prepare the model lipid membranes (liposomes). The effects of saturation degree of acyl chains of membrane lipids, ionic strength of medium and phase properties of saturated lipid, DPPC, on interactions were investigated by ITC and the phase transition diagrams were compared to understand the effects of different systems. Furthermore, fluorescence spectroscopy measurements were performed to confirm the solubilization results. Dynamic light scattering (DLS) measurements were performed throughout this study in order to characterize the lipid vesicles in terms of determination of particle size distribution. Differential scanning calorimetry (DSC) was used to understand the activity of bile acid deoxycholic acid on DPPC membrane because of change in thermodynamic state and transition temperature. As a result, the correlation between

the results of isothermal titration calorimetric assay and spectroscopic assays was observed.

This study indicates that the understanding of interactions between bile salts and lipid membranes are essential in order to develop the efficient colloidal delivery systems. Because it is also known that liposomes are used as carrier for active molecules as an another result of mimicking the biological membranes. However, gastrointestinal conditions affect the stability of liposomes. Therefore, it is also important that developing systems which mimic the gastrointestinal conditions such in the presence of bile salts or digestive enzymes in order to elucidate interactions of active molecules such as pharmaceuticals or nutraceuticals and biological membrane.

SAFRA TUZLARININ MODEL LİPİD MEMBRANLAR İLE ETKİLEŞİMİ

ÖZET

Safra tuzları diet ile birlikte alınan yağlar ve kolesterol ile misel yapı oluşturarak yağların sindiriminde ve bağırsaktan emiliminde önemli role sahip biyolojik yüzey aktif maddelerdir. Liposomlar, fosfolipidler gibi amfifilik özelliğe sahip moleküllerin sulu ortamda kendiliğinden küresel agregatlar oluşturmasıyla meydana gelen çift katmanlı kapalı yağ kesecikleridir. Biyolojik hücre membranları dinamik yapıları nedeniyle üzerinde çalışılması zor yapılardır. Liposomların yapı ve bileşimi açısından biyolojik membranlara benzemesi nedeniyle, liposomlar model lipid membranlar olarak araştırmalarda kullanılabilirler. Membran lipidler kimyasal yapılarına bağlı olarak fosfolipidler, sfingolipidler ve kolesterol olarak sınıflandırılırlar. Fosfatidilkolin ise membranda temel olarak bulunan ve fizyolojik pH'da nötral olmasını sağlayan fosfolipid sınıfıdır. Surfaktan özelliğe sahip safra tuzlarının biyolojik membranlarla etkileşimi temel olarak membrana katılma ve membran solubilizasyonu olarak ikiye ayrılabilir. Safra tuzları, lipid membranların yapı taşı olan amfifilik özellikteki fosfolipidlerden farklı olarak misel oluşturabilirler. Kritik misel konsantrasyonu (CMC) altındaki değerlerde safra tuzları lipid membran katmanlarına katılabilirler, bu değer üzerindeki konsantrasyonlarda ise membranların solubilizasyonuna neden olurlar.

Moleküller arası etkileşimleri incelemek amacıyla çeşitli spektroskopik ve kalorimetrik yöntemler olmak üzere biyofiziksel yöntemlerden yararlanılmaktadır. Safra tuzları gibi küçük moleküllerin lipidler gibi makromoleküllere bağlanmaları ve fiziksel etkileşimleri için yaygın olarak izotermal titrasyon kalorimetri, diferansiyel taramalı kalorimetri, floresans spektroskopisi ve dinamik ışık saçımı yöntemleri kullanılmaktadır. Bu nedenle, bu çalışmada bu yöntemlerin prensiplerinin öğrenilmesi ve daha sonrasında etkileşimleri incelemek ve sonuçları doğrulamak için bu yöntemlerin kullanılması amaçlanmıştır.

Safra tuzları ve model lipid membranlar arasındaki etkileşimler temel olarak izotermal titrasyon kalorimetri (ITC) yöntemi ile çalışılabilir. Safra tuzlarının sulu ortam ve lipid membranlar arasındaki katılımı bağlanma sabitinin bulunması ile incelenebilir, ancak model oluştururken safra tuzlarının negatif yüklü olması nedeniyle elektrostatik etkileşimler göz önünde bulundurulmalıdır. Safra tuzlarının membranlar üzerine solubilize edebilme etkisi ise lipid membranların kapalı küre formundan misel formuna geçişi faz diagramları oluşturularak gözlenebilir. Membranın safra tuzları tarafından doyurulduğu ve solubilize edildiği kritik safra tuzu konsantrasyonları ve safra tuzu/lipid konsantrasyonu oranları bu diyagramlar yardımıyla belirlenebilir. İzotermal titrasyon kalorimetresi ile etkileşimlerin tüm termodinamik parametrelerini tek bir deneyde elde edilebilir.

İzotermal titrasyon kalorimetri, moleküler etkileşimleri incelemek amacıyla kullanılan biyofiziksel metotlardan biridir. Küçük moleküller (ilaç aktif molekülleri, deterjan özelliğe sahip moleküller gibi ligandlar) ile makromoleküllerin (protein,

DNA, lipid ve polimerler gibi) etkileşimlerinin incelenmesinde, etkileşimlerin termodinamik profilinin çıkarılması amacıyla, bağlanma kat sayısı (k_B), entalpi (ΔH), Gibbs serbest enerjisi (ΔG), entropi (ΔS) gibi termodinamik parametrelerinin belirlenmesinde yaygın olarak kullanılan bir yöntemdir. İki molekül arasındaki fiziksel değişim veya kimyasal reaksiyonların termodinamik karakteristiklerinin tek bir deney ile ve direk olarak tespit edilebilmesini sağlaması, bu yöntemi diğerlerinden üstün kılan bir özelliktir. İlaç ve gıda endüstrisinde formül geliştirmek üzere bu yöntemden yararlanılmaktadır.

İzotermal titrasyon kalorimetre, sıcaklığın sabit tutulmasını sağlayan adyabatik ceket içerisinde referans ve örnek haznelerinden oluşur. Referans bölmesine tampon çözelti veya saf su konulur. Örnek haznenin üzerine etkileşimleri araştırılacak olan çözeltilerden birinin bulunduğu şırınga yerleştirilir ve şırıngadaki çözeltinin örnek hazne içerisine enjeksiyonu ile iki çözelti arasındaki etkileşim örnek hazne içerisinde gerçekleşir ve bu etkileşim sonucu ısı açığa çıkar veya absorblanır. Örnek haznesi içerisindeki çözeltinin, şırınga içerisindeki çözelti ile doymunluğa ulaşmasından sonra reaksiyon ısısı sabit kalır ve bu sabit ısı dilusyon ısısını vermektedir. Tampon çözelti, tuz konsantrasyonu ve pH ölçümler üzerinde etkili faktörlerdir. ITC ile yapılan ölçümlerde, çözelti konsantrasyonlarının yanı sıra, enjeksiyon hacmi ve sayısı da uygun olarak seçilmelidir.

Bu çalışmada, safra asidi olarak steroid yapısına bağlı iki hidroksil grubu ve bir karboksil grubu içeren deoksikolik asit and chenodeoksikolik asit kullanılmıştır. Safra tuzları, suda çözünebilir steroidal yapılardır. Kolesterol metabolizmasının son ürünleri olarak, kimyasal yapıları kolik asit türevleridir. Kimyasal yapıları, tetrasiklik hidrokarbon yapısına sahip rijit steroid yapı ve kısa alifatik zincirden oluşur. Safra tuzları, bu kimyasal yapıları nedeniyle klasik yüzey aktif maddelerden farklı özelliklere sahiptir. Safra tuzları, steroid yapısına bağlı hidroksil grup sayısına ve konjugasyona bağlı olarak farklı özellikler gösterirler. Deoksikolik asit, steroid yapısına bağlı C3 ve C12'de hidroksil grubu içerirken, chenodeoksikolik asitte hidroksil grupları C3 ve C7'ye bağlıdır. Liposomları oluşturmak için 1- palmitoil-2-oleil-sn-glisero-3-fosfokolin (POPC) ve 1,2- dipalmitoil- sn-glisero-fosfokolin (DPPC) kullanılmıştır. DPPC doymuş iki palmitik asit (C16:0) yağ zincirlerinden oluşurken, POPC doymamış oleik asit (C18:1) ve doymuş palmitik asit (C16:0) yağ asidi zincirlerini içerir. Deoksikolik asit ve chenodeoksikolik asitin, doymamış yağ asidi yapısına sahip POPC ve doymuş yağ asidi içeren DPPC liposomlar üzerindeki etkisi karşılaştırılmıştır. POPC membranların solubilizasyonu için saf su ve fosfat tampon çözeltisi olmak üzere iki farklı çözücü kullanılarak tuz iyonlarının solubilizasyon üzerine etkisi incelenmiştir. DPPC membranların solubilizasyonu için ise, sıvı-kristalin faz ile jel fazının etkileşimler üzerinde etkisini incelemek amacıyla 25°C ve 55°C olmak üzere DPPC'nin fazlar arası geçiş sıcaklığı altındaki ve üzerindeki değerler seçilerek ölçüm yapılmıştır. Kalorimetrenin örnek bölümündeki toplam safra tuzu ve lipid konsantrasyonu değerlerinden oluşturulan faz geçiş diyagramları bu etkiler göz önünde bulundurularak karşılaştırılmıştır. Lipid membranların doymunun ve solubilizasyonun gerçekleştiği koşullarda, kritik safra tuzu/lipid oranları (R_e^{sat} ve R_e^{sol}) ve su fazındaki safra tuzu konsantrasyonları (D_w^{sol} ve D_w^{sat}) faz diyagramlarından elde edilmiştir.

Ayrıca, membran solubilizasyon değerlerinin doğrulanması için floresans spektroskopisi ölçümleri yapılmıştır. Safra tuzlarının, lipid membranların katmanlı yapısı üzerine etkisi floresans şiddetinin zamana karşı ölçümü ile gözlenmiştir. İzotermal titrasyon kalorimetri ve floresans spektroskopisi yöntemleri ile elde edilen

sonuçlar arasında korelasyon olduğu bulunmuştur. Çalışmada, dinamik ışık saçınımı yöntemi (DLS) ile liposomların partikül büyüklüklerinin ölçümü gerçekleştirilerek partiküllerin karakterizasyonları sağlanmıştır. Ayrıca, ITC ile yapılan deneyler sonucu elde edilen veriler göz önünde bulundurularak oluşturulan farklı oranlarda POPC ve deoksikolik safra asidi içeren karışık lipid ve misel yapıların partikül büyüklükleri DLS ile ölçülmüştür. Böylece, surfaktan özellikte olan safra tuzlarının lipid membranların (liposomların) boyutlarına etkileri incelenerek membran solubilizasyon değerleri ve liposom yapıların stabiliteleri de incelenmiştir. Safra tuzlarının DPPC membranın faz geçiş sıcaklığı üzerine etkisi ise diferansiyel taramalı kalorimetre (DSC) ile incelenmiştir. Surfaktan özellikleri sebebiyle safra tuzları, lipidlerin termodinamik fazları üzerinde etkiye sahiptirler ve oda sıcaklığında jel fazında bulunan DPPC lipid molekülleri belirli konsantrasyonlarda deoksikolik safra asidinin sisteme ilavesi sonucu likit kristalin faza geçerek faz değişimi gösterirler. Bu faz geçişleri ise diferansiyel taramalı kalorimetre (DSC) yardımıyla sistemin ısı kapasitesindeki değişimin sıcaklığın fonksiyonu olarak ölçülmesi ile belirlenebilir. Termodinamik durumun ısı ile değişimi beraberinde lipid yapılarının konformasyonel değişimlerine sebep olduğundan DSC biyolojik sistemlerdeki etkileşimlerin etkilerini incelemek amacıyla yaygın olarak kullanılabilen bir diğer kalorimetrik yöntemdir.

Bu çalışma, etkili taşıyıcı sistemler geliştirmek amacıyla safra tuzlarının lipid membranlar ile etkileşiminin anlaşılmasının önemli olduğunu göstermektedir. Liposomlar asıl yaşam kapsülleri olan hücre membranlarına benzerliklerinin sonucu olarak taşıyıcı yapılar olarak kullanılmaktadırlar. Ancak, gastrointestinal koşullar ve sistemde bulunan veya salgılanan maddeler liposomların stabilitesini etkiler. Safra tuzlarının veya sindirim ezimlerinin varlığı gibi gastrointestinal koşulların taklit edildiği koşullara sahip sistemlerin geliştirilmesi farmasötikler ve nutrasötikler gibi biyoaktif moleküller ile biyolojik membranlar arasındaki etkileşimlerin ve etki mekanizmalarının anlaşılması için de önemlidir.

1. INTRODUCTION

This project, is divided into two parts, the first part of project has been about the understanding of the principles of techniques which have been intended to use in experiments and the second part of project has included the using these techniques in order to investigate interactions between surfactant molecules and model lipid membranes by means of studying membrane partitioning and solubilization.

It has been discovered that liposomes can be useful tools to study structural and functional properties of biological membranes because they resemble cell membranes in terms of structure and composition. Also, surfactants have been used to solubilize the membranes for the purpose of acquiring membrane isolated from proteins to understand the drug mechanisms on the membranes (Jones, 1999). On the other hand, the effects of drugs on the biological membrane can be studied by means of understanding interactions of surfactants with membranes because many pharmaceuticals have surface active nature such as surfactants. These two type of interactions can be accepted as analogues of each others (Schreier, 2000). Moreover, these of all fundamental interactions provide to understand how the efficient delivery systems can be developed because there have existed the applications of lipid-surfactant mixtures as colloidal drug delivery systems. Furthermore, bile acids have attention as a physiological surfactants because of having functional and structural advantages. They are biosynthesised from cholesterol in the liver and secreted within bile from liver, stored in gallbladder, and recirculated almost all (~95 %) 6-15 times per day in the intestinal system, and so they interact with membranes during this enterohepatic circulation (Sievänen, 2007; Mouaz et al., 2001). Also, their rigid steroid backbone structure is different by comparison with classical head-tail surfactants that lead the differences in the aggregation properties (smaller aggregates) of bile acids between other surfactants (Hildebrand et al., 2002). These features enable them to be attractive tools for drug design and delivery systems development by means of forming lipid-bile acid micellar mixtures. In order to investigate the interactions between lipid membranes and surfactant-like molecules,

there are several biophysical techniques such as spectroscopic methods and titration calorimetric method have been used by many researchers for last years (Goñi and Alonso, 2000; Heerklotz and Seelig, 2000).

In this project, isothermal titration calorimetry, differential scanning calorimetry, dynamic light scattering, and fluorescence spectroscopy have been chosen because they are widespread convenient techniques for monitoring the interactions between surfactants and model lipid membranes.

The primary goal of this project is to provide elucidating the interactions between bile acids and model lipid membranes. The investigation of interactions of biological surfactant, bile acids, with lipid membranes are essential in order to develop the efficient delivery systems as a consequence of studying. When this project has been planned, it has been considered that it is important to have knowledge about the fundamentals to evaluate results of experiments and to develop applications. Therefore, the mechanism of surfactants on lipid membrane systems has been reviewed as well as methods before studying the interactions.

1.1 Model Lipid Membranes

Biological membranes consist of lipid bilayers as an essential structure that billions of lipid molecules assembly to build up lipid bilayer membrane and a hundreds of different kinds of lipids form cell membrane. In all living systems, the membranes are most abundant cellular structure, and play an important role to compartmentalize living matter and to protect the genetic material. Because biological membrane is the essential capsule of life, it can be accepted to underlie the micro-encapsulation technology (Mouritsen, 2005).

Understanding of structure of membranes, which occur as fluid and soft interfaces, is important to elucidate the functions of cellular components, and thereby mechanism of action of bioactive molecules on the cell. According to Mouritsen, natural materials such as membranes are created by nature in a perfect functions, so they are promising systems for biomedical applications, for example, use of liposome in targeted delivery and gene therapy, and immobilization of enzymes or proteins to supported lipid membrane interfaces for developing biosensors and medical micro-devices (2005).

Model lipid membranes can be used to accomplish the difficulty of the study with natural membrane systems because of their dynamic structure. Developing model lipid membranes can be useful to mimic the natural cell membranes while studying the role of lipids in cellular interactions. Model membranes provide the understanding of the role of lipids in cellular uptake, the estimation of toxicity of drugs, and developing efficient drug delivery systems. In addition, interactions of peptides, polymers and nanocarriers with lipid membranes can be studied thanks to these models. Although the model membranes can not represent the fully identical characteristics with natural membranes, it has been found correlation between biophysical interactions and cellular uptake or therapeutic efficacy (Peetla, 2009).

The development of model membrane systems during last a few years can be noticed in Figure 1.1. The biological membranes have a basic function to provide environment by enclosing cell and organelles, but also play an important role in biological functions. They are varied depending on the cell type and they have dynamic organization to regulate conformational changes, signaling, trafficking, and recognition (Chan and Boxer, 2007). Therefore, as it was mentioned previously, several model systems have been developed, but essential lipid bilayer structure has been retained. Figure 1.1 has shown giant unilamellar vesicles, GUVs (a), Networks of GUVs connected by lipid microtubes (b), ruptured GUVs on solid supported bilayers (c), membrane nanodiscs (d), NanoSIMS image of supported lipid bilayers (e), ruptured cell membranes on solid support (f), bilayers tethered to a solid support containing ion channels (g), vesicles tethered to supported bilayers (h), multi-scale simulation image of membrane system (i) (Chan and Boxer, 2007). This figure can also explain the development process of the visualization techniques of biological membrane to give more information about composition, structures and dynamics of biological membranes.

Model membranes which are unilamellar or multilamellar vesicles, micelles, monolayers at an air-water interface, planar lipid bilayers, bilayered micelles and supported bilayers provide the simpler system to investigate membrane toxicity, membrane fluidity, permeability of these membrane models using different physicochemical techniques, including nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), isothermal titration calorimetry (ITC), electron spin resonance, fluorescence spectroscopy, X-ray diffraction. Also, for

mamalian model membranes, the effect of ions, heavy metals and drugs can be assessed and the effects of antimicrobial peptides, antibiotics, interaction of proteins with model membranes, and insertion of proteins in the model membranes can be studied for bacterial model membranes (Le et al., 2011).

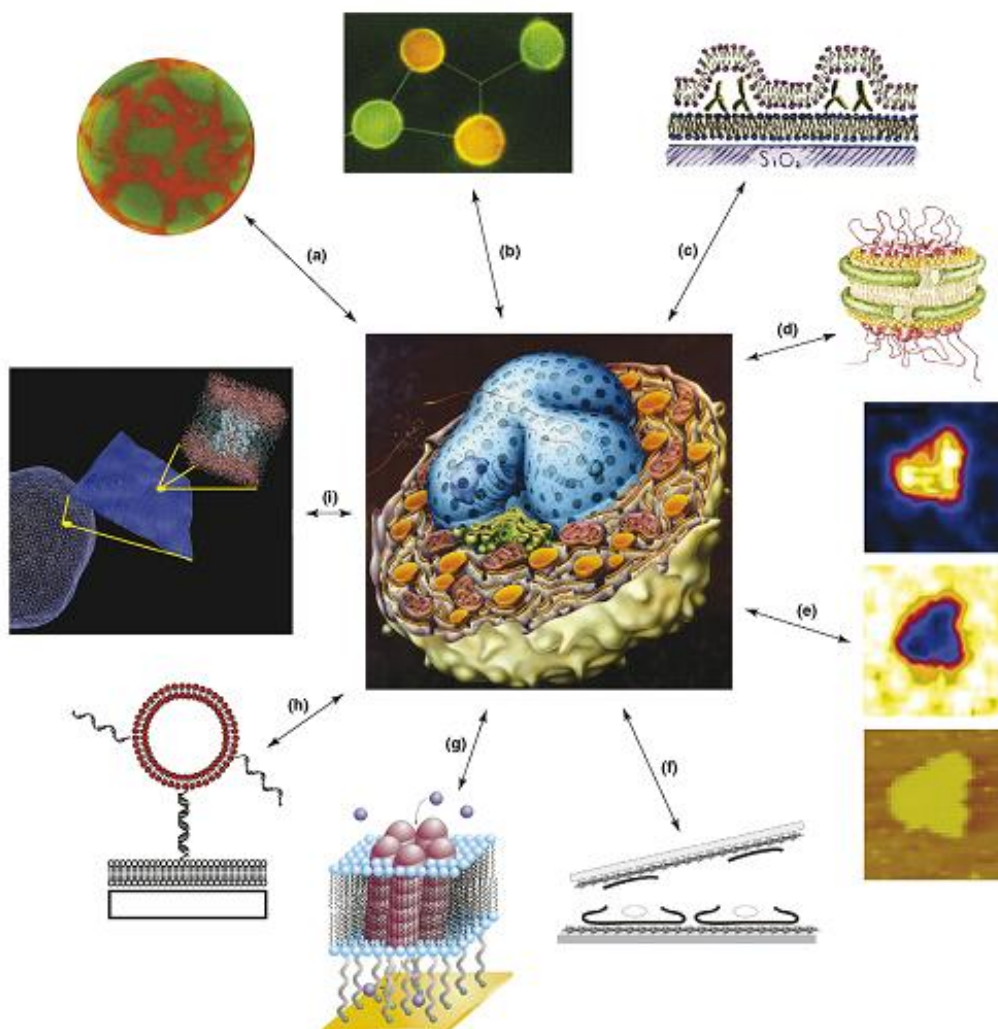


Figure 1.1 : Illustration of different model membrane systems (Chan and Boxer, 2007).

Membrane lipids can be classified as phospholipids (glycerol-based lipids), sphingolipids (ceramide-based lipids) and cholesterol (sterols) depending on their chemical structures. Phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and in a small amount of phosphatidylinositol (PI) and cardiolipin depending on their hydrophilic polar head group (Peetla, 2009). PC is major constituent of membrane that makes it neutral at physiological pH. Sphingolipids, which have hydrophobic ceramide backbone, provide great hydrophobicity to membrane. Sphingomyelin and glycosphingolipids

are the major sphingolipids in mammalian membrane. In addition, cholesterol affects membrane fluidity and lipid raft formation (van Meer et al., 2008; Le et al., 2011). Chemical structures of essential lipids formed to membrane is shown in the Figure 1.2.

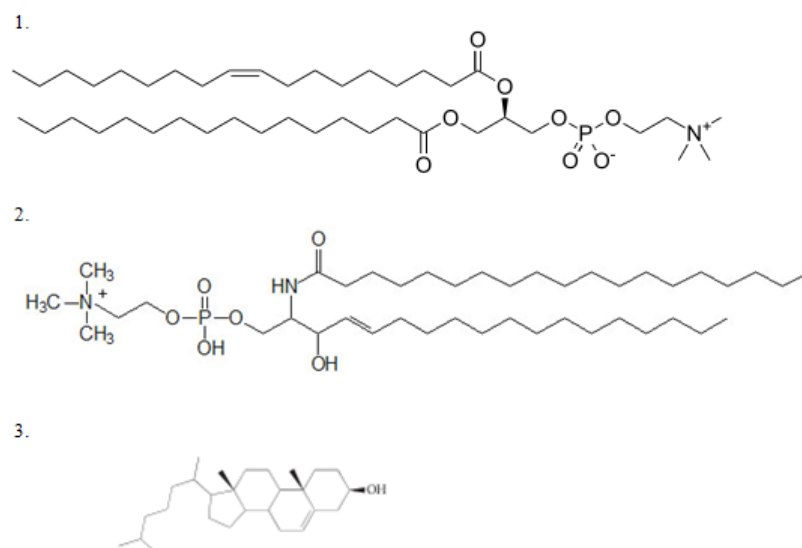


Figure 1.2 : Chemical structure of membrane lipids (1. Phosphatidylcholine; 2. Sphingomyelin; 3. Cholesterol).

Lipids in the membranes have functional roles: (i) the one of them is that they store the energy which is essential for membrane biogenesis, (ii) the another function is that amphiphatic lipids including hydrophobic and hydrophilic portion enable to form membranes spontaneously and compartmentalize the cell, (iii) the final function is that they can play a role as first and second messengers for signal transduction and molecular recognition (van Meer et al., 2008).

1.1.1 Liposomes- Phospholipid membrane vesicles

According to literature, “Phospholipid spherules (liposomes) as a model for biological membranes” authored by Dr. Weissman and Grazia Sessa in 1968 is the first academic reference including “liposome” as an accepted word by the scientific community (Kulkarni, 2005). Therefore, it can be inferred that liposomes were used as a model to investigate biological membranes before recognition of their several applications from drug and cosmetic formulations to diagnostics and food industry products (Zhang et al., 2002).

Liposomes or lipid vesicles are spherical self-assembled aggregates which are consisted of amphiphilic molecules such as phospholipids. Different varieties of

liposomes, including unilamellar or multilamellar vesicles, from very small to large, can be produced depending on the nature of amphiphilic lipids. Phosphatidylcholine (PC) forms a bilayer structure, whereas phosphatidylethanolamine (PE) can form micelles or inverted hexagonal structures depending upon the size of head group of phospholipids (Wang, 2005).

Amphiphilic molecules, phospholipids and surfactants (detergents) as ordinary examples, include polar (hydrophilic) and nonpolar (hydrophobic) parts, so these molecules aggregate spontaneously in both of polar and nonpolar solvents because of hydrophilic and hydrophobic interactions (Zhang et al., 2002).

The ability of amphiphiles (lipids) to form various kinds of structures and phases is termed as (lipid) polymorphisim. Packing parameter (P) of amphiphilic molecules determine the geometry of amphiphiles, and so the structure and phases of aggregates. P is defined in Eq 1.1.

$$P = \frac{v}{al} \quad (1.1)$$

where v stands for the volume of amphiphiles (hydrophobic part), a is a cross-sectional area of polar head group, and l is a length of nonpolar hydrocarbon chain (normal to cross-sectional area). According to Eq 1.1, the compatibility of size of head group and lenght of hydrophobic tail affect the geometry of aggregates. If P is below 1/3, spherical micelles are formed preferably; while P is above 1, inverted structures (micelles and hexogonal structure) form and bilayer structure is favoured for the value of P around 1 (Zhang et al., 2002).

The amphiphilic molecule form an effective cylindrical shape when cross-sectional areas of polar head group is almost equal to nonpolar region of molecule ($P \sim 1$). Then, these cylinders are arranged in paralel while forming two-dimesional monolayer consisted of one monolayer formed by polar head group and another formed by hydrophobic fatty acid chains. Bilayered sheet or lamella is composed of two of these monolayers. Naturally occured phospholipids as a major component of cell membranes is the typical example of these bilayered structure. However, when the cross-section areas of polar head group is different than nonpolar region of amphiphiles, cylindrical shape, and therefore planar lamellar bilayers are not formed. The size of head group, variation in the number of fatty acid chains, and electrostatic effects can cause the curved structures, including hexogonal phases and

micelles which are ranging from spheres to elongated rods generally. Also, if polar group is broader than nonpolar region, the structure tends to form positive curvature, whereas if nonpolar region is broader than polar head, the negative curvature is formed. The positive curvature forms micelles (L_I phase) and normal hexagonal phase (H_I phase), but the negative curvature cause reverse (inverted) micelles (L_{II} phase) and reverse hexagonal phase (H_{II} phase). In addition, the cubic phase is another nonbilayer phase. Temperature and pH are effective parameters for transition from bilayer to nonbilayer structure. (Kirby and Gregoriadis, 1999). Figure 1.3 indicates the structure of aggregates and phases depending upon the geometry of amphiphilic molecules due to packing parameter, P .

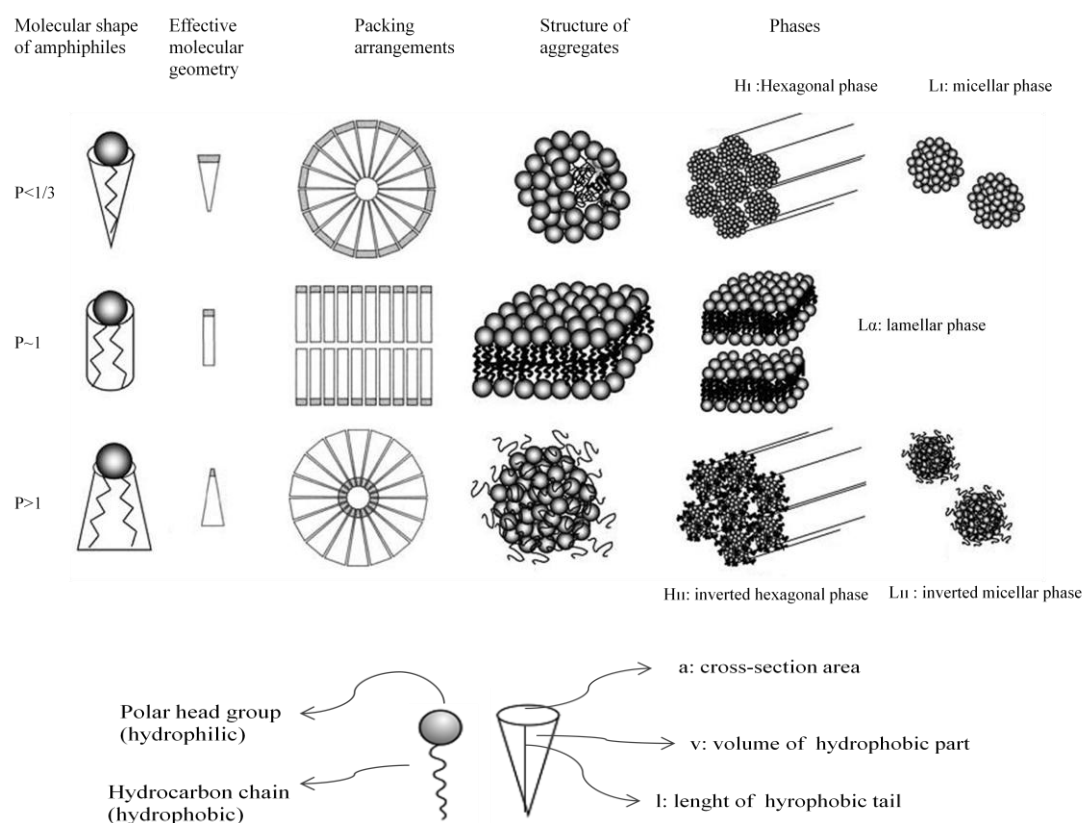


Figure 1.3 : Illustration of different molecular geometry and structure of amphiphiles depending on the value of packing parameter, P (adapted from Kirby and Gregoriadis, 1999).

Competition between forces due to colloidal and entropic nature provides the aggregation of lipid molecules by self-assembly process and stability of final structure is depend on type of lipid, composition, and environmental conditions such as temperature, degree of hydration, and pH. For instance, increase of temperature cause to transform a lamellar structure into a inverted hexagonal or cubic structure.

Also, if hydrophobic and amphiphilic molecules such as hydrocarbons, alcohols, detergents, and drugs are incorporated into lipid structures, the equilibrium between structures of aggregates can change, for example, inhibition of H_{II} structure by detergents. Cholesterol differs from other lipid molecules in respect of membrane curvature with a small polar head group, -OH, and steroid ring structure, so that inverted conical shape forms and promotes H_{II} structure (Mouritsen, 2005).

Liposomes can be classified differently depending on their preparation methods, their lamellarity (structure), size, and functionality. The preparation methods also affect the size and lamellarity of vesicles. Vesicles can be divided into three corresponding to lamellarity: multilamellar vesicles (MLV) consist of concentric bilayers, separated by aqueous regions; multivesicular vesicles (MVV) are formed from small non-concentric vesicles trapped into large vesicles; unilamellar vesicles (ULV) are constituted single bilayer. Unilamellar vesicles can be classified depending upon size: vesicles whose diameters ranging from 20 nm to 100 nm are named small unilamellar vesicles (SUV); vesicles from 100 nm to 1000 nm are referred to as large unilamellar vesicles (LUV); and large unilamellar vesicles possessing higher diameters than 1000 nm are known as giant unilamellar vesicles (GUV) (Kulkarni, 2005; Ramon and Danino, 2008). Type of lipids, length of hydrocarbon chain and temperature determine the thickness of bilayer that is approximately 4 nm. Moreover, the different types of vesicles have been developed based upon their interactions with environment that these interactions lead different applications of liposomes. According to functionality of liposomes, there are conventional liposomes, sterically stabilized liposomes, targetable liposomes, activosomes or polymorphic liposomes, and cationic liposomes (Zhang et al., 2002). For example, in recent years, different methods for functionalization of surfaces have been developed in order to target diseased tissue. Liposomes are functionalized with targeting ligands which are generally small ligands (e.g., vitamins, saccharides, and small peptides) bound covalently to hydrophobic anchor (e.g., lipid) in organic solvent. Another surface functionalization method is the post-insertion method that ligands (e.g., antibodies, peptides, and proteins) are coupled to lipid-PEG micelles. Also, post-functionalization provides conjugation on the preformed liposomes directly that complex and larger ligands (e.g., proteins and antibodies) are used (Jøllck et al., 2011).

1.1.1.1 Liposome formation

Formation of vesicles (closed bag-like structures) can occur in two steps that amphiphilic molecules such as phospholipids form a bilayer as an intermediate structure at first step, and bilayer closes to form vesicles (vesiculation of intermediate structure) in the second step (Figure 1.4) (Lasic, 1987; Antonietti and Förster, 2003). Bending energy to form curvature and edge energy of bilayer compete to form vesicles. Energy is required to form vesicles that is supplied by sonication, homogenisation, heating, etc. depending upon the type of lipids and presence of cholesterol to determine membrane curvature (Mozafari et al., 2008).

$$P = \frac{v}{al} = 1 + Hl + \frac{Kl^2}{3} \quad (1.2a)$$

$$H = \frac{1}{2} \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \quad (1.2b)$$

$$K = \frac{1}{R_1 R_2} \quad (1.2c)$$

where H is mean curvature, and K is Gaussian curvature (Antonietti and Förster, 2003).

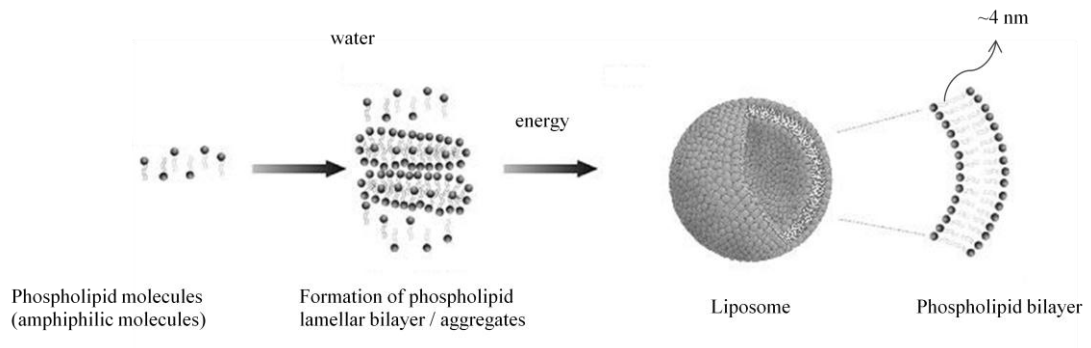


Figure 1.4: Liposome formation by self-assembly process from individual phospholipid molecules to bilayer membrane leaflets, and then transformation into liposomes (adapted from Mozafari et al., 2008).

1.1.1.2 Liposome preparation techniques

There are several methods to produce liposome in the laboratory-scale, also in the industrial scale. Lipid bilayers can be formed spontaneously by hydration of phospholipids. However, further processes are needed in order to obtain unilamellar vesicles in desired size, structure and trapping efficiency. Table 1.1 has summarized the preparation methods of liposomes in different size and structure.

Table 1.1: Methods for preparation of different size of liposomes (Zhang et al., 2002; Jesorka and Orwar, 2008).

Type of Liposomes	Size of Liposome	Methods of Preparation
MLV (Multilamellar vesicles)	<10 μm	Thin-film hydration (evaporation-dried, spray-dried or lyophilized lipid material) Thin-film hydration followed by freeze-thaw cycling
SUV (Small unilamellar vesicles)	20-100 nm	High-energy sonic fragmentation, extrusion, high-pressure homogenization, solvent injection
LUV (Large unilamellar vesicles)	100-1000 nm	Freeze-thaw cycling, swelling in non-electrolytes, de/rehydration, extrusion, detergent dialysis, reverse evaporation
GUV (Giant unilamellar vesicles)	>1000 nm	De/rehydration, electroformation, solid-film hydration, detergent dialysis

MLVs are produced by dissolving of phospholipids in organic solvent such as mixture of chloroform/methanol and then, drying by evaporation to form lipid-film as a common method for preparation of multilamellar vesicles. Vacuum (< millitorr) is applied for several hours in order to remove the residual organic solvent. Then, lipid films are hydrated with aqueous medium at the 5-10°C above the T_m (transition temperature) of lipid and homogenous lipid suspension is obtained by agitation mechanically (vortexing or shaking). Because MLVs have low-trapping volume by this method, freeze-thaw cycling is applied to improve trapping (encapsulation) efficiency and volume (freezing in liquid nitrogen or dry ice and thawing at temperature above the T_m of lipid) (Zhang et al., 2002). Extrusion provides unilamellar liposome had well-defined size, but trapped (encapsulated) content can leak out because they are forced through polycarbonate membranes. Also, LUV can be obtained by homogenization and microfluidization that provide advantages such as simplicity, large capacity, rapid, and enable to use high concentrated lipid suspensions. They are also convenient methods for scale-up. Microfluidization is a cost-effective, solvent-free continuous technique with good trapping efficiency (>20%). It can cause lipid degradation and wide-size distribution by comparison

with extrusion. Furthermore, sonication is a simple method to produce SUV by reducing size of liposomes. After sonication, vesicle suspension is passed through 0.45 μm membrane filter to remove titanium particles from probe (Zhang et al., 2002; Ramon and Danino, 2008; Jesorka and Orwar, 2008). Solvent injection methods such as ethanol and ether injections, having same concepts although different procedures, can generate 20-50 nm size of liposomes (SUV). In addition, reverse phase method (REV) is used to produce water-in-oil emulsions and is alternative method can form LUVs or MLVs with 50% trapping efficiency. Finally, solubilization of lipids in nonionic or ionic detergents, and then detergent is removed gradually by gel filtration column or by dialysis to form liposomes in the method of detergent dialysis. This method can be preferred for reconstitution of biological transmembrane proteins. However, size of liposome can not be controlled, and the type and composition of lipid, the nature of solute, and the type of detergent are parameters which affect the procedure as disadvantages of method (Zhang et al., 2002).

1.1.1.3 Liposome characterization techniques

Characterization of liposomes is important because of maintain the quality of liposomes during preparation, and storage. There are some characterization parameters to determine the quality which are visual appearance, size and size distribution of liposomes, lamellarity, trapped volume, trapping efficiency, solute release and stability. The size and size distribution can be determined by transmission electron microscopy such as cryogenic-TEM, freeze-fracture TEM, and by dynamic light scattering techniques. Dynamic light scattering can be used to determine stability.

Electron microscopy techniques are also utilized for determination of lamellarity as well as fluidity, coexistence of different structures, and transitions to other phases. Spectroscopic techniques such as nuclear magnetic resonance (P-NMR or F-NMR) can be used for lamellarity of liposomes.

Trapped volume can be measured by optical, fluorescent, and HPLC measurements. Internal volume also can be determined by electron spin resonance methods. Multilamellar vesicle formation can cause to lower trapped volume than theoretical value for unilamellar vesicles of same size. Measuring of solute entrapped in

liposomes provides information about trapped volume of liposome. Radioactive markers (radioactive sugar, ion, fluorescent dye, etc.) are used to determine solute trapping. To measure of trapping efficiency, the percentage of trapped solute with respect of total solute added should be calculated. Column chromatography, dialysis, and diafiltration can be applied to separate encapsulated solute inside liposome from external medium. It is assumed that remaining solute is encapsulated 100% inside liposome after separation. Thus, the molecule encapsulated in the liposome can be quantified by column chromatography. The amount of release of encapsulated molecule also should be determined. Two different type of release studies are present that one of them is characterization of the ability of liposome with certain lipid composition or retention of water-soluble markers and second method is monitoring the leakage rate of encapsulated molecule (or drug). Fluorescence markers such as calcein, carboxyfluorescein, and fluorescein dextrans and radiolabeled markers such as glucose, sucrose, DTPA, and inulin are used as water-soluble markers which are preferred to do not leak out from membranes and do not associate with membranes. When producing functional liposomes such as targetable or for delivery, marker is entrapped solute itself, for example cationic lipophilic drugs doxorubicin and camptothecins whose concentration can be determined spectroscopically. Moreover, membrane fluidity and permeability can be studied thanks to fluorescent markers.

Furthermore, surface potential is determined by zeta potential, and osmolality is determined by vapor-pressure osmometry. Fluorescence based pH indicators, NMR, fluorescence methods, Raman spectroscopy, and electron spin resonance methods can be used for phase transitions and phase separations (Zhang et al., 2002; Jesorka and Orwar, 2008; and Ramon and Danino, 2008).

Stability is a limiting parameter for liposome applications, for instance, shelf-life stability is important in clinical applications, and lifetime of chromatography and assay components is affected by instability of liposomes. The size stability and the ratio of lipid to encapsulated/membrane-bound molecule provide information about physical stability. Hydrolysis and oxidation of lipids, degradation by enzymes affect chemical stability, especially for unsaturated lipids. Leakage, aggregation, and binding reactions with other components in the medium are usual results of biological instability problems of liposomes (Jesorka and Orwar, 2008). The amount of leakage can be determined by spectrofluorimetry, using calcein and

carboxyfluorescein as fluorescent quencher. Also, ultracentrifugation, dialysis, molecular size chromatography, and ion exchange chromatography techniques can be used to monitor stability of liposomal formulations containing drug in terms of drug leakage on storage or in biological medium (Kirby and Gregoriadis, 1999).

1.1.1.4 Liposomes in drug delivery, medical and food applications

Liposomes are natural, biodegradable, nontoxic and nonimmunogenic lipid vesicles which can encapsulate or bind drug molecules into or onto their membranes, so they are good candidates for drug delivery systems. Physicochemical and colloidal characteristics such as composition, size, loading efficiency, and stability of liposomes, and biological interactions with cells are determinative factors of liposomes for using in drug delivery. Liposomes can be endocytosed by cells of mononuclear phagocytic system (MPS), mostly fixed Kupffer cells in the liver and spleen as a very useful way for liposome carrier systems. However, sometimes blood circulation time needs to be increased without uptake by MPS that specific ligand can bind to surface of liposome to target specific diseased cells. These are targetable liposomes such as PEG-coated, sterically stabilized liposomes. In accordance with composition and functionality of liposome, drug delivery can be designed in the different types of mode, for example, to enhance drug solubilization, to protect the sensitive drug molecules, to improve intracellular uptake, and to change the pharmacokinetics and biodistribution of encapsulated drug molecule (Zhang et al., 2002).

Liposomes can be used in medical applications because it may decrease undesired toxic effects of chemotherapy and improve treatment efficiency. They can be used in fungal infections and cancer therapy for human. Liposome-based vaccines are promising vehicles for preventative medicine. There have been studies on infectious diseases, anticancer therapy, and gene therapy as medical practices of liposomes (Zhang et al., 2002).

Liposomes in Food Industry

Applications of liposomes in medicine such as cancer treatments, in pharmaceuticals, drug delivery and cosmetics can lead the use of liposomes in food industry. Liposomes are efficient delivery systems for nutraceuticals such as proteins, enzymes, probiotics, vitamins, because of their lipophilic/hydrophilic properties,

colloidal size, stability, encapsulation potential (>20%), and protection of ingredient. Liposomes can encapsulate hydrophobic ingredients between bilayers, also hydrophilic ingredients in their interior. Liposomes can deliver, uniformly disperse, and release the active ingredient in a food matrix, also in a biological system. Vitamin C (ascorbic acid) can be entrapped within liposome with higher protection and stability by comparison with other methods. Milk and cheese production can utilise the liposome entrapped enzymes to accelerate ripening of cheese, to gain texture, to develop flavour, and to protect essential substrates. Incorporation of cholesterol can improve to stability of liposome in the these applications. Vitamins can be encapsulated by liposome in order to improve their retention. Lipid-soluble α -tocopherol (vitamin E), and water-soluble ascorbic acid (vitamin C) can be entrapped by liposome so that the antioxidative effect of vitamin E increase by synergistic effect. Moreover, enzyme stability can be incerased by entrapment in liposomes. Archaesomes, lipid-based nanoliposome including polar ether lipids, have higher resistance to low pH environment and bile salts, and beter thermostability against chemical and enzymatic oxidation and hydrolysis than liposomes, including ester phospholipids. Therefore, the using archaesomes provides advantages for protection of antioxidants and sensitive nutraceuticals such as PUFA (polyunsaturated fatty acids). In additon, minerals such as Ca^{+2} , Mg^{-2} , and Fe^{+3} can be delivered by liposomes (Ramon and Danino, 2008).

According to Mozafari, surfactant micelles, nanospheres, nanoparticles, nanoemulsions, nanococheles, liposomes, and nanoliposomes are encapsulation technologies that can be applied in the food industry, improving stability of entrapped material by protecting against environmental, enzymatic and chemical changes. Due to the nanotechnology, improvement of taste, flavor, color, texture, and consistency of foods, absorption and bioavailability of nutraceuticals, development of antimicrobials, new food packaging materials, nanosensors for traceability and monitoring conditions during storage and transport of food, and encapsulation of food components and additives can be achieved. In addition that liposomes and nanoliposomes as nanocarrier systems are studied by pharmaceutical, cosmetic and food industries in order to protect and delivery bioactive substances, they can be produced by using natural sources such as egg, soy, and milk lipids to be used in food-grade products (2008).

1.2 Surfactants

Before the interactions of surfactants with model lipid membranes is studied, the effects of surfactant on membran systems should be reviewed briefly.

Surfactant are surface-active, amphiphilic compounds that decrease surface tension by forming monolayer at the interface and they can form micelles, bilayer vesicles or other aggregates by self-assembly above the critical micelle concentrations, and also they are water-soluble below the critical micelle concentration. Synthetic detergents, physiological compounds including bile salts, lysolipids and some amphiphilic peptides, and other amphiphiles are classified as surfactants (Heerklotz, 2008).

A variety of intermediate structure, which are mixed vesicles, bilayered phospholipid fragments, cylindrical mixed micelles, lipid-rich spherical or elipsoidal mixed micelles, and lipid-poor mixed micelles, can be formed having the different ratio of surfactant to lipid. Also, some biophysical techniques can be used to characterize the these different structures. For instance, freze-fracture electron microscopy (FFEM) and cryo transmission electron microscopy (cryo-TEM) provide the visualization (imaging) of structures and transitions between the structures can be examined by fluorescence spectroscopy, photon correlation spectroscopy (PCS), small angle X-ray scattering (SAXS), and small angle neutron scatteing (SANS). Moreover, thermodynamic properties of lipid-surfactant interactions, the stability of lipid-surfactant mixed micelles, and stoichiometry can be determined by isothermal titration calorimetry (ITC) (Garidel and Lasch, 2007). The study of interactions between surfactants and phospholipids (or lipids) provides to understand the formation mechanism and thermodynamic stability of systems, and therefore their applications as a nano-carrier systems for delivery systems of drugs (bioactive molecules). Surfactant-lipid systems can enable to develop efficient delivery systems for drug by improving the solubility of drugs. There is an aspect of developing efficent drug deliver system by mixing surfactants with lipids depending on the ratio between these structures.

Surfactants and lipids are amphiphilic molecules constituted from hydrophilic head group (ionic or nonionic) and hydrophobic hydrocarbon chain. In aqueous medium, the polar group has a function to prevent phase separation, but water-hydrocarbon interface is unfavorable so that the system works to minimize the inteface and

amphiphilic molecules aggregates to generate the supramolecular structures depending on the geometry of molecules by self-assembly. This is referred to as hydrophobic effect. Hydrocarbon molecules cause the disruption of hydrogen bonds between water molecules and rearrangement of water molecules surrounding the hydrocarbon molecules. Therefore, configurational entropy decreases and as a result hydrophobic effect occurs (Garidel and Lasch, 2007).

According to Gibbs phase rules, monomers and micelles exist in equilibrium at certain concentration (at fixed temperature and pressure). This certain concentration value is termed critical micelle concentrations (CMC) for micelles and critical aggregation concentrations for others. Surfactant molecules are observed in micellar form above the CMC. However, they are found as monomers form below the this value.

1.3 Interactions of Surfactants with Model Lipid Membranes

Interactions of surfactants with lipid membranes can be considered in the two main processes which are the partitioning of surfactants into the lipid membranes (formation mixed vesicles) and solubilization of membranes (formation mixed micelles) by means of increasing concentration of surfactants ($C_{\text{surfactant}} > \text{CMC}$) (Heerklotz and Seelig, 2000; Garidel et al., 2007).

Lichtenberg introduced three-stage model to describe the phase transitions, vesicles to micelles, that includes mixed vesicular stage, coexistence stage which mixed vesicles and mixed micelles exist together, and mixed micellar stage, also surfactant monomers and aggregates are in equilibrium (Keller et al., 1997; Lichtenberg et al., 2000; Hildebrand et al., 2002). According to the this model, surfactant molecules are inserted into the lipid vesicles until saturation of vesicles, a critical surfactant/lipid ratio (R_e^{sat}). If the concentraion of surfactant increases, mixed vesicles turns into mixed micelles, and lipid-rich mixed micelles, mixed vesicles, and surfactant monomers coexist until solubilization of vesicles, a critical surfactant/lipid ratio (R_e^{sol}). This region is called coexistence region in the phase diagram. Above the value of R_e^{sol} , solubilization of vesicles are completed and mixed vesicles are transformed into mixed micelles finally. Mixed micelles, detergent-rich mixed micelles and surfactant monomers exist together in this region (Keller et al., 1997; Hildebrand et al., 2002). In Figure 1.5, the phase boundaries of the vesicle-to-micelles

transition are illustrated. Diagram is drawn as lipid concentration, L and total detergent concentration, D_t . R_e indicates a the ratio of surfactant concentration in the aggregates (D_e) to lipid concentration (L). D_t^{sat} and D_t^{sol} are phase boundaries of saturation and solubilization, respectively. D_w symbolizes the hypothetical surfactant concentration. If the system is adaptable to Gibbs' phase rule, phase boundaries are intercepted one point in the ordinate of diagram (detergent concentration), and detergent monomers are found in equilibrium with micelles and vesicles. Also, this intersection point is slightly below than CMC of detergent (Heerklotz et al., 2009).

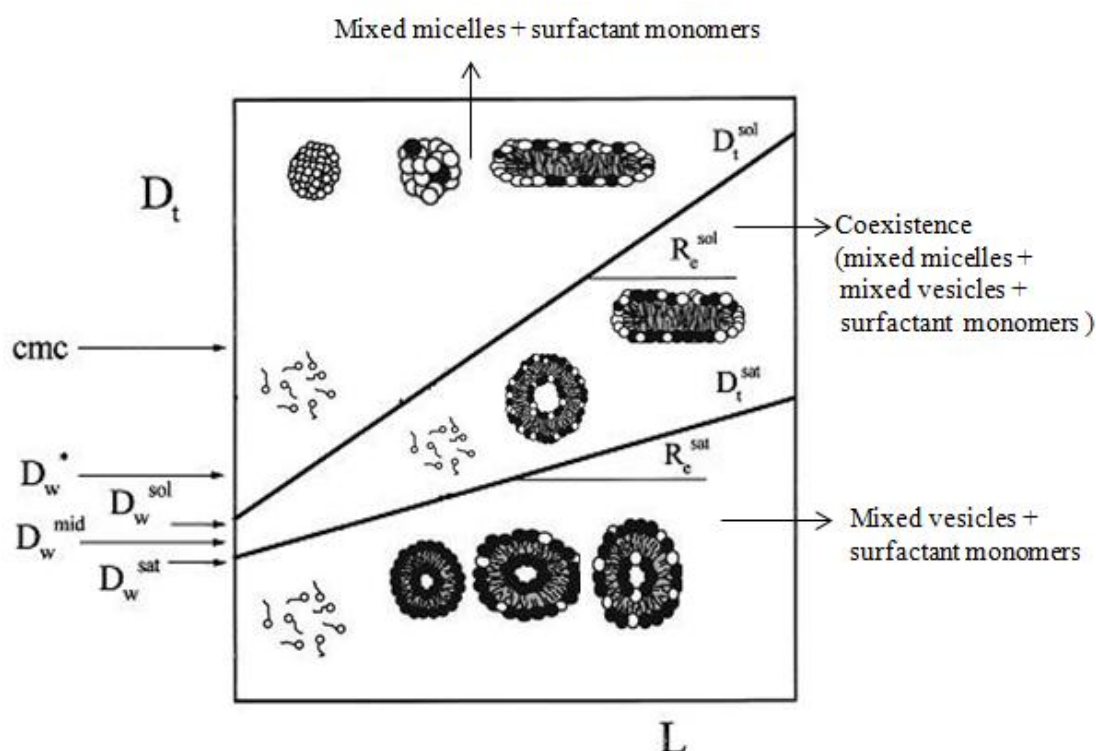


Figure 1.5 : Schematic phase diagram of lipid/surfactant system (adapted from Lichtenberg et al., 2000; Garidel et al., 2007).

Surfactant molecule can partition into the membrane and aqueous phase differently from amphiphilic molecules forming the membrane (Henriksen et al., 2010). When the concentration is below the critical micellar concentration (CMC), surfactant molecules partition into lipid membrane, and the increase of the surfactant concentration gradually until CMC value leads to change of physical properties of membranes, accompanied with thermodynamical changes. Membrane-water partitioning of surfactant can be modelled to quantify the binding by determination the partition coefficient (Heerklotz and Seelig, 2000; Heerklotz, 2008). While determination of the partition coefficient of the surfactant in the equilibrium between

aqueous phase and membrane, the charges of surfactants should be considered, for example, negatively charged bile acids (Garidel and Lasch, 2007).

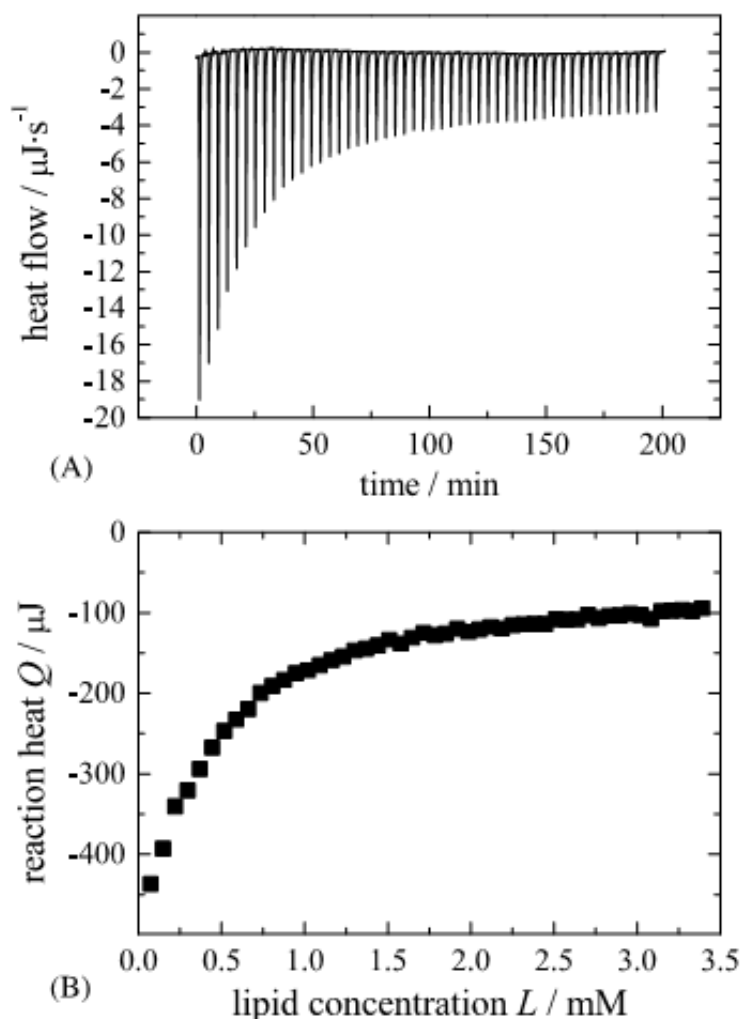


Figure 1.6: Membrane partition experiment by ITC. Titration of SPC dispersions into Sodium cholate solution at 30°C. (A) Raw heat spikes for each injections versus time and (B) The interaction heat versus lipid concentration in the cell (Hildebrand et al., 2003).

Membrane partitioning experiments can be ideally performed by means of ITC because it offers the determination of thermodynamic parameters in the only one experiment (the advantages of this technique will be reviewed next chapter of the thesis) by titrating of liposome vesicle solution into surfactant monomer solution in the ITC sample cell ($C_{\text{surfactant}} \ll \text{CMC}$). At the beginning, the negatively charged bile acids are incorporated into lipid membrane, so the charge of membrane increase gradually and incorporation of bile acids decrease by electrostatic interactions, also the free bile acids for insertion into liposomal membrane in the ITC sample is

reduced. As a result, the heat because of partitioning of biosurfactants, bile acids, becomes constant, and heat of dilution of lipid vesicles are obtained at that level (as it is seen in Figure 1.6 B).

$$P = \frac{x_b}{x_w} \quad (1.3)$$

where P is a partition coefficient, x_b is the mole fraction of bound surfactant bound in the mixed vesicles, and x_w is the mole fraction of free surfactant in aqueous phase. As it is given in eq. 1.3. P is described as the ratio of x_b and x_w .

Since bile acids are negatively charged biosurfactants, electrostatic repulsion between charged surfactant molecules and vesicles has to be taken into account for calculating P. By using Kuchinka and Seelig approach, surface charge density, σ , can be determined by following eq.1.4:

$$\sigma = z e_0 \frac{\frac{D_b}{L A_L}}{1 + \frac{D_b A_D}{L A_L}} \quad (1.4)$$

e_0 is the elementary electric charge, z is the electric charge of surfactant (bile acid), D_b is the concentration of bound surfactant, L is the lipid concentration in the mixture, A_L is the average surface area of lipid molecule, and A_D is the average surface area of surfactant molecule. A_L is approximately $65 \text{ \AA}^2$ for DPPC and POPC molecule and A_D is about $40 \text{ \AA}^2$ (Garidel et al., 2007).

The surface potential, ψ_0 can be calculated using Graham equation:

For 1:1 electrolytes:

$$\psi_0 = -k_B \frac{T}{e_0} a \cosh \left(\frac{\sigma^2}{4\epsilon_0 \epsilon_r R T 1000 c_{el}} + 1 \right) \quad (1.5)$$

where k_B is the Boltzmann constant ($1.3806488 \cdot 10^{-23} \text{ J/K}$), T is the absolute temperature, e_0 is the elementary electric charge, ϵ_0 is the permittivity of free space, ϵ_r is the dielectric constant of water, R is the gas constant ($8.3144621 \text{ J/(mol.K)}$), and c_{el} is the electrolyte concentration.

P can be determined by using ψ_0 :

$$P = P_0 e^{\left(\frac{z e_0 \psi_0}{k_B T} \right)} e^{\left(-\rho \frac{(1-x_b)^2}{R T} \right)} \quad (1.6)$$

P_0 is the intrinsic partition coefficient and ρ is the nonideality parameter that assumes nonideal mixing of surfactant in the lipid membrane.

Bound surfactant concentration can be quantified in terms of total detergent concentration and molar water concentration:

$$Db = \frac{1}{2} (D_t - L) - \frac{W}{2P} + \sqrt{\frac{1}{4} (D_t + L)^2 - \frac{1}{2} (D_t - L) \frac{W}{P} + \frac{1}{4} \left(\frac{W}{P}\right)^2} \quad (1.7)$$

In the eq. , W is the molar water concentration (55.5 mol⁻¹).

According to Gouy-Chapman theory, surfactant concentration at lipid membrane surface, D_w:

$$D_w = D_{wbulk} e^{\left(-\frac{ze_0}{k_B T} \psi_0\right)} \quad (1.8)$$

In eq. 1.8 , D_{wbulk} = D_t - D_b

During isothermal titration calorimetric assays, injection of lipid vesicles solution leads to change of bound surfctant concentration in the ITC sample cell. The change of bound surfactant concentration is given in the following eq.1.9 :

$$\frac{\Delta D_b}{\Delta L} = \frac{D_b(n+1) - D_b(n)}{L_{(n+1)} - L_{(n)}} \quad (1.9)$$

Heat change observed from ITC is:

$$\Delta Q = \left(\frac{\Delta D_b}{\Delta L} \Delta H^T + \Delta H_{dil}\right) L_{t(syr)} v_{inj} \quad (1.10)$$

L_{t(syr)} represents total lipid concentration in the syringe, v_{inj} is the injection volume. ΔQ is calculated with ΔH^T, which is transfer enthaply of inclusion of surfactant molecules into lipid membranes, and also considering ΔH_{dil}, heat of dilution of lipid vesicles solution. ΔH_{dil} can be obtained by titration of lipid solution into buffer in the sample cell (Garidel and Lasch, 2007).

Non-linear least square model is used to fit the parameters, P and ΔH^T. Also, P_{av} denotes the average partition coefficient which is the middle injection number of partition experiment. Gibbs free energy changes, ΔG_#^T are calculated by ΔG_#^T = -RTlnP_# (# means “0” or “av”, so all thermodynamic parameters can be calculated for intrinsic and average partition coefficient). Entropy term, TΔS_#^T, is calculated by means of Gibbs-Helmholtz equation:

$$T\Delta S_{\#}^T = \Delta H^T - \Delta G_{\#}^T \quad (1.11)$$

Membrane solubilization means dissolution of lipid membrane bilayers with increasing of surfactant concentration in the solution. The change of temperature, pressure or composition result in the phase transitions (Heerkoltz et al., 2009).

Various mixed vesicles and micelles structures are occurred during vesicle-to-micelle transition. Surfactant molecules are incorporated into lipid vesicles at low surfactant concentrations, as a result mixed vesicles are formed. Surfactant molecules can be also transported into the inner layer of vesicles which this process can be called as flip-flop. Increasing of the surfactant concentration in the solution cause the saturation of lipid membranes that accompanied by increasing the size of vesicles. After certain surfactant concentration, D_t^{sat} , lipid vesicles can not carry surfactant molecules into layers and starts to disintegrate. Further increasing of surfactant concentration cause to form mixed micelles from mixed vesicles. Surfactant concentration is reached to certain concentration, D_t^{sol} , mixed vesicles are transformed into mixed micelles completely. Moreover, different structures and phases of mixed micelles such as lipid-rich mixed micelles and surfactant-rich mixed micelles can be formed (Garidel et al., 2007; Garidel and Lasch 2007). Heerklotz points out that there are different pathways to show various structures that consist of local and principal structures (2008).

Composition-induced changes can be determined and phase diagrams can be established by isothermal titration calorimetry (Heerkoltz et al., 2009). Membrane solubilization experiments by ITC are performed by injection of highly concentrated surfactant solution ($C_{\text{surfactant}} \gg \text{CMC}$) into lipid vesicular solution in the ITC sample cell (Figure 1.7). As a consequence, the heat of transition of surfactant micelles into the lipid vesicles and phase diagrams depending on the concentration of lipids and surfactants in the solution can be determined.

The effective surfactant to lipid ratio, R_e which determines the composition of mixed aggregates, is:

$$R_e = \frac{D_B^{\#}}{L} \quad (1.12)$$

The total surfactant concentration is given as:

$$D_t = D_w + D_b \quad (1.13)$$

where D_w is the surfactant concentration in the aqueous phase, D_b is the bound

surfactant concentration in the lipid membrane. In the phase diagram, D_w indicates the inteceptions of phase boundaries for saturation and solubilization. The relationship between the hypothetical surfactant concentration, D_w , and R_e is described by eq. 1.4:

$$D_t^\# = R_e^\# L + D_w^\# \quad (1.14)$$

$R_e^\#$ can be determined from the slope of phase boundaries where # means saturation and solubilization of lipid membranes by surfactant. Also, $D_w^\#$ can be determined by extrapolation of phase boundaries to ordinate, $L=0$ mM.

This linear equation is adapted for highly concentrated lipid solutions. Deviation from linearirt can be observed form low concentrated lipid solutions, so that the another equation was derived to describe phase boundaries for this kind of systems by Roth et al (2000).

$$D = D_w^* + [\alpha R^{sol} + (1 - \alpha) R^{sat}] L - \frac{A_w}{\alpha^{0.5} L^{0.5}} \quad (1.15)$$

where α is the ratio between lipid concentration in micellar form and total lipid concentration. A_w is the fitting parameter.

When the mixed vesicles are tranformed in to mixed micelles completely, which means $\alpha=1$:

$$D = D_w^* + R^{sol} L - \frac{A_w}{L^{0.5}} \quad (1.16)$$

When it is assumed that $\alpha_{min} \ll 1$, the equation for determining saturation phase boundary becomes as following eq. 1.17:

$$D = D_w^* + R^{sat} L - \frac{A_w}{\alpha_{min}^{0.5} L^{0.5}} \quad (1.17)$$

Roth et al approach is compatible for low lipid concentrations. They thought that there is a effect of size of micelles on the shape of phase bounadries. However, there is stil distinction between D_w^{sat} and D_w^{sol} as a experimental result (Garidel et al., 2007).

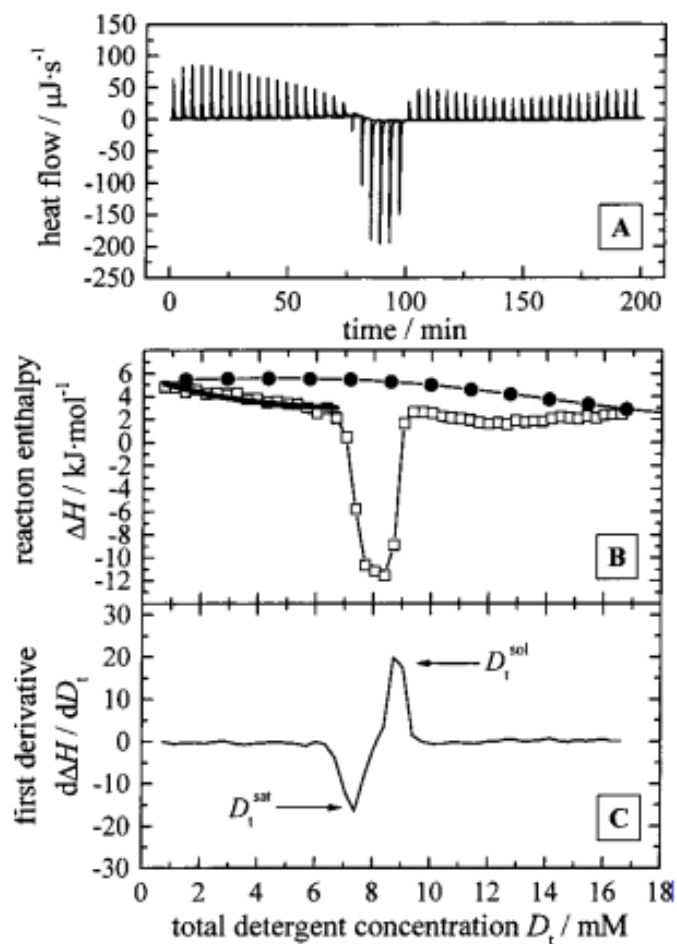


Figure 1.7: Membrane solubilization experiment by ITC. Titration of highly concentrated sodium cholate solution into DPPC dispersion at 60°C. (A) Raw heat spikes for each injections versus time, (B) The interaction heat versus surfactant concentration, and (C) The first derivative of heat in terms of total detergent concentration, D_t^{sat} and D_t^{sol} can be determined (Hildebrand et al., 2002).

1.4 Bile Acids

Bile salts are the salts of bile acids which are group of water-souble steroids with distinctive physicochemical and biological characteristics. Bile salts are biosyntesized from cholesterol in the hepatocytes of the liver and stored in the gallbladder under high concentration (>300 mM) (Stamp and Jenkins, 2004; Monte et al., 2009). Because they are the end products of cholesterol metabolism, their structure is derived from cholic acid. Bile acids are formed from two connecting group, a rigid steroid nucleus (A, B, C, D rings as it is given in Figure 1.8) and a short aliphatic chain. The steroid nucleus consists of tetracyclic hydrocarbon perhydrocyclopentanophenanthrene, including three six-member rings (A, B, and C)

and one five member rings (D). In higher vertebrates, there is a cis configuration between A and B rings, so the steroid nucleus is curved, whereas some of them have flat structure (*allo-* bile acids) because of trans configuration in lower vertebrates. Depending on the side chain structure, they occur in the various species. C_{24} bile acids, major part of the bile, exist in higher vertebrates. Three structural features of bile acids such as side-chain structure, stereochemistry of A and B rings, and the distribution of number, position and stereochemistry of $-OH$ groups in steroid nucleus are different in different species. C_{24} bile acid, 5- β configuration with 3- α , 7- α , and 12- α hydroxyl groups, is a structure of most evolved mammalian bile acid (Mukhopadhyay and Maitra, 2004).

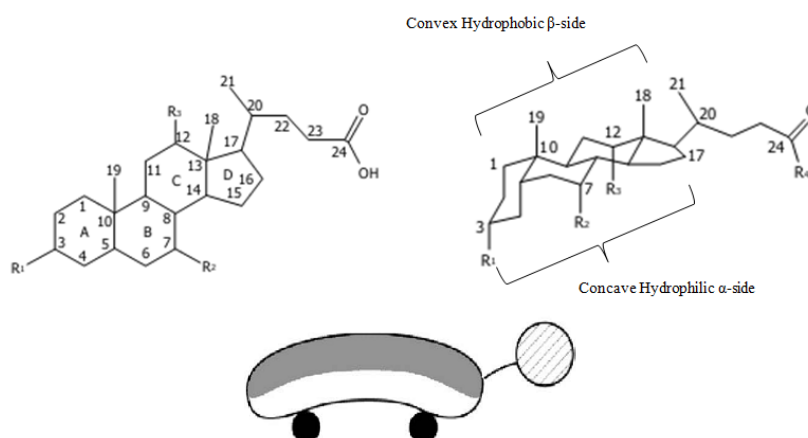


Figure 1.8: Chemical structure of bile acids and schematic illustration of dihydroxybile acid.

Bile salts are biological surfactants which play an important role for fat digestion and absorption through intestinal wall by forming mixed micelles with lipids, fats and cholesterol. Mixed micelles enable lipases to access interior of lipid droplet by increasing the surface area of fats (Mukhopadhyay and Maitra, 2004; Stamp and Jenkins, 2008). They can form micelle because of hydrogen binding as well as hydrophobic effect. Also, bile salts have different structure than classical amphiphiles which consists of lipids, hydrophilic polar head group and hydrophobic nonpolar hydrocarbon chain (Madenci and Egelhaaf, 2010). Bile salts have hydrophilic concave lower side (α -face) and hydrophobic convex upper side (β -face) that cause a facial structure and position and orientation of hydroxyl groups and hydrophobic methyl groups determine their structure. The hydroxyl groups are oriented towards α -side, except ursodeoxycholic acid, and methyl groups at C18 and C19 are oriented towards β -side. Also, carboxyl chain contributes the hydrophilic

character of bile acids (Monte et al., 2009). Moreover, since bile salts have rigid steroid structure, a weak separation between hydrophilic and hydrophobic sides in aggregates can occur with remaining of some hydrophobic part in aqueous solution and hydrophilic part in micelles (Madenci and Egelhaaf, 2010). The characteristics of bile salts as mentioned above provide unique features to bile acids.

As a result of cholesterol catabolism, cholic acid and chenodeoxycholic acid are produced as primary bile acids. Then, they are conjugated with glycine (75%) and taurine (25%) that these conjugated forms (glycolate and taurocholate) have higher water solubility under physiological conditions. There are general three properties of primary bile acids are being end-product of cholesterol catabolism, secretion into bile largely in a conjugated form, and being membrane-impermeable, water-soluble, and amphiphilic molecules of conjugated bile salts which can form mixed micelles with lamellar lipids (Mukhopadhyay and Maitra, 2004). Some physicochemical properties of bile acids are given in the Table 1.2 and chemical structures of most known bile acids are given in the Figure 1.9.

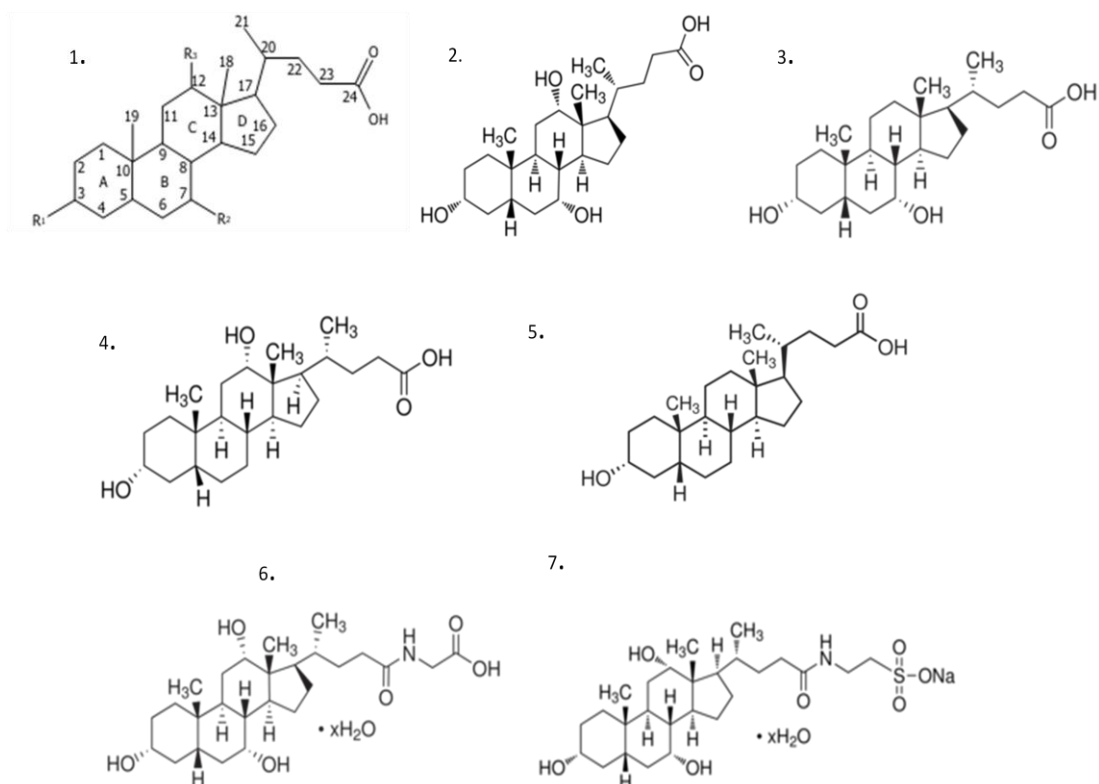


Figure 1.9: Chemical structures of certain bile acids (1. General structure of bile acids, 2. Cholic acid, 3. Chenodeoxycholic acid, 4. Deoxycholic acid, 5. Lithocholic acid, 6. Glycocholic acid, 7. Taurocholic acid sodium salt hydrate).

Table 1.2: Some physicochemical properties of bile acids.

Bile acid		R groups	Solubility in water (μM)	CMC (mM)	cmp H	pKa
Cholic acid	Primary- Trihydroxybile acid	$\text{R}_1, \text{R}_2, \text{R}_3, \text{R}_4$: OH	$273^{\text{e,f}}$	$13^{\text{a,e}};$ $11^{\text{e}}; 18.4^{\text{d}}$	6.65^{e}	5.2^{a}
Chenodeoxycholic acid	Primary- Dihydroxybile acid	R_1, R_2 : α - OH; R_4 : OH	$27^{\text{e,f}}$	$9^{\text{a,e}}; 3^{\text{c}};$ $7^{\text{d}}; 4^{\text{e}}$	7.22^{e}	6.2^{a}
Deoxycholic acid	Secondary- Dihydroxybile acid	$\text{R}_1, \text{R}_3, \text{R}_4$: OH	$28^{\text{e,f}}$	$10^{\text{a}};$ $1.8 \pm 0.2^{\text{b}};$ $5^{\text{c}};$ $5.3^{\text{d}}; 3^{\text{e}}$	7.08^{e}	$6.2^{\text{a}};$ 6.58^{c}
Lithocholic acid	Secondary- Monohydroxybile acid	R_1, R_4 : OH	0.05^{f}			
Glycocholic acid	Glycine conjugated trihydroxybile acid	$\text{R}_1, \text{R}_2, \text{R}_3$: OH R_4 : NHCH_2CO O^-	32	$12^{\text{a}};$ $11^{\text{a,e}};$ $7.5^{\text{b}};$ 7.1^{c}		3.8^{a}
Taurocholic acid	Taurine conjugated trihydroxybile acid	$\text{R}_1, \text{R}_2, \text{R}_3$: OH R_4 : NHCH_2 CH_2SO_3^-	Vey soluble ^e $100\text{mg/ml}^{\text{c}}$	$10^{\text{a}};$ $6^{\text{a,e}};$ $7.0 \pm 0.5^{\text{b}};$ $3-11^{\text{c}}$		$<2^{\text{a}};$ 1.4^{c}

Abbreviations: cmc, critical micellar concentration; cmpH, critical micellization pH and $\text{cmpH} = \log \text{cmc} + \text{pKa}^{\text{BAPs}}$.

^a values from Stamp and Jenkins, 2008.

^b values from Ollila and Slotte, 2001.

^c values from <http://www.sigmaaldrich.com>

^d values from Simonovic and Momirovic, 1997. The CMC values refer sodium salts of bile acids and determined by micro-calorimetre at pH 7.9.

^e values from Roda et al., 1983; Hofmann and Mysels, 1992. CMC values of bile acids also in 0.15M Na^+ solution.

^f values from Hofmann and Roda, 1984. pKa values of all unconjugated bile acids are approximately 5.0

Bile includes water (80%) and bile acids, phospholipids, cholesterol, other lipophilic molecules, bilirubin and other pigments, proteins, heavy metals, and drug metabolites that are insoluble in urine (Sievänen, 2007; Stamp and Jenkins, 2008). 4-800 mls of bile are secreted in a per day and it is secreted through bile duct into intestine only when food is taken and it passes from stomach to duodenum (cholecystokinin stimulates the gallbladder). Bile acids exist in the conjugated form in that part, and they are impermeable to the cell membranes. They form mixed micelles with dietary lipids (cholesterol, phospholipid bilayers, fatty acids which are products of digestion) at the lower end of the small intestine. Lipids may diffuse into

the epithelium to the portal veins, and micelles move towards to distal ileum that ~95% of bile acids are reabsorbed and sent to liver with portal vein. This process is known as enterohepatic circulation and it can cycles several times (6-15) daily (from the gallbladder to the ileum, to portal vein, and back to the liver). Approximately 5% of bile acids are excreted from the body in the deconjugated form which are lithocholic and deoxycholic acid. They are called as secondary bile acids that are degraded by colonic bacteria. Therefore, this is only time that cholesterol is also excreted from the body. Enterohepatic circulation provides not to synthesise of new bile acids for each meals by returning ~95% of bile acids (Hofmann, 1999; Stamp and Jenkins, 2008).

Bile salt molecules aggregate to form micelles by self-assembly like classical amphiphiles but the transition range is broader than classical amphiphiles. They form small size aggregates in comparison to classical amphiphiles. The change of CMC values of bile acids with medium conditions such as ionic strength, pH, and temperature shows similarity with classical amphiphiles. For example, the increase of ionic strength causes to decrease values of CMC because aggregation happens favorably. Also, bile salts are ionized when pH is above pK_a . If pH is around pK_a , dissociated or undissociated molecules aggregate to form micelle, and CMC decrease. Moreover, the CMC values of bile acids can alter depending on the type of bile acid (Madenci and Egelhaaf, 2010).

Also, lipid mixed micellar shape changes with respect to lipid to bile ratio. Mixed micelles transform into rodlike micelles by increasing of lipid/bile acid ratio (Marrink and Mark, 2002). Recent studies showed that bile acids are ordered according to radial shell model. Polar lipids are ordered radially, their hydrophilic head groups tend towards into aqueous phase and bile acid molecules locate between polar head groups perpendicularly (Hjelm et al., 1995; Marrink and Mark, 2002; Stamp and Jenkins, 2008).

Furthermore, bile acids are strong cytotoxic molecules at higher concentrations and they can be used as sensors because they can detect and control their concentrations in the body by themselves. Also, they have a role in carcinogenesis. Their toxicity is related to hydrophobicity of them. For instance, lithocholic acid, which has one hydroxyl group bound to steroid nucleus, has high toxicity. However, deoxycholic and chenodeoxycholic acid, with two hydroxyl groups, and cholic acid with three

hydroxyl group are less toxic (Stamp and Jenkins, 2008). The hydrophilicity of certain bile acids can be given in this order: ursodeoxycholic acid>cholic acid>chenodeoxycholic acid>deoxycholic acid>lithocholic acid, taurine-conjugated>glycine conjugated>free bile salts. The hydrophilicity and hydrophobicity may determine their other physicochemical properties, but it is still controversial situation (Monte et al., 2009).

In addition to the primary role of bile acids is solubilization and transport of lipids and lipid-soluble molecules such as vitamins, they can regulate many physiological pathway, synthesis of bile acids, and thereby modulating of cholesterol homeostasis as their other physiological functions.

1.4.1 Bile acid biosynthesis

The biosynthesis of bile acids is illustrated from cholesterol to primary bile acids, cholic acid and chenodeoxycholic acid in Figure 1.10. Firstly, cholesterol is oxidized to 7 α -hydroxycholesterol (5-cholesten-3 β ,7 α -diol) by cholesterol 7 α -hydroxylase. Then, this molecule is turned to 4-cholesten-7 α -ol-3-one and OH derivative by oxidation and isomerization reaction. Cholic acid and chenodeoxycholic acid are synthesized in the hepatocytes in the liver. They are conjugated with glycine and taurine by amidation reaction that catalyzed by acyltransferase. Hydroxy group at C7 is cleaved to produce secondary bile acids, deoxycholic acid from cholic acid and lithocholic acid from chenodeoxycholic. Deconjugation and dehydroxylation in the colon is happened by colonic bacteria. The final products enter the enterohepatic circulation and reach the liver, and then they are re-conjugated with either glycine or taurine.

Finally, Hoffman states that “Bile acids are good in the infant because they enhance lipid absorption, and thereby promote growth; the enterohepatic circulation of bile acids is bad in the adults because it downregulates hepatocyte low-density lipoprotein receptor activity, and thereby elevates plasma cholesterol level; and the defects in bile acid metabolism are ugly because they cause morbidity and death.” in his article (1999).

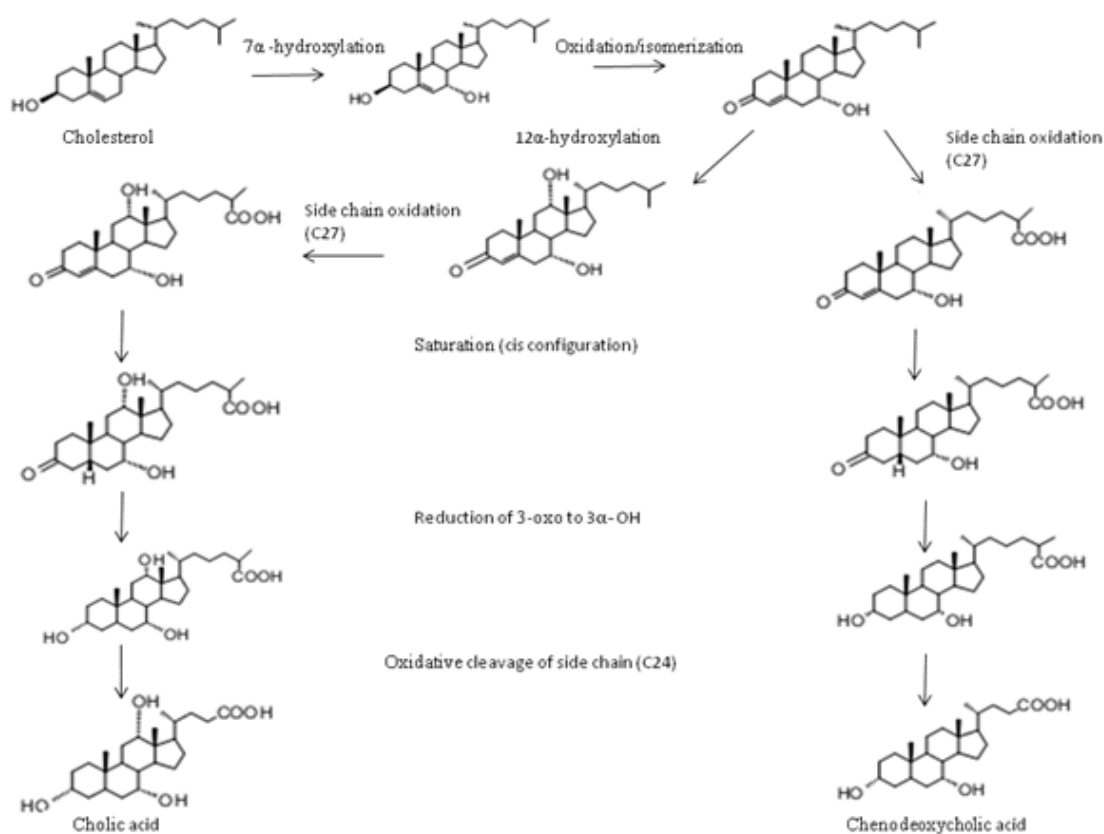


Figure 1.10 : Biosynthesis pathway of bile acids from cholesterol (Mukhopadhyay and Maitra, 2004).

1.4.2 Bile acids in delivery systems for drugs and food ingredients

Bile acids have various applications, especially in pharmaceutical industry, depending on their specific properties and functions in the human body.

Recirculation of bile acids makes them interesting tools in designing of pharmaceutical molecules and prodrugs. Because the transportation route of bile acids in the human body, from small intestine into portal circulation, from portal circulation into the hepatocyte, from hepatocyte into the bile, and back to small intestine, is the one of the most effective recycling routes, various transport proteins take a part in this enterohepatic circulation. Sievänen indicates that targeting to specific enzymes or membrane transporters has occurred as a new approach for prodrug design nowadays, and the active nutrient transport systems have been a target to improve the bioavailability of drugs. Also, bile acids have rigid steroidal backbone, enantiomeric purity, availability, and low cost in addition to their transport capacity and organ specificity in enterohepatic circulation, so they can be efficient molecules as bile-acid drug hybrid molecules and bile acid derived prodrugs for pharmaceutical industry (2007).

Also, there is another important feature of bile acids that displacement of drugs from the cyclodextrin carrier by bile acids. Holm points that competitive binding to cyclodextrin and displacement of drug from cyclodextrin cavity can be accomplished by lipophilic products from meals and gastrointestinal secretions (2009). Cyclodextrins have a cage-like supramolecular structure with hydrophilic outer surface and lipophilic cavity that can encapsulate the drug molecules in their cavity region forming inclusion complexes in order to improve the water-solubility, stability, and bioavailability as well as non-inclusion complexes (Loftsson et al., 2005). Cyclodextrins are absorbed in the intestinal system slightly, it is thought that bile salts can bind competitively to cyclodextrin to remove active molecules such as xenobiotics from complex. Therefore, the use of cyclodextrins to enhance solubility of drugs for oral drug development requires to elucidate the interactions between bile salts and cyclodextrin (Holm et al., 2011).

Moreover, it is known that liposomes can be utilized to enhance absorption of poorly water soluble drugs because they resemble liposomal lipid bilayers and biomembranes. Phase transition studies of liposomal vesicles can provide to find out the mechanism of oral absorption of liposomes under stimulated gastrointestinal conditions, such as presence of bile salts. The biological bile salts have an important function to enhance absorption of dietary lipids and poorly water soluble drugs (lipophilic molecules) and to decrease membrane toxicity because of bile acid monomers, since bile salts can form mixed micelles with phospholipids in gastrointestinal system. After transdermal delivery take advantage of the use liposome containing bile salts, transfersomes, they can increase transmembrane absorption because they assemble mixed micelles promptly in the gastrointestinal tract (Chen et al., 2009).

In addition, there have been applications for delivery of food ingredients in surfactant-based micellar solutions that aim to enhance solubility and stability of some poorly soluble phenolic compounds like curcumin (Sahu et al., 2008), carotenoids (Sugawara, 2001) and anthocyanins (Mulinacci et al., 2001) and improve their cellular uptake. Interactions of four different antioxidants (p-hydroxybenzoic acid, syringic acid, sinapic acid and quercetin) with polymeric surfactant, Pluronic[®] P104, and solubilization of these phenolic antioxidants in surfactant-based micelle solution were investigated. Dynamic light scattering was used to determine size of

micellar structures, UV-Vis spectrophotometry was used to determine the solubilization of phenolic compounds, and location of phenolic compounds in micelles were determined by NOESY spectra (Parmar et al., 2011). Amri et al. has stated that resveratrol, non-flavonoid polyphenolic compound, has antioxidant, antiinflammatory, cardioprotective and anti-tumor activities, but its therapeutic applications are limited because of its low bioavailability, poor solubility, limited stability, and rapid metabolism. Therefore, formulations to improve bioavailability of resveratrol has been presented by Amri et al. (2012). The enhancement of solubilization of resveratrol in different bile acid micellar solutions (cholic acid, keto-derived cholic acids, keto-derived deoxycholic acid and taurocholic acid) were studied by Atanackovic et al. They determined the CMC values of bile acids by conductometry and analyzed the solubilization of resveratrol by HPLC in the study in order to find efficient bile acid micellar solution which has more solubilization effect on resveratrol and less membranolytic activity (2009).

Various studies about formulations containing bile salts, phospholipids, and fatty acids have existed in the literature. These studies include the effects of bile salts on the lipid vesicles, containing phospholipids, sphingomyelin, cholesterol, and etc., in particular their ability of partition and solubilization on the lipid membranes, also depending on environmental conditions such as temperature and ionic strengths (van Erpecum and Carey, 1997; Garidel et al., 2000; Mouaz et al., 2001; Nagadome et al., 2001; Ollila and Slotte, 2001; Hildebrand et al., 2002,; Hildebrand et al., 2003, 2004; Hildebrand et al., 2003; Garidel et al., 2007; Andrieux et al., 2009; Matsuoka et al., 2010). The mechanism of vesicles transitions into micelles or vice versa (phase transitions between different forms of micelles and vesicles) has been investigated by different techniques such as isothermal titration calorimetry, differential scanning calorimetry, dynamic light scattering, small angle neutron scattering, right angle scattering, nuclear magnetic resonance spectroscopy (^{31}P NMR), surface tension measurements, fluorescence spectroscopy, electron microscopy techniques, pressure perturbation calorimetry, turbidimetry measurements, and quantification by HPLC (Keller et al., 1997; Lopez et al., 1998; Majhi and Blume, 2001, 2002; Tan et al., 2002; Heerklotz et al., 2004; Hildebrand et al., 2004; Keller et al., 2006; Andrieux et al., 2009; Heerklotz et al., 2009; Krylova et al., 2010). In particular, isothermal titration calorimetry is a powerful experimental tool and can be preferred to research

the actions of classical surfactants such as sodium dodecyl sulfate (Keller et al, 2006; Tan et al., 2002), octyl glucoside (Keller et al., 1997) or surfactants like molecules such as anbiotic peptide (surfactin) (Heerklotz and Seelig, 2001), antimicrobial peptides (mastoparans) (Henriksen and Andresen, 2011), and lysolipids which are products of lipid hydrolysis (Henriksen et al., 2010).

Since bile acids have potential use in drug development and delivery system associated with being naturally occuring surfactant and having different structure than ordinary surfactants, the investigation of interactions between bile acids and lipid vesicles has been aimed in this project. Interactions between bile salts and lipid membranes have been studied in terms of partitioning of bile salts into lipid membrane and solubilization of lipid membranes, thereby determination of phase transition diagrams. Synthetic phospholipids, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), have been used to form lipid vesicle and deoxycholic acid has been chosen to investigate its action on lipid membranes. Thermodynamic characteristics of interactions have been represented by isothermal titration calorimetry, principally. Because deoxycholic acid is negatively charged, electrostatic interactions have been considered while calculating partition coefficients. The ratio of bile acid to lipid (R_e^{sat} and R_e^{sol}) and bile acid concentration (D_t^{sat} and D_t^{sol}) at saturation and solubilization conditions of lipid membranes by bile acids have been deduced from the phase diagrams, indicating the regions of mixed micelles, mixed vesicles, and coexistence. Dynamic light scattering and fluorimetric measurements have been performed to confirm the data from calorimetric assay.

2. EXPERIMENTAL TECHNIQUES

This part of project includes the descriptions of calorimetric (isothermal titration calorimetry and differential scanning calorimetry) and spectroscopic techniques (dynamic light scattering and fluorescence spectroscopy) in order to study the effects of bile salts as biological surfactants on model lipid membranes.

Isothermal titration calorimetry, differential scanning calorimetry, dynamic light scattering, and fluorescence spectroscopy have been reviewed in terms of their principles, instrumentation, and their applications in the pharmaceutical and food industries. Also it has been aimed to evaluate the techniques from the point of membrane studies.

Isothermal titration calorimetry offers advantages to the researchers because it directly characterizes the molecular interactions presenting the all thermodynamic parameters in a single experiment. Differential scanning calorimetry is also used to determine thermodynamic characteristics, and in particular, to monitor the change in the transition temperature of lipids. Particle size and size distributions are measured by dynamic light scattering method to characterize the vesicles obtained before attending to the other assay, also the solubilization of model lipid membranes can be monitored by determination of the average particle size. Furthermore, the solubilization of model lipid membranes can be studied by fluorescence spectroscopy determining the lamellarity of vesicles.

2.1 Isothermal Titration Calorimetry (ITC)

Calorimetry is a universal technique since every reaction can cause heat changes. Therefore, every process can be monitored by isothermal titration calorimetry. Also, the application of this technique is important to develop formulations for pharmaceutical and food industry thanks to its ability for determination of molecular recognition.

2.1.1 Principle of isothermal titration calorimetry

Chemical and physical processes occur with heat changes depending on type of process which may be an endothermic (absorption of heat from surrounding) or exothermic (release of heat to surrounding). Isothermal titration calorimetry (ITC) is a calorimetric method to measure reaction heat thereby to find the enthalpy of reaction (ΔH , kcal/mol or kJ/mol) and reaction rate ($\delta n/\delta t$, moles/sec) (as a function of time or injection number). Therefore, it can be used to determine thermodynamic characteristics of molecular interactions, especially in biological processes such as binding interactions between ligands (surfactants, ions, etc.) and macromolecules (lipids, proteins, DNA, RNA, etc.). ITC can be accepted as practical method because the entire thermodynamic profile of molecular interactions can be obtained directly. Equilibrium binding constant (K_B), enthalpy (ΔH) can be obtained as the concentration of titrant-titrate complex is known. Also, Gibbs free energy (ΔG), entropy (ΔS), heat capacity (ΔC_p) as other characteristics which are used to identify the systems can be calculated from thermodynamic equations.

$$\Delta G(T) = \Delta H(T_R) + \int_{T_R}^T \Delta C_p dT - T\Delta S(T_R) - T \int_{T_R}^T \Delta C_p \frac{dT}{T} \quad (2.1)$$

If ΔC_p is temperature-independent:

$$\Delta G(T) = \Delta H(T_R) - T\Delta S(T_R) + \Delta C_p \left[T - T_R - T \ln \left(\frac{T}{T_R} \right) \right] \quad (2.2)$$

$$\Delta C_p = \frac{d(\Delta H)}{dT} = T \frac{d(\Delta S)}{dT} \quad (2.3)$$

Moreover, there is a relationship between ΔG and binding constant:

$$\Delta G = -RT \ln K_B \quad (2.4)$$

where R is a gas constant and T is the absolute temperature. According to van't Hoff equation:

$$\int_{T_1}^{T_2} d \ln K_B = \int_{T_1}^{T_2} \frac{\Delta H}{RT^2} dT \quad (2.5)$$

$$\int_{T_1}^{T_2} \Delta G = - \int_{T_1}^{T_2} \Delta S dT \quad (2.6)$$

The second derivative of ΔG gives the ΔC_p . van't Hoff analysis is the non-calorimetric approach of binding reactions. However, this approach is failed in some situations that temperature range of experiments are very limited and the enthalpy-

entropy compensation between large and strongly temperature-dependent ΔH and ΔS induce K_B becomes temperature-independent usually (Jelesarov and Bosshard, 1999). In addition, kinetic parameters (K_m and k_{cat}) of enzyme-catalyzed reactions can be determined by isothermal titration calorimetry.

$$Q = n \Delta H_{app} = [P]_{total} V \Delta H_{app} \quad (2.7)$$

$$\text{Power} = \frac{dQ}{dT} = \left(\frac{d[P]_{total}}{dT} \right) V \Delta H_{app} \quad (2.8)$$

$$\text{Reaction rate} = \left[\frac{1}{V \Delta H_{app}} \right] \frac{dQ}{dT} \quad (2.9)$$

2.1.2 Instrumentation of isothermal titration calorimeter

Isothermal titration calorimeter consists of reference cell, sample cell and an adiabatic jacket of surrounding of reference and sample cells to keep the temperature of cells at the same temperature. Reference cell contains buffer or water and keeps at experimental temperature. Syringe is positioned above the sample cell properly so that one of the interaction solutions into syringe can be titrated into sample cell where another interaction solution is included and reaction takes place in the sample cell. Components of isothermal titration calorimeter (ITC) and the raw data output from an ITC experiment are shown in Figure 2.1.

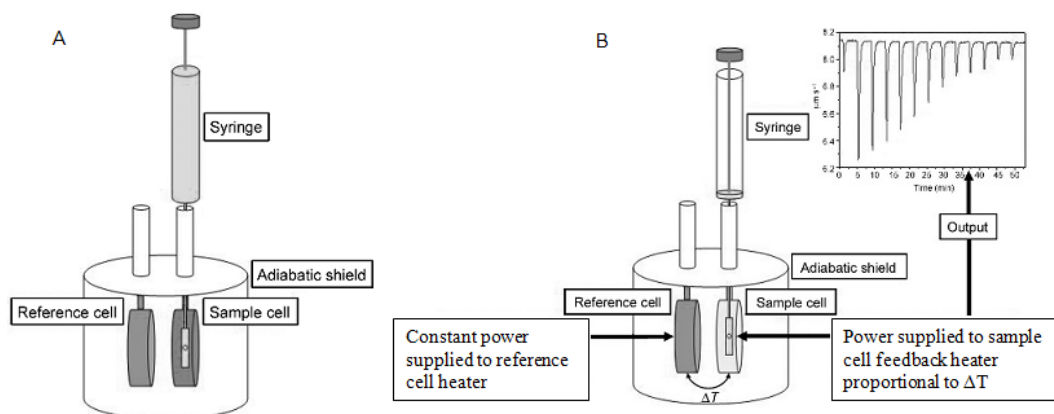


Figure 2.1: Schematic diagram of isothermal titration calorimetry. (A) Before starting of titration, (B) Completing of titration (Thomson and Ladbury, 2004).

ITC is worked in power compensation (isothermal) mode in order that temperature, which increases or decreases because of the reaction in the sample cell during titration, is kept constant between sample and reference cell. If the reaction in the sample cell is endothermic, sample cell needs the energy to maintain the temperature

identical with reference cell. This power is presented against to time. As it is illustrated in the Figure 2.2, the reaction is exothermic in the sample cell and the raw heat data ($\mu\text{cal/s}$) is given as a plot of power against time (minute). Each peak indicates the injection of titrant into sample cell and titrant concentration into sample cell increases until saturation of component in the sample cell with component in the syringe (titrant) occurs. Then, heat of dilution is measured that this heat is observed because the component in the syringe is diluted when it is titrated into sample cell, any binding interactions between these two components is not monitored in this stage. Therefore, control experiment should be performed by injection of titrant into buffer solution in the sample cell of calorimeter. In the lower part of Figure 2.2, integration of each injection as an integrated raw data output (kcal/mole of injectant) is plotted against molar ratio of components.

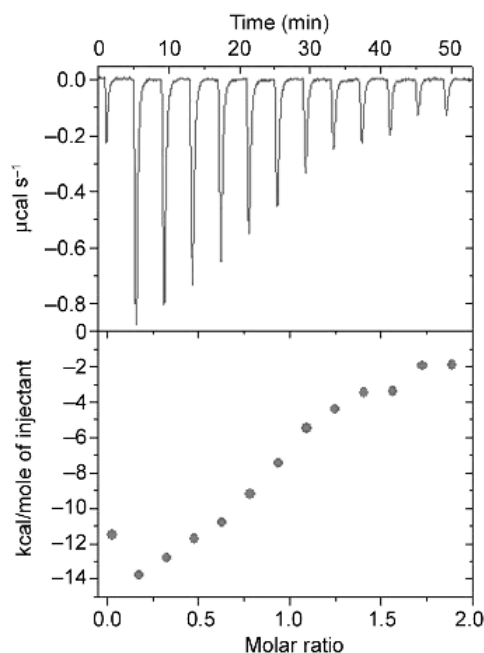


Figure 2.2: Raw data and integrated raw data output from ITC experiment (Thomson and Ladbury, 2004).

Optimum conditions should be chosen in order to obtain correct values of K_B , n , ΔH , ΔG , and ΔS from ITC experiment. Heat change can be measured for each injection and for following injections which develop a curved thermogram, a plot of heat change against injection number or $[\text{ligand}] / [\text{macromolecule}]$, under these conditions. The curved thermogram depends on macromolecule concentration and equilibrium binding constant, K_B . If interaction is strong which means K_B value is larger than 10^8 M^{-1} , low macromolecule concentration is enough to produce proper

curvature in the thermogram. However, concentration should be high enough that can be measured. On the contrary, if interaction between ligand and macromolecule is weak, $K_B < 10^4 \text{ M}^{-1}$, high macromolecule concentration is needed for required thermogram but heats should not become too high. When ITC experiment is performed, there are several steps to achieve appropriate results. These steps are (i) planning the experiment, (ii) preparing solutions for experiment, (iii) collecting the raw ITC data, (iv) doing the control experiment, (v) correcting the raw ITC data, (vi) nonlinear regression to determine thermodynamic parameters, (vii) interpretation of data (Freyer and Lewis, 2008).

After the appropriate concentrations of titrant and titrate solutions are identified, it should be regarded that these solutions must be prepared in the same buffer, salt concentrations and pH to ensure heat of dilution signals are lower than binding signals. Moreover, the number and volume of injections should be determined. Choosing correct buffer is important point to decrease heat of ionization and optimize the ITC experiments with respect to correct the raw ITC data. In addition, solutions should be degassed before they are loaded in the syringe and sample cell to prevent bubble formation which causes spurious heat signals (Freyer and Lewis, 2008).

Isothermal titration calorimetry provides the binding constant (K_B), the number of binding sites (n), enthalpy (ΔH), Gibbs free energy (ΔG) and entropy (ΔS) in a single experiment and experiment is conducted rapidly and easily. Also, chemical modification or immobilization is not needed as such in fluorescence-based assays or other spectrophotometric assays. Turbid, colored solutions or suspensions can be used while investigating of interactions between these components (Ladbury, 2004). These properties can be regarded as advantages of using isothermal titration calorimetry method to study biomolecular interactions.

2.1.3 Applications of isothermal titration calorimetry

ITC can be applied in various research areas, for example, polymer chemistry, biochemistry, cell biology and nanotechnology. Ghai et al. has reported that more than 500 articles, in which ITC was used, were identified in 2010 (2012). In literature, the using of ITC to study biological and bio-molecular recognition interactions, ligand binding, protein-receptor, lipid-lipid, and lipid-small molecular

interactions, enzyme activity, drug discovery is available. In particular, the binding interactions of guest molecule with cyclodextrin can be given as a well-known example of ITC. Also, interaction of micellar systems such as drug-surfactant, polymer-surfactant, and protein-surfactant have been studied widely relating to development of pharmaceutical formulations. Investigation of polyelectrolyte complexes and DNA interactions are also other study subject of ITC. Finally, nanoparticles can be characterized by ITC to create efficient nanoparticle formulations and study interactions with living systems (Bouchemal, 2008). In addition to diverse applications of ITC such as in interactions of proteins, proteins-membranes, in studies of enzyme kinetics, and designing of drugs, ITC can be used in food science. Tannins which are heterogenous mixture of polyphenols can bind to proteins and these tannin-protein interactions as non-sepecific interactions can be studied by ITC (Ghai et al., 2012). Also, ITC can be used while investigating the interactions of (-)epigallocatechin gallate with lipid bilayers (Sun et al., 2009) and the interactions between catechin as a green tea flavanoid and β -casein as milk protein (Yuksel et al., 2010), also binding of bile salts to dietary fibers and chitosan (McClements and Thongngam, 2005). Moreover, sensory science as a section of food science can utilize ITC by means of searching the thermodynamics of interactions of sweeteners, and also inhibitor with syththetic receptor model to correlate the data from sensory evaluation (Chen et al., 2011).

Finally, ITC can be utilized in order to investigate lipid membrane-surfactant interactions, for examples (i) to measure the value of critical micelle concentration (CMC) of surfactant, (ii) to determine partition equilibrium between lipid membrane and monomeric surfactant, (iii) to study membrane solubilization with high concentration of surfactant ($>CMC$) and to form membrane-surfactant phase diagrams, (iv) to study membrane formation upon dilution of mixed lipid-surfactant micelle (Heerklotz, 2000).

All in all, ITC can be used to understand the mechanism of binding as a result of providing binding activity, enthalpy, and entropy values of binding interactions between two components. Furthermore, ITC is a useful method for drug discovery studies. ITC method not only provides a general approach to determine molecular interactions giving thermodynamic parameters of interaction but also to probe the catalytic activity of enzymes and to understand kinetic behaviors of enzymes.

2.2 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) is a calorimetric method to measure heat capacity (C_p) of system as a function of temperature. DSC can be used to study thermal transitions and conformational changes of biological systems. Heating causes the change of thermodynamic state that can be accompanied by conformational change.

DSC gives three types of information that can be sequenced as (i) absolute partial heat capacity of a molecule, (ii) entire thermodynamic parameters such as enthalpy change (ΔH), entropy change (ΔS), heat capacity change (ΔC_p) regarding to heat induced transition, and (iii) partition function and the population of intermediate states, also their thermodynamic parameters (Freire, 1995).

2.2.1 Principle and instrumentation of differential scanning calorimeter

Differential scanning calorimeter consists of two cells that one of them is reference cell filled with buffer and another one is sample cell. Reference cell and sample cell are surrounded by adiabatic jacket. DSC works in differential mode to measure the difference in heat capacities of sample solution and buffer. (Power compensation type DSC measures electrical power difference between sample and a reference cell) Since temperature increases during measurement, additional electrical power ($\mu\text{cal/s}$) is required to keep the temperature constant between two cells. Additional electrical power is direct indicator of thermal transition of solution in sample cell. Transition is detected as a sharp peak (Figure 2.3).

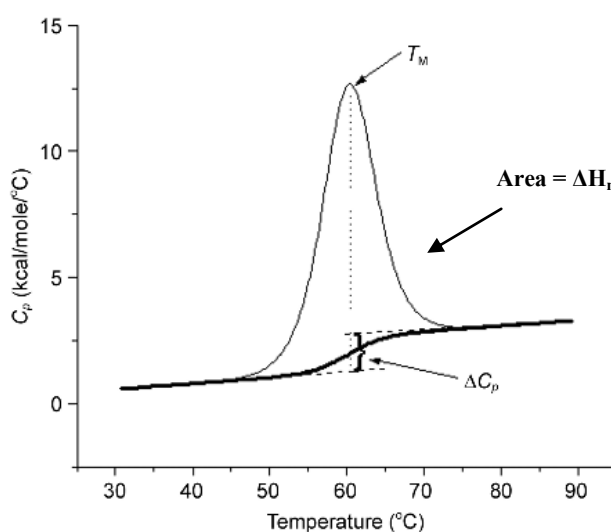


Figure 2.3: Typical thermal transition curve from DSC.

Furthermore, typical DSC experiment provides overall thermodynamic parameters. T_m , which is defined as transition temperature (transition midpoint), is the center temperature of peak and heat capacity (C_p) is at maximum at this temperature. Calorimetric transition enthalpy (ΔH°_m) is obtained by integration of curve that is plotted excess heat capacity, C_p ($\text{kcal mol}^{-1} \text{ }^\circ\text{C}^{-1}$) against temperature ($^\circ\text{C}$). Difference in the baseline gives a measure of heat capacity change (ΔC_p) that is associated with transition (Figure 2.3).

$$\int C_p dT = \Delta H^\circ \quad (2.22)$$

The excess heat capacity (ΔC_p) is composed of two factors that first component is intrinsic excess heat capacity which is caused by sum of populated molecular species during transition between states and second component, which is referred as transition excess heat capacity, occurs because transition causes increase the heat capacity of system. δC_p^{int} is lower than δC_p^{trans} . If it is not much smaller than δC_p^{trans} , it can cause an error on evaluation of $\Delta H_{vh}/ \Delta H^\circ_m$ ratio and two-state process (Jelesarov and Bosshard, 1999).

$$\Delta C_p = \delta C_p^{int} + \delta C_p^{trans} \quad (2.23)$$

When data is converted into C_p/T and then plotted against temperature, entropy change, ΔS can be determined by calculating the area under the plot. Also, ΔH and ΔS provide to calculate ΔG at any temperature.

$$\Delta S = \int \frac{C_{p,xs}}{T} dT \quad (2.24)$$

$$\Delta G = \Delta H - T\Delta S \quad (2.25)$$

In addition, calorimetric enthalpy which is obtained from DSC can be compared with van't Hoff enthalpy (ΔH_{vh}). If ΔH_{vh} is equal to calorimetric enthalpy (ΔH°_m), it means that two-state transition progresses and investigating of temperature-dependence of equilibrium provides revealing thermodynamic data. Also, the ratio of van't Hoff enthalpy to calorimetric enthalpy gives quantitative information to understand nature of transition (O'Brien and Haq, 2004). Van't Hoff enthalpy is model-dependent whereas calorimetric enthalpy is independent from model and ΔH_{vh} postulates that transition occurs in two-state process. If $\Delta H_{vh}/ \Delta H^\circ_m$ is higher than 1, it means that intermediate states dominate. A ratio of $\Delta H_{vh}/ \Delta H^\circ_m < 1$ indicates irreversible transition (Jelesarov and Bosshard, 1999).

However, the ratio of $\Delta H_{vh}/\Delta H_m^\circ$ should be interpreted carefully to prevent misunderstanding while determining the transition state. Experimental uncertainties such as existence of intermediate state of protein folding/unfolding transitions can cause misinterpretation (Freire, 1995).

2.2.2 Deconvolution of DSC data/ Statistical-mechanical approach

Deconvolution analysis is applied in order to elucidate number of states populated during thermal unfolding and thermodynamic parameters for each states. According to Freire, most effective deconvolution algorithms include multiple cycling through each individual transition step combined with nonlinear least squares optimization and conclude global nonlinear least square optimization. Deconvolution analysis should include several steps that are (i) calculation of excess heat capacity, ΔC_p that all molecules should be found in native state at the initial temperature, (ii) ΔC_p should be fitted by using nonlinear least squares method in terms of equation of $\Delta C_p = \sum_{i=1}^N \Delta H_i \frac{\partial P_i}{\partial T} + \sum_{i=1}^N P_i \Delta C_{p,i} = \Delta C_{p,tran} + \Delta C_{p,bl(p,int)}$ where P_i refers the population (probability) of state, i , ΔH_i gives the enthalpy of the i^{th} state relative to native state and ΔC_p is temperature derivative of ΔH . Sum of squared residuals (SSR) between calculated and experimental values should minimize when best set of parameters applied. Then, quality of fit should be evaluated in terms of standard deviation of fit and residual analysis (iii) estimation of initial parameter values, (iv) variational analysis of baseline estimate to select best baseline parameter set combinations, (v) error estimation of fitting parameters, but these errors are because of optimization procedure that concentration uncertainties and baseline subtraction are not included (Freire, 1995).

Researchers have formulated statistical-mechanical aspects of deconvolution analysis with the purpose of redefining the partition function of system to clarify interaction between molecules and to use standard expression of statistical thermodynamics, $\frac{\partial \ln Q}{\partial T} = \frac{\Delta H}{RT^2}$. If separate DSC scans provide unfolding enthalpies, unfolding heat capacity change and unfolding free energies of each components of complex, statistical analysis become easy. Also, global statistical analysis without priori information of thermodynamic parameters can be performed, but this kind of statistical analysis can cause error due to exist large number of parameters and

become interdependent of some parameters, also can be called multiple minima problem (Jelesarov and Bosshard, 1999).

Although DSC is useful method to determine all thermodynamic parameters, protein stability studies may be need high concentration of proteins such as at least 1mg/ml and aggregation of denatured protein and self-association of native protein can be observed if concentrations are not selected properly for DSC studies (Bruylants, et al., 2005).

Reversibility of transition should be checked by doing experiment with at least two scans of sample. The curve should be identical in two scans except direction of peaks (being endothermic). It is important to evaluate the equilibrium thermodynamics that they cannot be applied to irreversible systems. Buffer scan should be performed in addition to these two sample scans (Freire, 1995).

T_m can be utilized to compare relative stabilities of reversible systems under thermodynamic control. Increase of T_m indicates remaining of stability of system, but opposite situation is indicator of destabilization of system (O'Brien and Haq, 2004).

2.2.3 Applications of differential scanning calorimetry

DSC is an indirect method to determine binding affinities, but it can be used for ultratight binding as a major advantage of this technique. Binding constant (K_m) at T_m can be calculated for reversible two-state transition. It assumes that binding to native form of protein and binding sites on native protein is totally saturated. Increase in T_m demonstrates the binding of ligand to protein in native state. ΔH_B° , ΔS_B° , and ΔG_B° are obtained by DSC for ligand-protein binding. It is not easy to correlate between changing in T_m and binding affinity because of interaction with denatured state of protein (Bruylants, et al., 2005). Protein-protein, protein-ligand, protein/drug- DNA interactions can be investigated by DSC. Also, protein/drug-lipid, DNA-lipid and detergent-lipid interactions in micellar systems as non-specific bindings can be identified/detected by DSC (O'Brien and Haq, 2004; Bruylants, et al., 2005).

Moreover, DSC can be considered as a significant technique to analyze stability of membranes and lipids (Bruylants et al., 2005). Lipid model or biological membrane can occur in different phases because of temperature-induced transition (interconversion). DSC is rapid, straightforward, and nonperturbing physical

technique for thermodynamic characterization of thermotropic phase transitions of membranes (Lewis et al., 2007). DSC can present detailed information about physical and energetic properties of drug substances so that it is recommended to use in the earliest stage of drug development. Also, United States Pharmacopeia explains how DSC analysis should be done to determine the transition temperatures and sample purity (Clas et al., 1999). It can be used to investigate biomolecular interactions as well as isothermal titration calorimetry for drug design although it can be much applied to study stability of biological macromolecules, protein stability, lipid phase transitions, nucleic acid ‘melts’, surfactant micellization and stability of liquid biopharmaceuticals (O’Brien and Haq, 2004; Bruylants et al., 2005).

2.3 Combined Application of ITC and DSC

As it is shown in Figure 2.4, DSC can be used in company with ITC and other methods, which provide structural information of complex, so that overall thermodynamic profile of folding (association) can be determined. In particular, combination of ITC and DSC is beneficial while analyzing complex systems such as protein-DNA association.

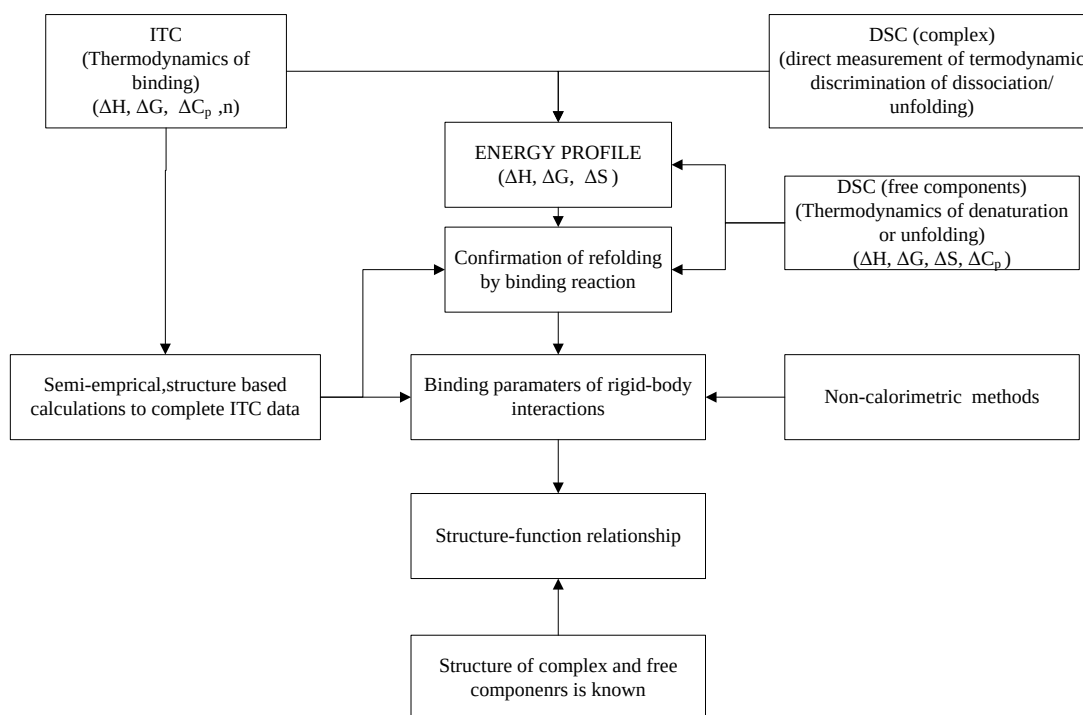


Figure 2.4: Schema of combination of ITC and DSC to obtain energy profile (Jelesarov and Bosshard, 1999).

The thermodynamic profile of interactions can be obtained by calorimetric methods such as ITC and DSC are supplementary data of structural data that the integration of thermodynamic and structural data guide the drug development while optimizing binding to target molecules (Chaires, 2008). Ghai et al. state that combination of molecular dynamic simulations of binding interactions and experimental analysis of interactions such as by ITC and SPR can be potential tool to correlate the interaction data (2011).

To sum up, experimental data which are yielded by calorimetric techniques should be supported by other non-calorimetric methods like biochemical, structural and biophysical methods in order to confirm the results. Understanding of fundamentals of calorimetric techniques will lead to develop efficient drugs as an application. Therefore, determining of thermodynamic characteristics is important in order to provide insight into stability of macromolecules (such as biomolecular association) and interactions between macromolecules (biomolecules) and ligands.

2.4 Dynamic Light Scattering (DLS)

Dynamic light scattering, which is also known as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS), is a method that measures the average size and size distribution of particles in a suspension.

The part of light is scattered when it passes through the solution which contains particles, based on α ,

$$\alpha = \frac{\pi D}{\lambda} \quad (2.26)$$

α is a dimensionless size parameter that is equal the ratio of πD to λ . πD is circumference of particle and λ is a wavelength of incident radiation. Also, α describes the type of scattering, for example, if $\alpha \ll 1$, it is named as Rayleigh scattering, $\alpha \sim 1$: Mie scattering, and $\alpha \gg 1$: geometric scattering (Brar and Verma, 2011).

Light scattering techniques can be classified as a static light scattering and dynamic light scattering. Static light scattering, which is also referred as Rayleigh or Multi-angle light scattering, measures the particle size directly and molecular mass. Therefore, it can be used to determine state of particles and to measure masses. Also,

measuring of stoichiometry of complexes is possible. Moreover, information about particle structure can be obtained by SLS. It can be combined with DLS or FFF (Field-Flow Fractionation) to determine particle shape. Average molecular weight is found by Zimm Equation. Dynamic Light Scattering measures the diffusion rate of the particles that provides the size of particles based on Stokes radius or hydrodynamic radius of the particle (Brar and Verma, 2011).

2.4.1 Principle of dynamic light scattering

The principle of DLS technique is scattering of light by particle diffusion. Because of diffusion, particles move and cause wavelets which yield changed electric field at the detector. While proceeding of brownian motion in the sample solution, the electric field and intensity at detector fluctuate. It means that DLS measures time-dependent fluctuations in scattering intensity because of Brownian motion (constant random motion) of particles in suspension. Therefore, electromagnetic radiation (wave) can absorb or emit energy and momentum. Energy difference between incident and scattered photons can be neglected because brownian motion causes the small frequency change whereas the change in momentum has an importance in dynamic light scattering (Hallett, 1994).

Magnitude of scattering wave vector (Q) is

$$|Q| = \frac{4\pi n}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad (2.27)$$

According to equation, if light is scattered at small angle (θ), it gives information about gross dynamic and structural properties of scatters, but while light is scattered at high angle provides this information at a finer scale. Also, when DLS is compared with other scattering techniques, in terms of particle sizing ranges, it is seen that DLS has a greater scale than others.

Fluctuating signal is a primary problem in data recovery in DLS to get quantitative information. Small particles produce fast fluctuations whereas large particles and aggregates cause slower fluctuations. Autocorrelation analysis can be performed to determine the rate of fluctuations.

$$g^{(2)}(\tau) = \frac{C_1(\tau)}{\langle I(\tau) \rangle^2} = 1 + |g^{(1)}(\tau)|^2 \quad (2.28)$$

$C_1(\tau)$ is intensity autocorrelation function at τ , instrumental delay time.

The theory has two assumptions that the first is assumed that particles are moving in Brownian motion in solution and second assumption is that particles are spherical.

$$g^{(1)}(\tau) = e^{-\tau D Q^2} \quad (2.29)$$

In equation, D is a diffusion coefficient. This equation is valid only in the presence of small monodispersed particles. Because solution can contain a size distribution of scatters, equation turns into following:

$$g^{(1)}(\tau) = a \sum_{i=1}^m \omega_i e^{-\tau D_i Q^2} \quad (2.30)$$

for continuous distribution of particle sizes, equation becomes:

$$g^{(1)}(\tau) = a \int_0^\infty G(\Gamma) e^{-\Gamma \tau} d\Gamma \quad (2.31)$$

where Γ is equal DQ^2 . Equation gives the distribution of relaxation time, $G(\Gamma)$, thanks to complete Laplace inversion, thereby the distribution of particle size by using Stoke-Einstein relation (Hallett, 1994).

Stoke-Einstein equation is,

$$D = \frac{k_B T}{6\pi\eta a} \quad (2.32)$$

$$D = \frac{\mu_q k_B T}{q} \text{ (for charged particles)} \quad (2.33)$$

The drawback of DLS method is concentration of suspension. The concentration should not be high to obtain single-scattering (High concentration of solutions cause multiple scattering) (Hallett, 1994). Also, scattered light should be sufficient to provide statistical analysis of correlation function (Nobmann et al., 2007).

2.4.2 Instrumentation of dynamic light scattering

Dynamic light scattering instrument basically consist of the light source, the focusing lens to converge the light a waist of sample cell, the detector to detect the scattered wavelets from sample which is positioned 90° to light source classically, and the correlator to convert the fluctuations of scattered light into electrical signal (Figure 2.5).

The fluctuations of scattering intensity is detected by photon detector. After statistics of scattering signal are analyzed by a correlator, the correlation function is turned into size distribution of particles in solution (Nobmann et al., 2007).

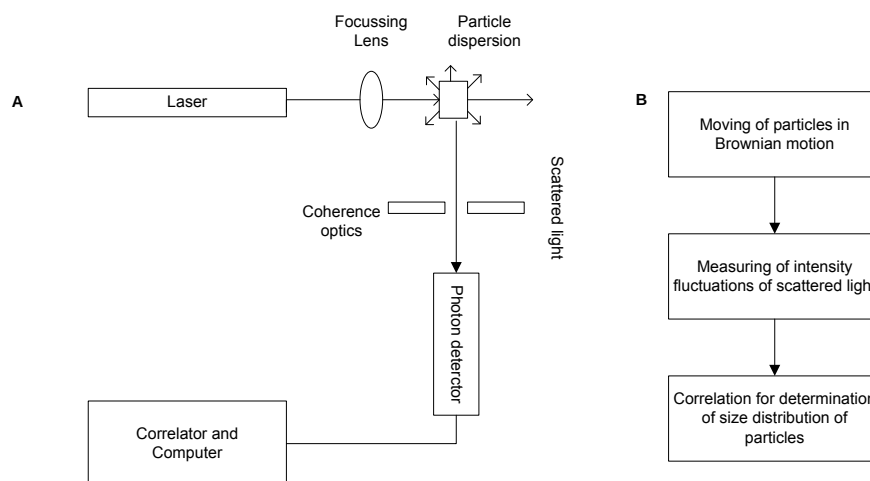


Figure 2.5: Schematic diagram of dynamic light scattering instrument.

Light-scattering techniques are widely-used for determination of nanoparticles. Estimation of particle size and particle-size distribution is important for characterization of nanoparticles and their applications. Dynamic light scattering is the one of the several techniques, for example, electron microscopy, atomic force microscopy, Fourier-transform infrared spectroscopy, X-ray photoelectron spectroscopy, powder X-ray diffraction, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy, nuclear magnetic resonance which are used to characterize particles (Brar and Verma, 2011).

DLS provides advantages that are (i) samples including broad distribution of particles can be analyzed; (ii) very small amounts of higher mass species can be detected; (iii) analysis does not involve chromatographic separation or dilution that aggregates are not affected (they are not lost).

On the other hand, there are limitations of using DLS, for instance, (i) the size of the particles cannot be estimated accurately because it is assumed that particles are spherical, (ii) heterogenous size distribution can induce misevaluation of size of the particles, and (iii) the quantification of amount of aggregats may be difficult because interference can affect scattering intensity, and in turn the size of particles (Brar and Verma, 2011).

2.4.3 Applications of dynamic light scattering

DLS can be regarded as a convenient technique for food applications. In recent years, increase of using nanomaterials as food ingredients and packaging material makes the detection and characterization of these particles important. Generally,

microscopic methods have been used with this purpose. According to Brar and Verma, isotopic labeling techniques should be designed. Particle size and distribution, surface area, surface charge and topography, composition and purity, hydrophobicity and solubility, chemical reactivity and bioactivity, and dispersion and aggregation state are important parameters when nanoparticles/particles are evaluated as a safe or not in food product to be sure that they do not induce toxicity. Huge variety of nanoparticles utilized in food industry, their possible interactions with food components (proteins, lipids, carbohydrates, and flavors) make difficulties for measurements of these particles. When studying particles in food, the medium should be identical with real system of food that makes measurement complicated (2011). Therefore, backscattering may have advantage while investigating concentrated high-molecular weight samples and diluted low-molecular weight samples (Brar and Verma, 2011; Hallett, 1994).

2.5 Fluorescence Spectroscopy

2.5.1 Principle of fluorescence spectroscopy

Photons, which are absorbed to the electronically excited-state, can cause emission of radiation (light) while returning to the ground-state. This process can be named radiative decay process, whereas nonradiative decay means that excess of energy is transferred into vibration, rotation, and translation of surrounding molecules. Emission of light is defined as a term of luminescence and is divided into two categories that are fluorescence and phosphorescence in terms of nature of excited-state. Fluorescence is emission of light from singlet-excited state so that the emissions are allowed and the emission rates are generally 10^8 s^{-1} . The emission of photon in fluorescence occurs rapidly as compared with phosphorescence. The emission of photon is forbidden and the rate of emission is $10^3\text{-}10^0 \text{ s}^{-1}$ because electron in the excited orbital is found in triplet state.

Fluorescent substances are named as fluorophores that occur from aromatic molecules such as quinine, fluorescein, rhodamine B, and pyridine (Lakowicz, 2006). Intensity is an emission of a population of n excited fluorophores ($I = nE$). Emission life-time is in the range from picosecond to nanosecond. Fluorophore molecules have some characteristics that are intensity, position of emission wavelength and life-time and each of fluorophore has its own properties. Intrinsic fluorophores are aromatic

amino acids and cofactors whereas extrinsic fluorophores can be linked to macromolecules (peptides, proteins, membranes, or DNA) covalently or not. In addition to these, structure of fluorophore and surrounding affect fluorescence properties of fluorophores (Albani, 2007). Fluorescent molecules can be used to study with physicochemical, biochemical and biological systems. The one of the usage area of fluorescent molecules is vesicle systems in order to investigate the characterization of bilayer in terms of microviscosity, order parameters; phase transitions; effect of additives; permeability and internal pH studies (Valeur, 2002).

Jablonski diagrams demonstrate process between absorption and emission of light and they are used to explain several molecular process which occur in excited-states. As it is shown in Figure 2.6, electrons pass to singlet excited state, S_n ($n>1$), from singlet ground state, S_0 , because of absorption of photons (light), and then different transitions can occur while returning of excited molecule to the ground state, S_0 . The one of these transitions is internal conversion that the molecule at excited-state S_n returns lowest excited state, S_1 . The molecule at excited-state S_1 can pass the ground-state, S_0 through different competitive process, for example, (i) emission of photon with a radiative rate constant, k_r , which is named as fluorescence, (ii) dissipation of part of absorbed energy as heat with a rate constant, k_i , (iii) transfer of some of energy to near molecules with constant rate, k_q , which is collisional quenching, or with a constant rate, k_t , (iv) inter-system crossing is that transition occurs to triplet-excited state, T_1 which is lower than the excited-state, S_1 with a rate constant, k_{isc} . Also, excess energy of molecule at triplet excited-state, T_1 , can transfer in a similar way such as (i) emission of photon with a rate constant, k_p , which is called phosphorescence, (ii) dissipation of nonradiative energy with a rate constant, k_i , (iii) transfer of energy to molecules located nearby with a constant rate, k_q' (by collision) and k_t' (energy transfer at distance) (Albani, 2007).

Stokes shift explains that energy of emissions are less than the energy of absorption so that fluorescence occur at lower energies (or longer wavelengths). Generally, rapid decay to lowest vibrational level of S_1 induces Stokes shift. Further loss of excitation energy by thermalization is the another reason of Stokes shift. Moreover, solvent effects, excited-state reactions, complex formation and/or energy transfer cause Stokes shift (Lakowicz, 2006). Fluorescence quantum yield is a ratio of number of emitted photons to number of absorbed photons.

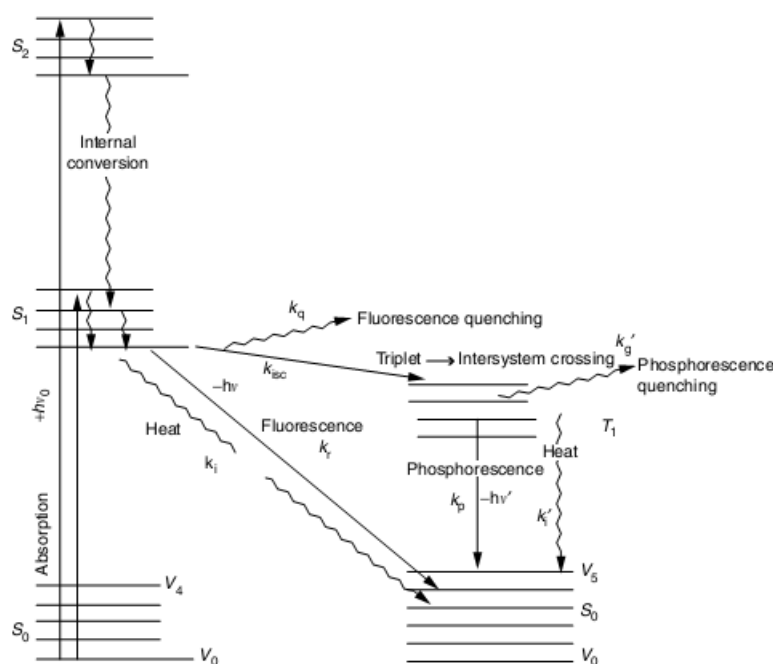


Figure 2.6: Molecular electronic transitions diagram- Jablonski Diagram (Albani, 2007).

Fluorophores which have highest quantum yield such as rhodamines give the brightest emissions (Lakowicz, 2006).

$$k = k_r + k_{isc} + k_i \quad (2.34)$$

k is the sum of kinetic constants of different transitions occurring from excited-state.

$$\Phi_F = \frac{k_r}{k_r + k_i + k_{isc}} \quad (2.35)$$

Fluorescence life-time gives information about availability of fluorophores in terms of time. The life-time of excited state is equal to mean time that molecule spends in excited-state before returning the ground-state. This time ranges from nanoseconds to picoseconds (Albani, 2007).

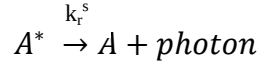
$$\tau_F = \frac{1}{k_r + k_{isc} + k_i} \quad (2.36)$$

k_r is a constant rate of transition because of radiative decay process so that radiative lifetime, τ_F provides radiative lifetime. Internal conversion and inter-system crossing affect the excited-state lifetime of fluorophore.

Fluorescence life-time can be measured by three methods that are Strobe, Time correlated single photon counting (TCSPC) which are time-domain measurements,

and multifrequency and cross-correlation spectroscopy which determines life-time in frequency domain. As it is mentioned previously, Jablonski diagram can illustrate the meaning of the fluorescence quantum yield and lifetime.

Fluorescence intensity is the amount of emitted photons per unit time and per unit volume of solution (mol/sL or einstein/sL).



$$i_F(t) = k_r^s[A^*] = k_r^s[A^*]_0 e^{-t/\tau_s} \quad (2.37)$$

According to eq. 2.37, intensity and quantum yield are proportional.

Fluorescence quenching is a one of the process that applied to decrease intensity of fluorescence. External molecules can quench fluorescence intensity, and quantum yield by two different mechanism that are classified as dynamic (collisional) quenching and static quenching.

When quencher binds to fluorophore molecules, nonfluorescent complexes can form in the ground-state that this process is referred as static quenching. Intensity and quantum yield decrease whereas fluorescence life-time does not change because there are still fluorescence molecules less than before quenching.

When quencher and fluorophore collide, fluorophore energy decreases because of collision. This process is called as collisional quenching or dynamic quenching. Oxygen, halogens, amines, and electron-deficient molecules like acrylamide are acted as collisional quencher. Fluorescence life-time of fluorophore in the excited-state reduces. Also, fluorescence intensity decreases depending on the Stern-Volmer relation.

$$\vartheta = k_q[Q][F] \quad (2.38)$$

$$\Phi_{F(Q)} = \frac{k_r}{k_r + k_i + k_{isc} + k_q[Q]} \quad (2.39)$$

$$\frac{\Phi_F}{\Phi_{F(Q)}} = 1 + k_q[Q] \frac{1}{k_r + k_i + k_{isc}} \quad (2.40)$$

$$\frac{I_0}{I} = 1 + k_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (2.41)$$

where Q is a concentration of quencher, k_q is a bimolecular quenching constant, τ_0 is a mean unquenched life-time of fluorophore, and K_{sv} is a Stern-Volmer quenching constant. K_{sv} shows sensitivity (accessibility) of fluorophore to the quencher. Lower

values of K_{sv} indicates that the fluorophore molecule is inaccessible to quencher and if values of K_{sv} are higher, it means that the fluorophore molecule can be found free in the solution or on the surface of biomolecule (Lakowicz, 2006).

Fluorescence anisotropy can be explained by principle that polarized light causes the photoselective excitation of fluorophores. Fluorophore molecules are oriented randomly in isotropic solutions, but fluorophores can be excited selectively by polarized light that absorption transition dipole of fluorophore molecules is parallel to electric vector of excitation. Partially oriented population of fluorophores (it can also be referred as photoselection) and partially polarized fluorescence emission occurs because of this selective excitation.

For vertically polarized exciting radiation, fluorescence anisotropy (r), and polarization (P) are defined:

$$r = \frac{I_v - I_H}{I_v + 2I_H} \quad (2.42)$$

$$P = \frac{I_v - I_H}{I_v + I_H} \quad (2.43)$$

Protein-protein associations, motional properties of membranes and immunoassays of substances can be determined by fluorescence anisotropy measurements. Fluorescence anisotropy provides information about size and shape of proteins or the rigidity of various molecules.

2.5.2 Instrumentation of fluorescence spectroscopy

In Figure 2.7, spectrofluorometer has been illustrated schematically. Fluorescence spectrometer consists of light source (Xenon lamp), the emission and excitation monochromators to select emission and excitation wavelength in which is desired to study, and concave-shaped gratings provide to decrease stray light, and detector (photomultiplier tube). Polarizers are required in order to measure the fluorescence anisotropy. Also, the sample chamber can be surrounded by the optical module, which include shutters in order to remove the exciting light or block the emission channel, and the beam splitter to reflect part of the excitation light to the reference cell. Optical filters are placed in order to eliminate undesired wavelengths in the excitation light or scattered light from the emission channel. (Lakowicz, 2006).

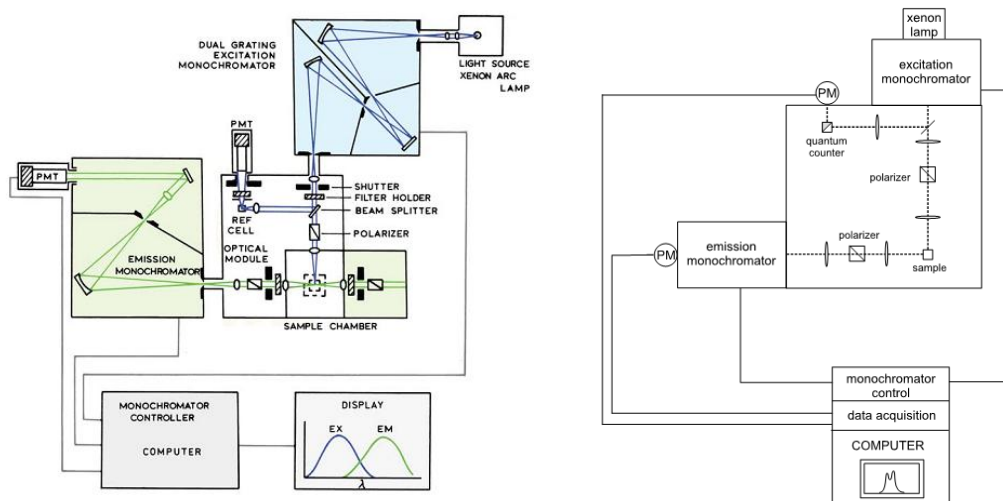


Figure 2.7: Schematic diagram of spectrofluorometer.

2.5.3 Applications of fluorescence spectroscopy

Fluorescence spectroscopy can be used while studying with physicochemical, biochemical and biological systems. Elucidating of molecular processes such as interactions between solvent molecules and fluorophores, rotational diffusion of biomolecules, distances between sites on biomolecules, conformational changes, and binding interactions can be provided by fluorescence spectroscopy (Lakowicz, 2006). Particularly, fluorescence spectroscopy can be used in order to investigate unfolding/refolding transitions of proteins. In some cases, fluorescence profile can indicate the general loss of protein structure and in other cases, information about stable intermediates in folding/unfolding transitions of proteins can be provided by fluorescence assays. Intensity of the emission, the average energy or wavelength of emission, and the polarization are fluorescence parameters which should be searched in order to characterize the protein structure. Time-resolved mode can be used to get more detailed and well-resolved information (Royer, 1995).

The characterization of membranes can be studied by fluorescence spectroscopy. Motional, structural, and organizational properties of membranes can be determined by different fluorescence techniques. The rate of motion of fatty acyl chain, the head-group of region of phospholipid, other lipid components and membrane proteins as motional properties of membranes can be studied by fluorescence anisotropy, which is utilized for measuring rotational diffusion and orientation, intensity and life-time measurements and fluorescence quenching that these processes can be used for determination of lateral diffusion. Structural properties of membranes are

orientational characteristics of lipid components. Organizational properties are classified as the distribution of lipids in the plane of the membrane (phase separation) and across the membrane bilayer, and the distance from the surface or depth in the bilayer. Intensity and life-time measurements can provide information on phase separations. Surface density and lipid-lipid/ protein-protein associations are studied by fluorescence quenching and fluorescence energy transfer techniques. Fluorescence anisotropy method can be used for determination of distance from surface. Surface properties which include surface charge and dielectric properties of membranes can be studied by measurement of intensity and life-time and solvent effects, respectively (Stubbs and Williams, 1992).

Fluorescence detection as an analytical method has several applications which are analysis of air and water pollutants, oils, food, drugs; monitoring of industrial processes and observation of species of clinical relevance (Valeur, 2002).

Surfactant binding to the model lipid membranes and transformation of lipid bilayers or vesicles to lipid-surfactant mixed micelles can be investigated by calorimetric (isothermal titration calorimetry and differential scanning calorimetry) and spectroscopic (fluorescence spectroscopy and dynamic light scattering) techniques. Therefore, it has been intended to use these four techniques while investigating the interactions between biological surfactants, bile acids, and lipid membranes. Lipid membrane partitioning and solubilization studies have performed by using these techniques as a second part of this project.

The thermodynamic profile of interactions of bile acids with model lipid membranes has been obtained by ITC. Also, the change in T_m has been monitored by DSC to provide information about the effect of bile acids on lipid membranes. DLS have been used for characterization of liposomes. Finally, in order to confirm the experimental results obtained from ITC, membrane solubilization has been performed by DLS and fluorescence spectroscopy.

3. MATERIALS AND METHODS

3.1 Materials

The bile acids, chenodeoxycholic acid and deoxycholic acid were purchased from Sigma-Aldrich (Denmark). Lipids were obtained from Avanti Polar Lipids (USA). Potassium hydrogen phosphate (K_2HPO_4), and potassium dihydrogen phosphate (KH_2PO_4) were used to prepare phosphate buffer solution in the certain concentration depending on the experiments. The pH of solutions were adjusted with NaOH and HCl solutions. Milli-Q water (Milli-Q[®], Merck Millipore, USA) was used for preparation of buffer solutions and samples. The substances were used without further purification. Chemical structures have been shown in the Figure 3.1 and some properties of materials have been presented in the Table 3.1.

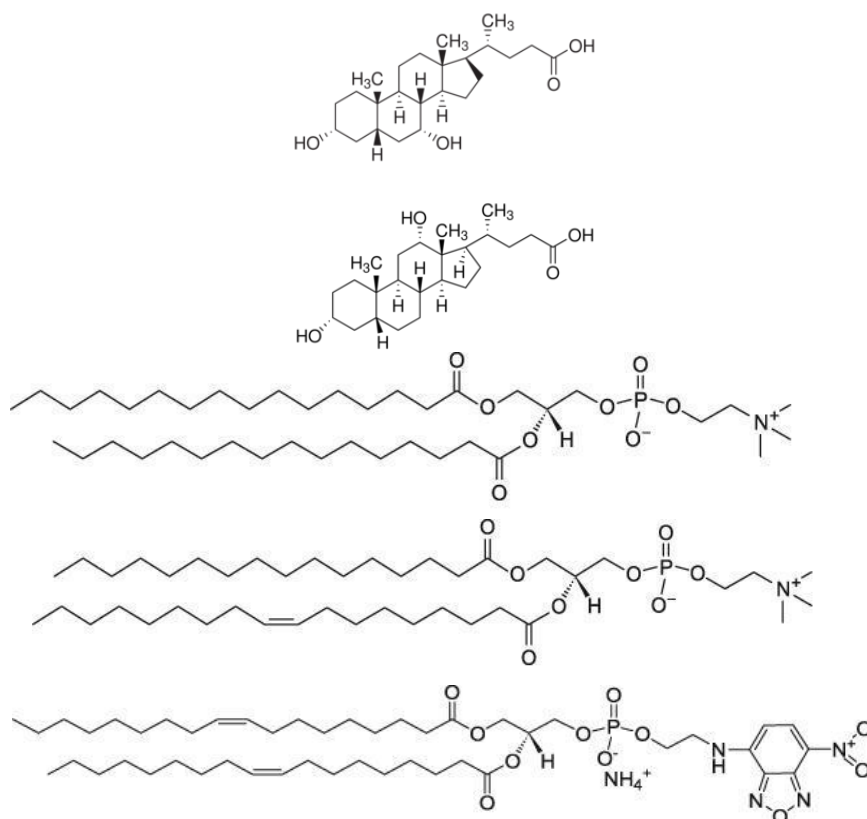


Figure 3.1: Chemical structures of Chenodeoxycholic acid, Deoxycholic acid, DPPC, POPC, and NBD-PE (from top to bottom).

Table 3.1: Properties of materials used in the experiments.

Molecules	Chemical name of molecules	Properties of molecules
DPPC (PC16:0/16:0)	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine	Mw:734.039g/mol Tm:41°C
POPC (PC 16:0/18:1)	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine	Mw: 760.076 g/mol Tm: -2°C
NBD-PE (PE 18:1)	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt)	Mw 924.155 g/mol Ex/Em: 460/535 nm
DCA	Deoxycholic acid	pKa: 6,8 CMC: 5mM solubility in water: 0.24 g/L

3.2 Methods

The general procedure of sample preparation is given in the Figure 3.2. The procedure of liposome preparation can be modified depending on the liposome composition and the properties of phospholipids which were used in the experiments. However, this methodology was followed to characterize the liposomes and to obtain results.

3.2.1 Preparation of liposomes

POPC and DPPC, unsaturated and saturated phospholipids (respectively) were used in order to prepare liposomes. When preparing the vesicles which consist of one type of lipids, phospholipid were dissolved in the buffer solution without lipid film formation. Then, phospholipid solution in the liquid-crystalline phase was stirred by magnetic stirrer that heating is required for saturated DPPC lipid whereas the POPC vesicles were hydrated and mixed at room temperature. Keeping above the transition temperature of lipids is important during hydration with buffer. After five cycles of freeze-thaw was completed, MLVs were obtained. In order to obtain homogenized LUVs, the phospholipid solution was extruded by Avanti Mini Extruder (Figure 3.3). 100 nm polycarbonate membrane and filter supports were used and at least 21 passes were applied to achieve proper particle size distribution of LUVs. While preparing lipid films, lipid were dissolved in CHCl₃-CH₃OH (9:1 v/v) mixture. Then, organic solvent was evaporated under N₂-flow (Reacti-Vap Evaporation unit, Thermoscientific) during 20 minutes to form a film, and then lipid films were kept under vacuum overnight. After rehydration of lipid films with buffer solution, same procedure was applied as explained above.

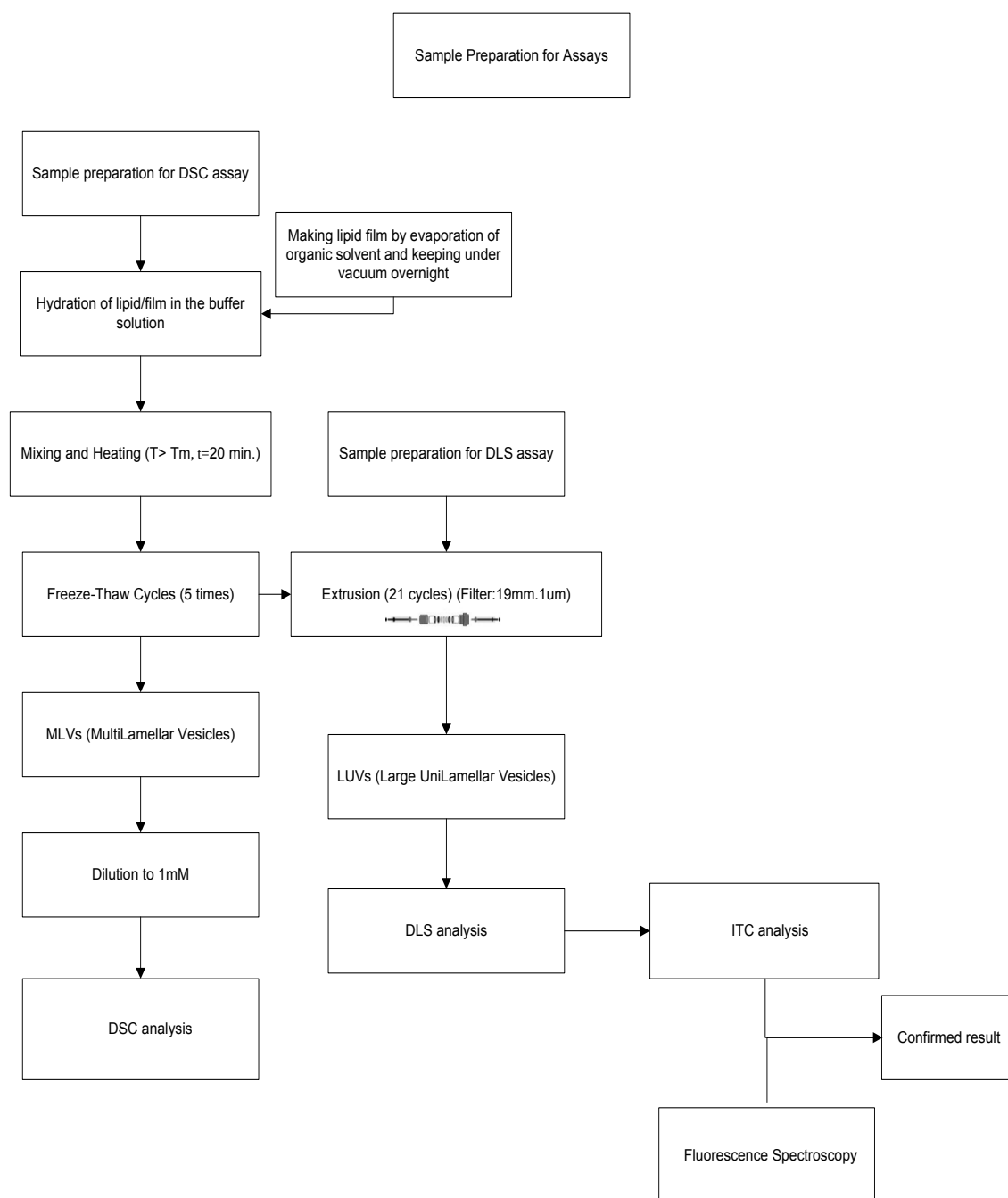


Figure 3.2 : General procedure for preparing of samples to analyze.



Figure 3.3 : Representation of using of Avanti Mini Extruder to make homogenized vesicles.

3.2.2 Differential scanning calorimetry

DSC measurements were performed with Nano DSC (TA Instrument, USA) (Figure 3.4). The scanning rate was 1°C/min. Temperature was increased from 20°C to 65°C during experiment. The results were given as a thermal transition curve, temperature (°C) against to heating rate ($\mu\text{J/s}$) or molar heat capacity (kJ/mol.K). More than one heating-cooling cycles were performed to obtain accurate results.

DPPC MLVs solutions were prepared as liposome concentration would be 1 mM at the final concentration, including 0,25 mM and 0,5 mM bile acid, and without bile acid. 10 mM HEPES buffer including 150 mM NaCl at pH 7,4 was used to hydrate the vesicles. The change in transition temperature of DPPC MLVs was observed between samples including bile acid at different concentrations and only DPPC MLVs solution.



Figure 3.4 : Nano DSC (TA Instrument, USA).

3.2.3 Isothermal titration calorimetry

ITC measurements were performed with Nano ITC-2G (TA Instruments, USA) as it is shown in the Figure 3.5. To characterize the lipid-small molecules partitioning, interactions between small molecules such as hormones, drugs, alcohols, and metabolites and biological membranes can be studied by using ITC (Chellani, 1999). Also, ITC is a convenient method to measure the critical micellar concentration of surfactants, to perform membrane solubilization at higher concentration of surfactants, and so to construct the phase diagrams of surfactant-lipid mixtures (Heerklotz and Seelig, 2000).

The volume of sample cell is approximately 1,0 mL, the mixing rate is 400 rpm, the syringe, 250 μL volume, was used in the experiments. Reference cell was filled with milli-Q water. The injection aliquotes, injection time and injection interval were chosen depending on the nature of experiments, membrane partitioning and solubilization, in order to get good baseline and curved thermogram as a result of

each injections. Triple measurements were performed to check the reproducibility and obtain accurate results.



Figure 3.5 : Nano ITC -2G (TA Instruments, USA).

3.2.3.1 Membrane partitioning assays

0,2 mM bile acid solution was prepared with 50 mM phosphate buffer at pH 8.0. DPPC and POPC liposomes were produced as describing in the section 3.2.1. Then, 50 mM DPPC LUVs and POPC LUVs solutions were titrated into 0,2 mM bile acid solution at constant temperature, 25°C. The process of partitioning of bile acids to model lipid membrane (liposome) have been demonstrated in the Figure 3.6. At the beginning, highly concentrated liposome solution was loaded in the syringe and very low concentrated bile acid monomer solution ($C_{\text{bile}} \ll \text{CMC}$) was filled in the sample cell. A series of small injections (5-10 μL) were performed. The peak of power was measured for each injection, and then these heat peaks are integrated to obtain heat response of system by ITC using power-compensation mechanism.

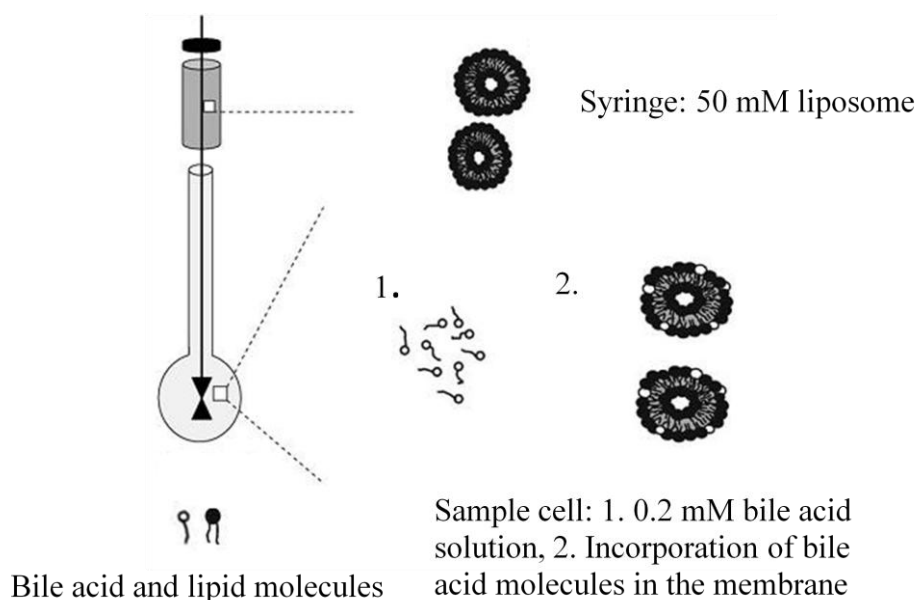


Figure 3.6: Illustration of membrane-partitioning experiments by ITC. There is a solution of phospholipid vesicles in the syringe and bile acid solution ($C_{\text{bile}} \ll \text{cmc}$) in the sample cell.

3.2.3.2 Membrane solubilization assays

Firstly, 50 mM bile acid solution was titrated into 50mM phosphate buffer solution, pH 8.0 at 25°C in order to determine critical micellar concentration (CMC) of bile acid as well as heat of dilution (ΔH_{dil}). The Figure 3.7 has illustrated the process of titration of highly concentrated bile acid solution into buffer solution. The value of CMC is important to perform membrane-solubilization experiments. The heat of dilution is also necessary information to calculate the accurate enthalpy of reaction provided by ITC.

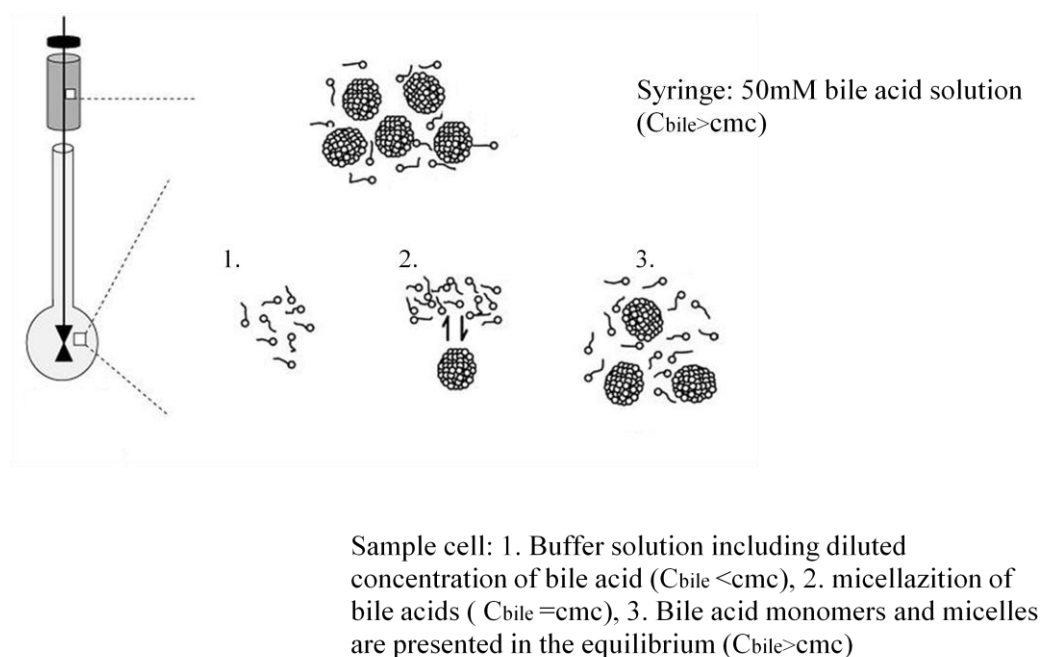


Figure 3.7 : Illustration of the measurement of cmc of bile acid solution (and also heat of dilution) by ITC. Bile acid micelles are filled in the syringe ($C_{bile} >> CMC$) and buffer solution in the sample cell.

POPC (0.5; 1; 2; 5; 10 mM) and DPPC (2; 5; 10 mM) were dissolved in 50 mM phosphate buffer, pH 8.0 and LUVs were prepared using standard liposome preparation procedure and lipid vesicles solutions were titrated with 50 mM bile acid (Deoxycholic acid) solution at 25°C and 55°C (55°C was applied only for experiments using DPPC). The critical detergent/lipid ratios were calculated for each system in order to indicate required concentrations to saturate and solubilize the DPPC and POPC lipid membranes. Also, vesicle to micelle transition diagrams were drawn as a graphic with lipid concentration against total bile acid concentration. To study the solubilization of lipid membranes by bile acid, POPC LUVs solutions

were prepared with phosphate buffer and water at pH 8.0 for both of solutions and ITC experiments were performed at 25°C, liquid-crystalline phase of lipids. Also, DPPC LUVs solutions were made with only phosphate buffer at pH 8.0, and the membrane solubilization studies were performed at 25°C and 55°C, in the gel phase and liquid-crystalline phase, respectively.

The stages of model lipid membrane solubilization has been shown in the Figure 3.8.

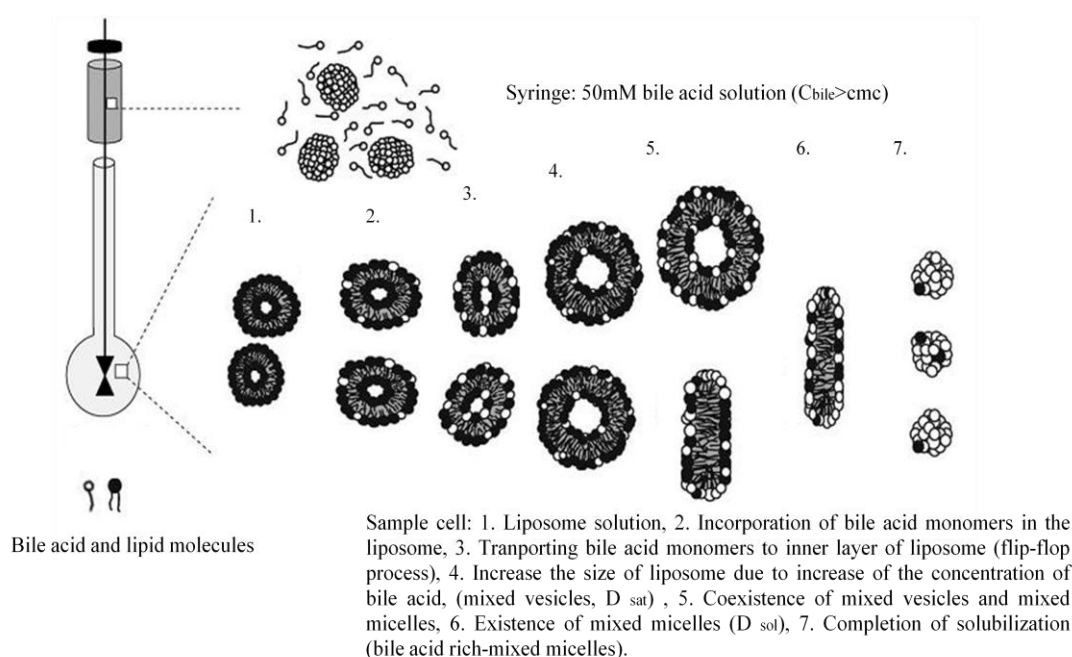


Figure 3.8 : Illustration of the solubilization of lipid membranes by bile acid. High concentrated bile acid solution is placed in the syringe ($C_{bile} \gg CMC$) and phospholipid vesicle solution in the sample cell.

3.2.4 Dynamic light scattering

The particle sizes of obtained LUVs after extrusion were measured by ZetaPals Zeta Potential Analyzer (Brookhaven Instruments Corporation, USA) (Figure 3.9). It enables to characterize the vesicles in the solution in terms of size and size distribution, and to continue the further experiments such as ITC and fluorescence spectroscopy assays. Temperature is 25 °C, scattering angle is 90° while performing dynamic light scattering measurements. The mean effective diameter (D_H) and polydispersity index (PDI) were recorded as a result of measurement. According to type of experiments, the results are given in terms of surface area or volume.

In addition, DLS measurements of phospholipid (POPC and DPPC) and bile acid systems were performed in order to confirm the solubilization results which had been obtained by isothermal titration calorimetry. The mixture of phospholipid and bile acid was prepared and the particle sizes were measured under the same conditions with calorimetric method.



Figure 3.9: ZetaPals Zeta Potential Analyzer (Brookhaven Instruments Corporation, USA).

3.2.5 Fluorescence spectroscopy

Fluorescence measurements were performed by FS920 steady-state fluorimeter (Edinburg Instruments, UK) (Figure 3.10). NBD-PE, fluorescent labeled phospholipid, was chosen to study for fluorescence quenching. Fluorescence intensity was measured with λ_{ex} at 458 nm λ_{em} at 516 nm because of maximum excitation and emission wavelengths of NBD-PE. Dithionite ($\text{S}_2\text{O}_4^{2-}$) was used to quench fluorescence intensity.

NBD labeled lipid vesicles were prepared, including 0.5 mol % NBD-PE in total lipid solutions. Lipid and bile acid solutions were mixed by the certain ratio of bile acid to lipid in accordance with the results of ITC assays. Then, 1M sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) was added as a quencher and the initial fluorescence intensity was measured and after that the signal became constant in a few minutes. After addition of dithionite, the decrease of fluorescence was observed and being stable of signal, 10 % Triton-X 100 solution was added to sample cuvette so as to disrupt the lipid membrane. The results of fluorescence intensity were given as graphic of sample/reference ratio against time.



Figure 3.10 : FS920 steady-state fluorimeter (Edinburg Instruments, UK).

4. RESULTS AND DISCUSSION

4.1 Determination of The Effect of DCA on T_m of DPPC by DSC

Transition temperature was determined by differential scanning calorimetry (DSC). Thermal transition curves of 1mM DPPC MLV and same concentration of liposome containing 0,25 mM and 0,5 mM deoxycholic acid, and also buffer solution were obtained in the temperature range from 20 to 65°C. The heating rate was 1°C/min. Main transition temperature (T_m) of saturated DPPC is 41,4°C which is higher than 37°C (Andrieux, 2009). In Figure 4.1, it can be observed that the main transition ($P_{\beta'} \rightarrow L_{\alpha}$) temperature of pure DPPC MLVs solution is 41,14°C and there is pre-transition ($L_{\beta'} \rightarrow P_{\beta'}$) temperature as a small peak before main transition at 35,6°C. Also, transition peaks have been shown as a result of cooling cycles in Figure 4.2. The transition temperature has shifted by addition of native surfactant, deoxycholic acid. Also, the shapes of peak have become smaller and broader by comparison with the peak of pure DPPC liposome. Furthermore, the peak of pre-transition has disappeared by adding deoxycholic acid to the lipid membrane gradually.

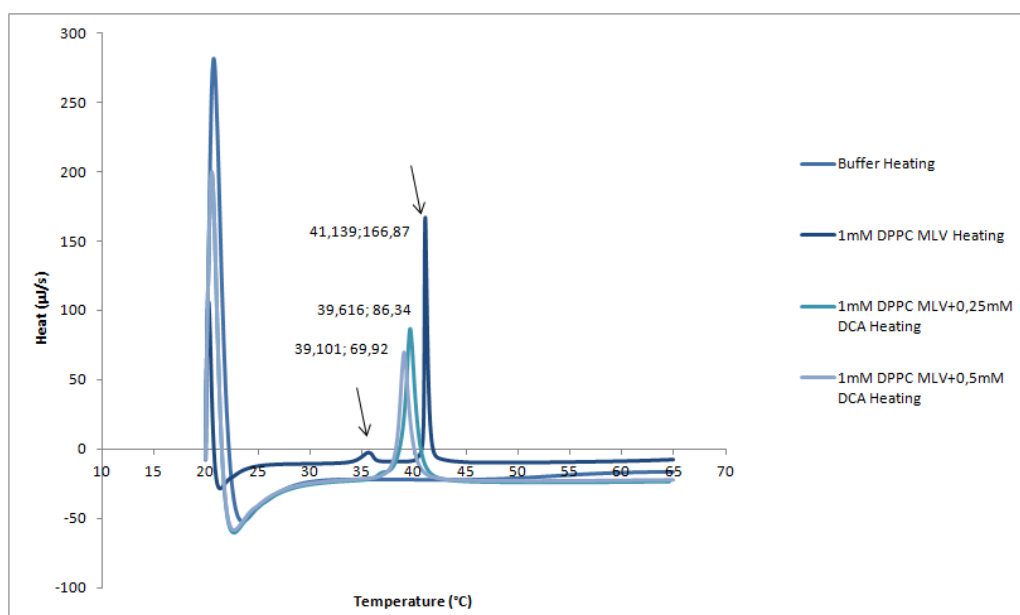


Figure 4.1: Transition curves of DPPC MLV solutions and containing bile acid from DSC as a result of heating.

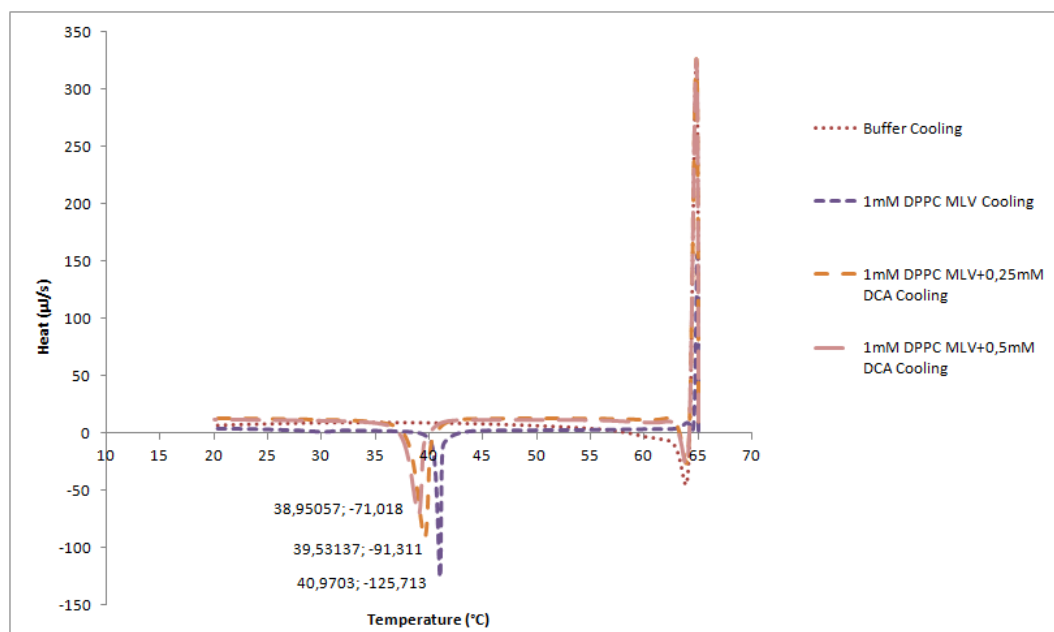


Figure 4.2: Transition curves of DPPC MLV solutions and containing bile acid from DSC as a result of cooling.

In addition, transition curves presented in the both of graphics indicate that released or absorbed heat accompanied to change in thermodynamic state of lipid has decreased by increasing the concentration of bile acid because the amount of lipid in that particular state has become less and less energy is needed to disorganize lipids. Surfactant molecules cause the change of lipid vesicle phases and this alteration occurs with change in transition temperature of phase.

As a result, the effect of bile salts on the saturated DPPC vesicles can be determined by DSC. When the heat is applied to DPPC vesicles, including different concentration of bile acid, the transformation of mixed vesicles in gel phase into mixed micelles occurs. Because DPPC exists in the gel-phase, insertion of bile acids into the lipid membrane becomes slowly and using isothermal titration calorimetre may not be practicable.

4.2 Determination of Thermodynamic Parameters of Interactions of Bile Acids, DCA and CDCA, with Membrane Lipids by ITC

Thermodynamic profile of interactions between bile acids and phospholipid membranes was obtained by isothermal titration calorimetry (ITC). Partitioning of bile acids into membranes and solubilization of lipid membranes by bile acids were investigated to determine the thermodynamic parameters such as partition

coefficient, change in enthalpy and entropy, and the Gibbs free energy, of interactions.

4.2.1 Membrane partitioning assays

50mM DPPC LUV and POPC LUV solutions were loaded into 250 μ L syringe and 10 μ L aliquots were injected into 0,2 mM ($C_{\text{bile acid}} \ll \text{CMC}$) bile acid solutions in the sample cell. The surfactant in the sample cell has become less available for incorporation into lipid vesicles while carrying on the titration. When the heat of interaction remains the constant, this heat corresponds to heat of dilution of lipid vesicles.

In Figure 4.3, calorimetric traces has indicated that partitioning of deoxycholic acid into DPPC membrane causes the exothermic heat. Also, the decline of concentration of deoxycholic acid to incorporate the membrane has led to decrease of enthalpy of reaction and become constant after certain lipid concentration in the sample cell. The mol ratio of deoxycholic acid to DPPC in the ITC sample cell has become approximately 0,033 when the reaction heat has started to be constant. The integration of reaction peaks provides the curve of heat changes of reaction versus injection number (Figure 4.3).

In Figure 4.4, calorimetric traces has indicated that partitioning of chenodeoxycholic acid into DPPC membrane causes the exothermic heat for the first three injections. The Chenodeoxycholic acid/DPPC mole ratio is $\sim 0,026$ when the reaction heat reaches the constant value.

Enthalpy values (J/mol) of incorporation of deoxycholic acid and chenodeoxycholic acid from aqueous phase into lipid membrane, consists of DPPC, are positive at 25°C (Table 4.1). Partitioning coefficient was calculated as $146816955 \pm 0,206$ for partitioning of chenodeoxycholic acid into DPPC lipid membrane and the partitioning coefficient of deoxycholic acid into DPPC lipid membrane was $3545520 \pm 0,354$. Chenodeoxycholic acid can be inserted into membrane stronger than insertion of deoxycholic acid because of structural difference between chenodeoxycholic and deoxycholic acid. The absence of hydroxyl group at C12 can provides higher affinity to partition into lipid membrane.

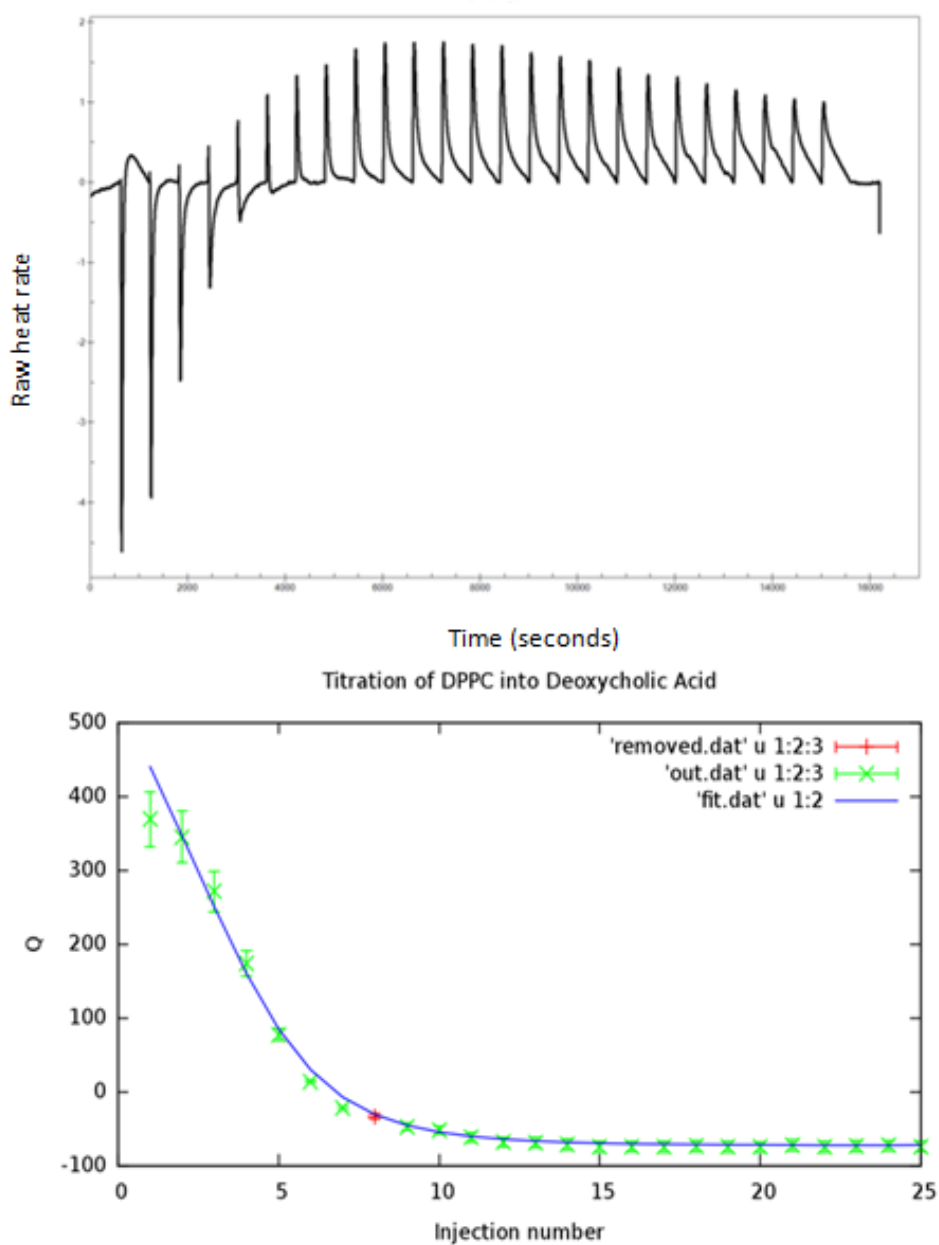


Figure 4.3: Titration of 50mM DPPC LUV solution into 0,2mM deoxycholic acid solution with 25x10 μ L injections at 25°C. The raw heat rate versus time (top), heat of interaction versus injection number (bottom).

Table 4.1: Thermodynamic parameters of partitioning of bile acids into DPPC lipid membrane in phosphate buffer pH 8.0 at 25°C.

Bile Acids	z	ΔG_{ins} (J/mol)	H(10 ¹¹ J/mol)	K
DCA	2,52 $\pm$ 0,54	(-37400 $\pm$ 2572)	2,47 $\pm$ 0,49	3545520 $\pm$ 0,354
CDCA	3,79 $\pm$ 0,41	(-46600 $\pm$ 3914)	1,91 $\pm$ 0,53	146816955 $\pm$ 0,206

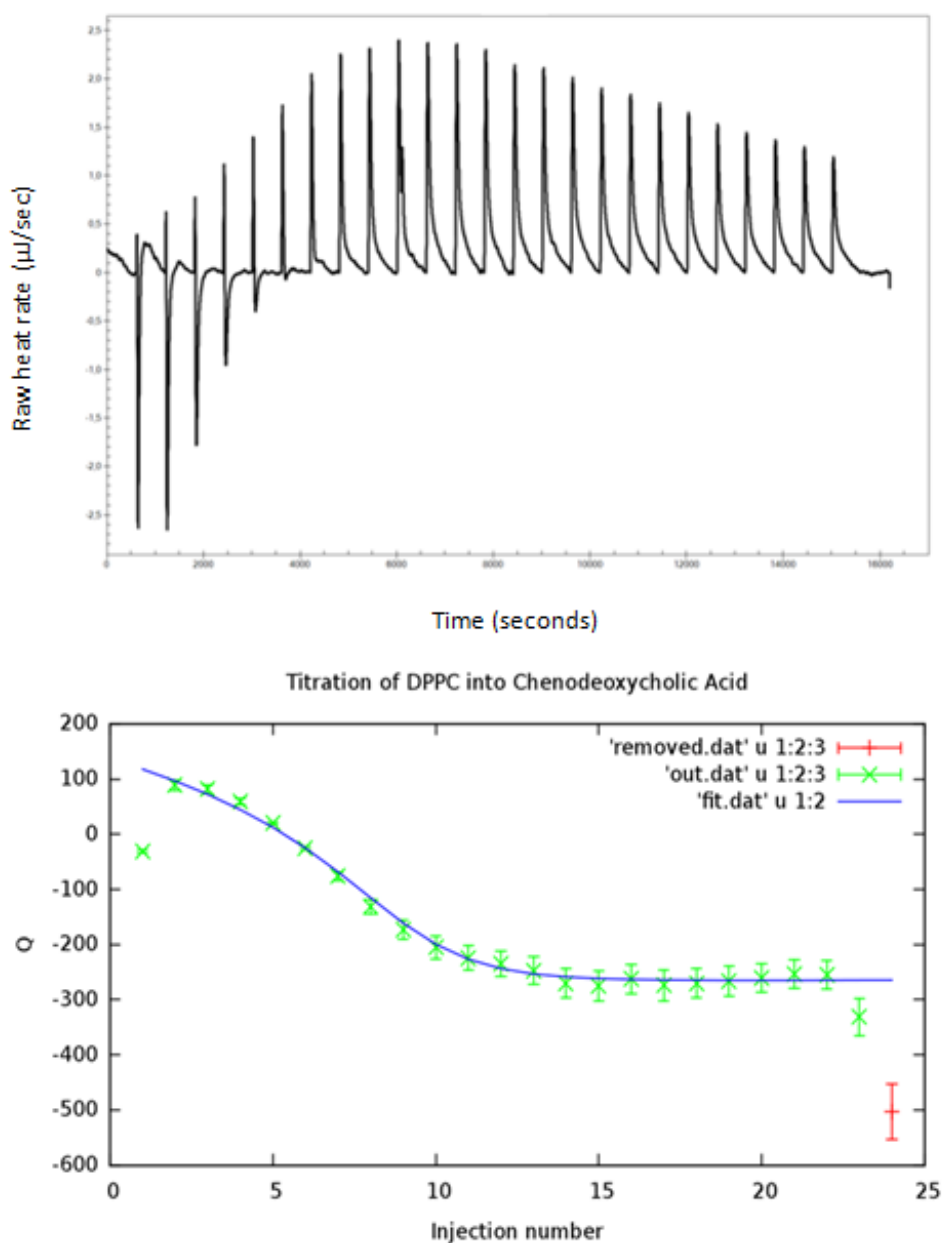


Figure 4.4: Titration of 50mM DPPC LUV solution into 0,2mM chenodeoxycholic acid solution with 25x10μL injections at 25°C. The raw heat rate versus time (top), heat of interaction versus injection number (bottom).

Heat traces regarding to partitioning of deoxycholic acid into POPC membrane have indicated endothermic peaks at 25°C in Figure 4.5. The lipid concentration has increased and available deoxycholic acid to partition into lipid membrane has decreased in the ITC sample cell with injections of POPC LUV solutions into deoxycholic acid solution that this causes the smaller reaction peaks and resulting constant reaction heat, heat of dilution of POPC vesicles. For POPC/DCA system, the mol DCA/ POPC ratio has been ~0,038 after 10 injections of 10μL POPC into ITC sample cell and the reaction heat has become stable.

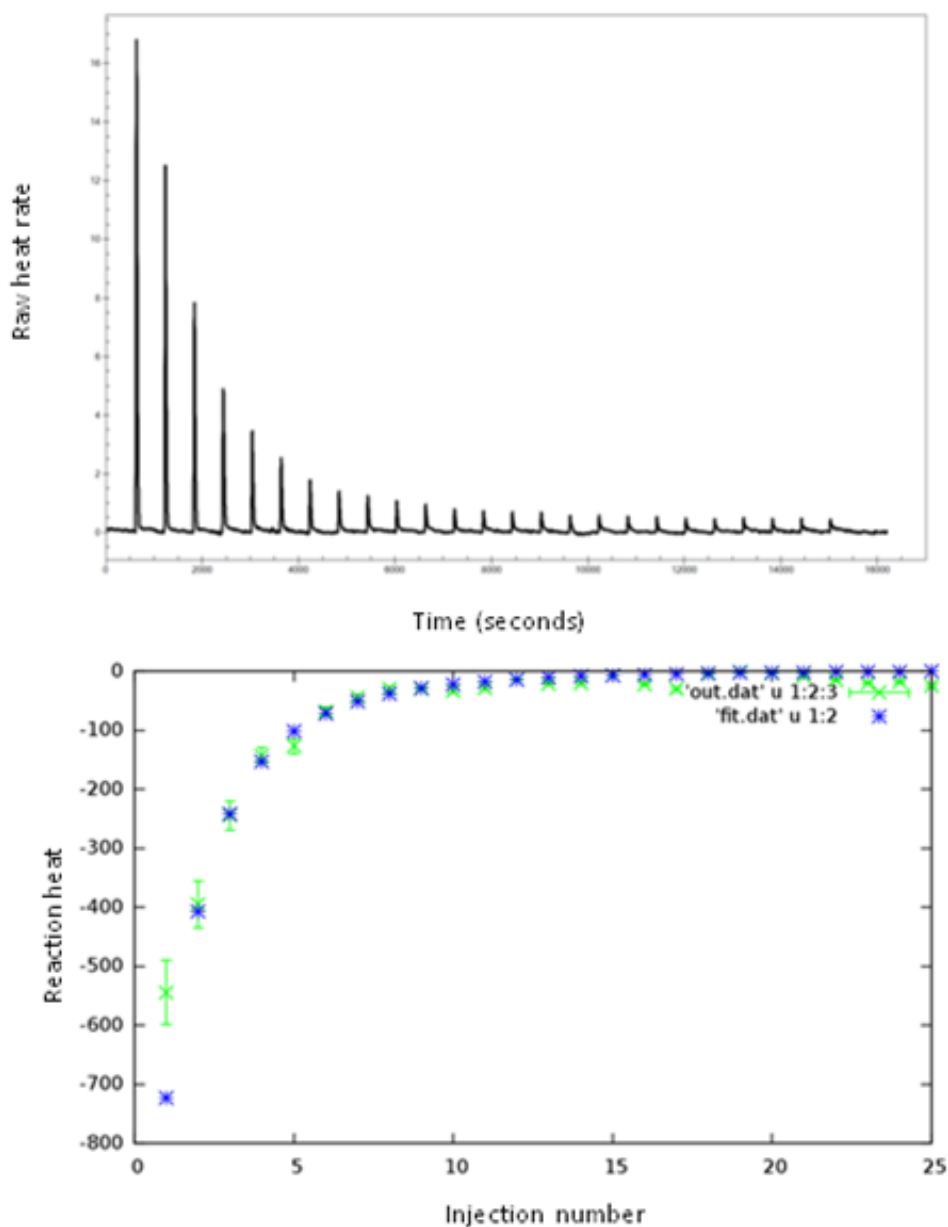


Figure 4.5: Titration of 50mM POPC LUV solution into 0,2mM deoxycholic acid solution with 25x10 μ L injections at 25°C. The raw heat rate versus time (top), the heat of interactions versus injection number (bottom).

The partitioning of chenodeoxycholic acid into POPC membrane has caused endothermic heat peaks at 25°C (Figure 4.6). Also, when the concentration of POPC reached to certain concentration in the sample cell, the reaction heat has started to be constant. The moles ratio of chenodeoxycholic acid to POPC in the ITC sample cell has become $\sim 0,023$.

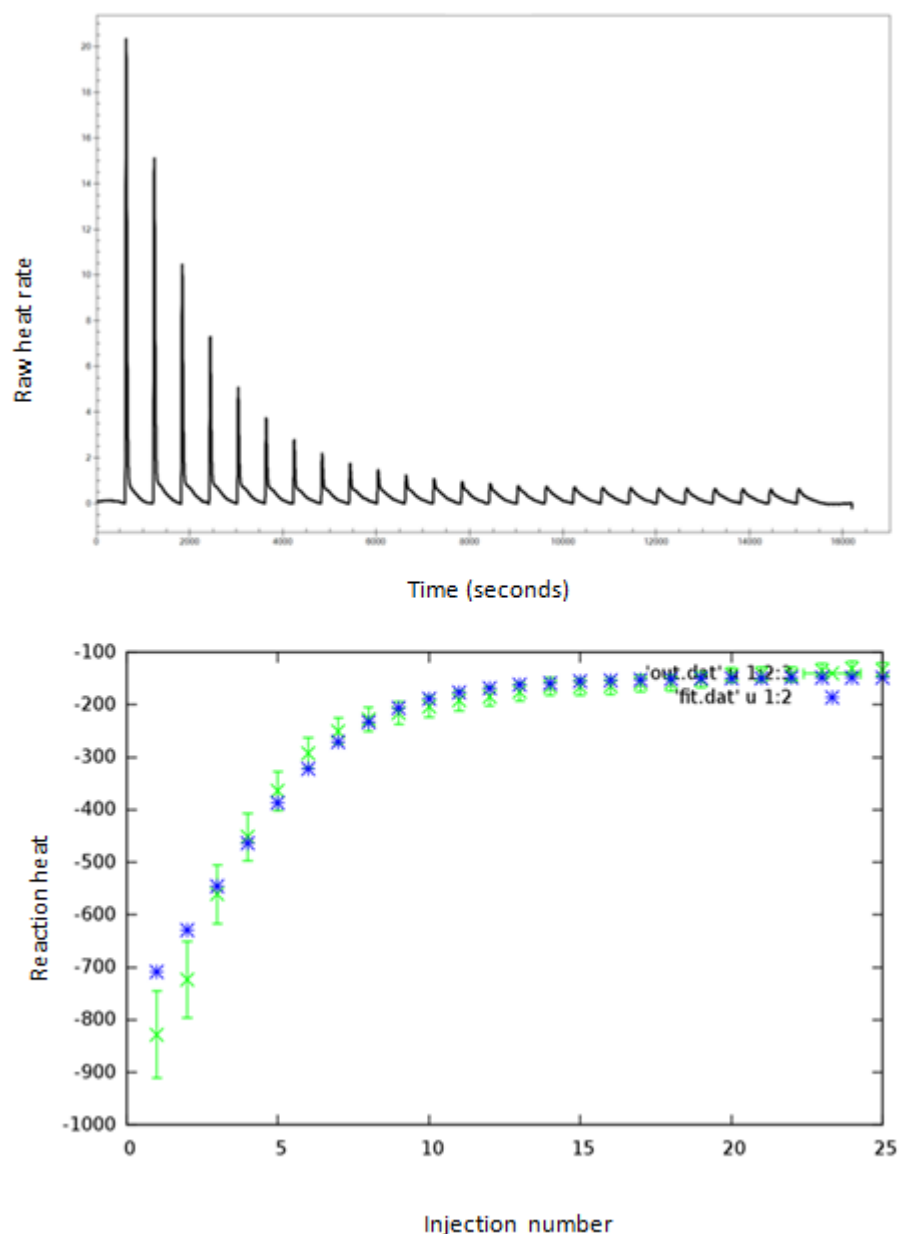


Figure 4.6: Titration of 50mM POPC LUV solution into 0,2mM chenodeoxycholic acid solution with 25x10 μ L injections at 25°C. The raw heat rate versus time (top), heat of interaction versus injection number (bottom).

Enthalpy values (J/mol) of partitioning of deoxycholic acid and chenodeoxycholic acid from aqueous phase into POPC lipid membrane are negative at 25°C that has been shown in Table 4.2. Partitioning coefficient of chenodeoxycholic acid into POPC lipid membrane was calculated as $774056 \pm 0,708$ whereas the partitioning coefficient of deoxycholic acid into POPC lipid membrane was found as $652400 \pm 0,684$. The lower partitioning strength to POPC lipid membrane were observed by comparison with the partitioning into DPPC lipid membrane.

Table 4.2: Thermodynamic parameters of partitioning of bile acids into POPC lipid membrane in phosphate buffer pH 8.0 at 25°C.

Bile Acids	z	ΔG_{ins} (J/mol)	H(10^{11} J/mol)	K
DCA	2	-33189±942	-1,41±0,15	652400±0,684
CDCA	2	-33613±854	-2,16±0,45	774056±0,708

4.2.2 Membrane solubilization assays

4.2.2.1 Determination of critical micelle concentration (CMC)

50mM deoxycholic acid was injected into the sample cell, containing the phosphate buffer pH 8.0 at 25°C as 50x5μL aliquots. In Figure 4.6, the curve of raw heat rate versus time has indicated that the each injection of deoxycholic acid produces heat exothermically.

Also, it can be observed that the heat of demicellization of bile acid has not shown the sharp decrease. This means that bile acids can form aggregates in a broad concentration range (Garidel et al., 2000). Micelles are formed by self-association of 4-40 bile acid molecules (Hofmann and Mysels, 1992).

The first derivation of the curve of reaction heat versus bile acid concentration provides CMC value of deoxycholic acid. The value of CMC was found as 6 mM approximately for deoxycholic acid in phosphate buffer at pH 8.0. It is obvious that pH affects the CMC of surfactant as well as structure of bile acid with number, position and orientation of hydroxyl groups. Increase of pH leads to increase of solubility of bile acids, more anions exist in the solution, and the concentration of anions is raised to CMC, micellization occurs.

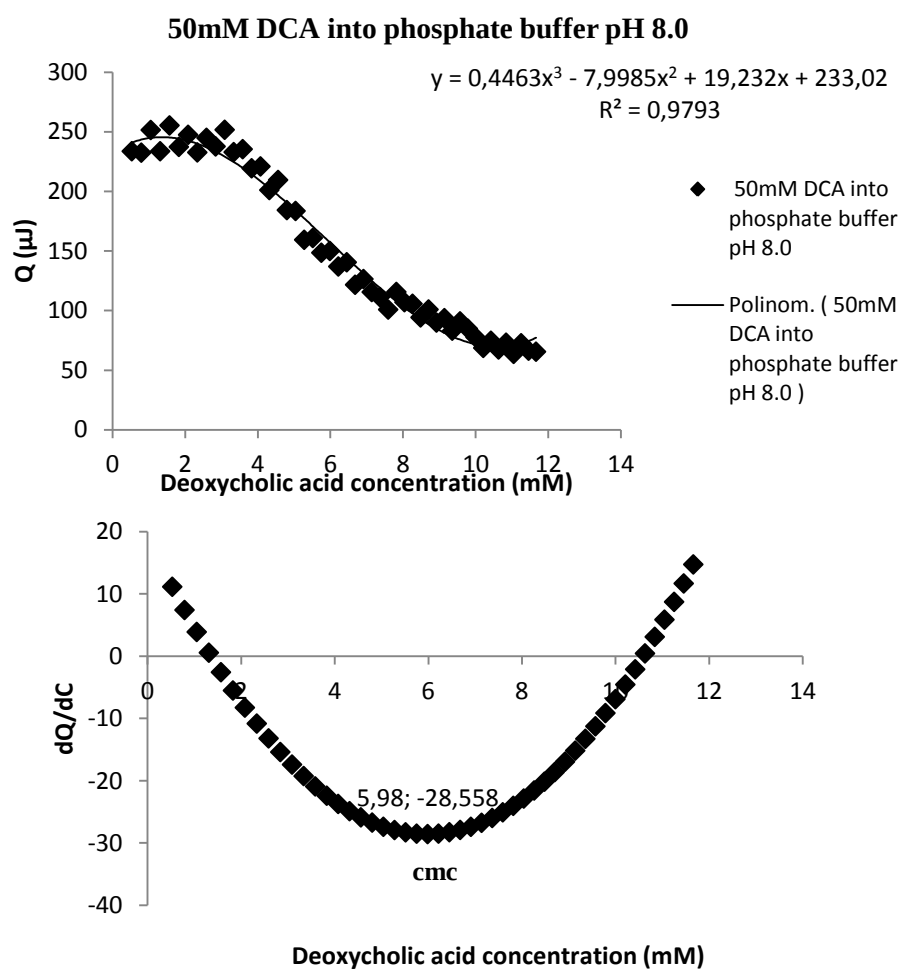
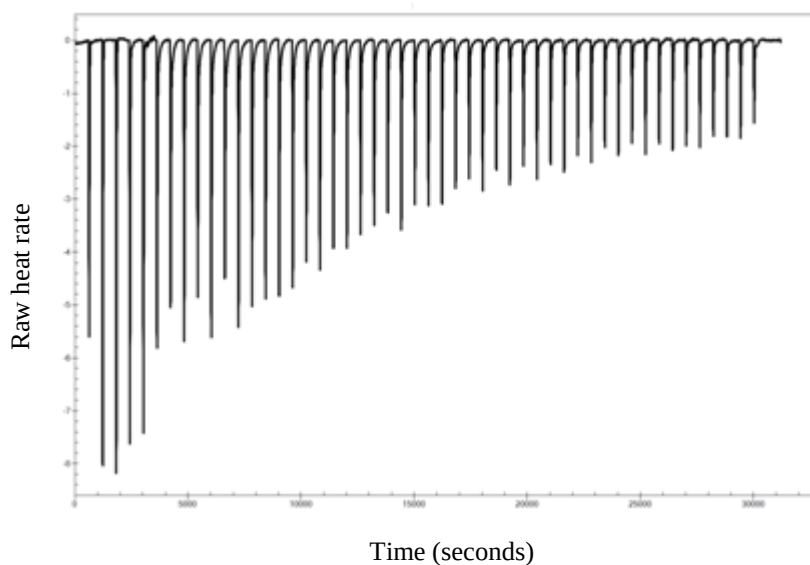
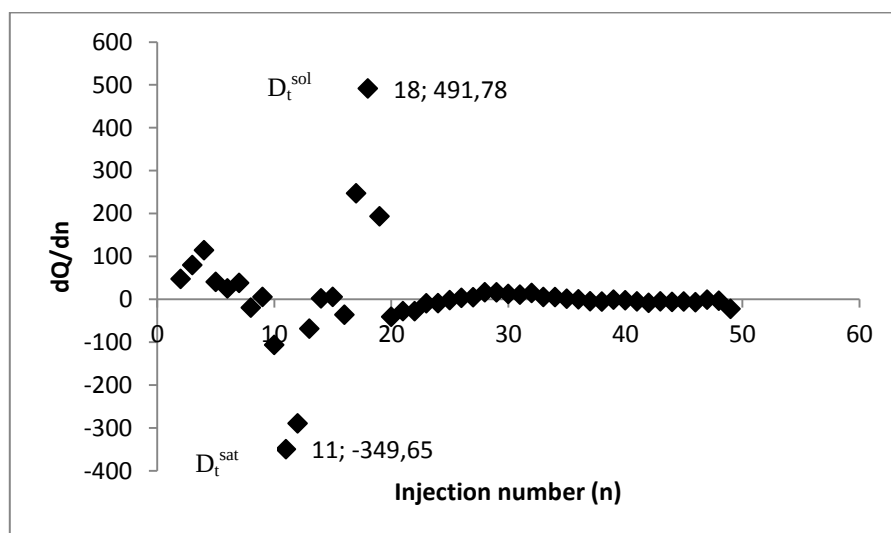
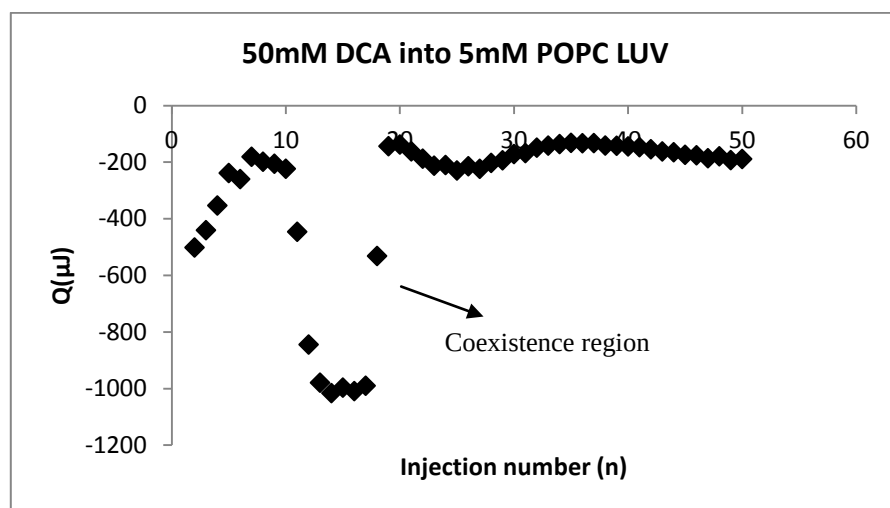
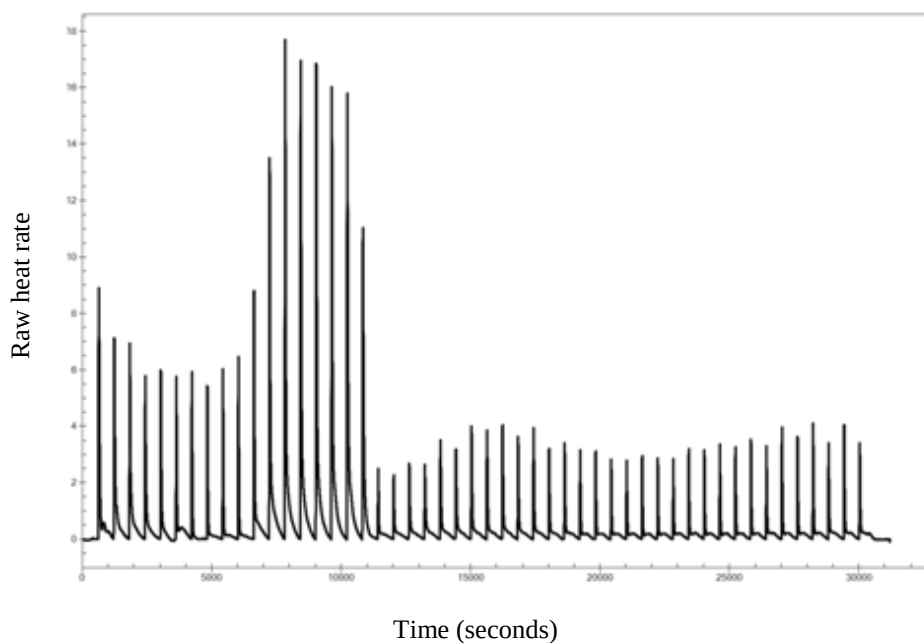


Figure 4.7: Determination of CMC: 50 injections of 50 mM deoxycholic acid into phosphate buffer pH 8.0 at 25°C. Raw heat rate versus time (top), the reaction heat versus deoxycholic acid concentration in the ITC sample cell (middle), the first derivative of reaction heat to deoxycholic acid concentration (bottom).

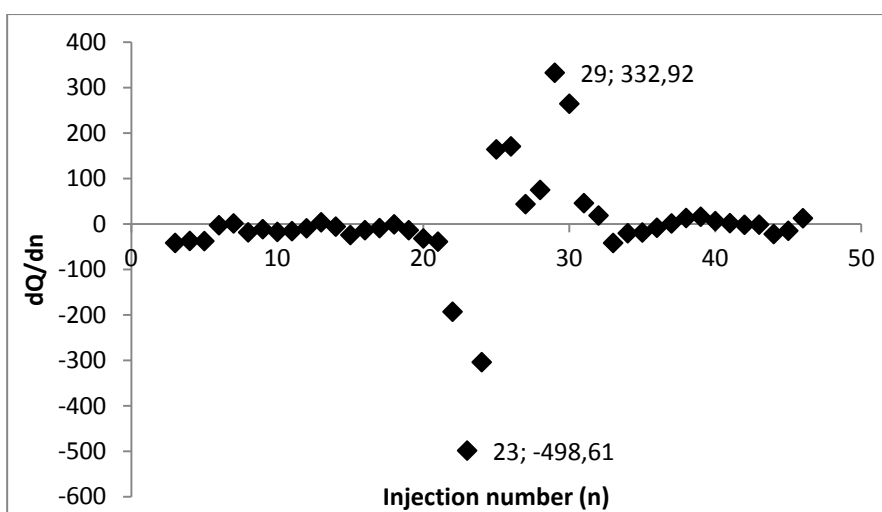
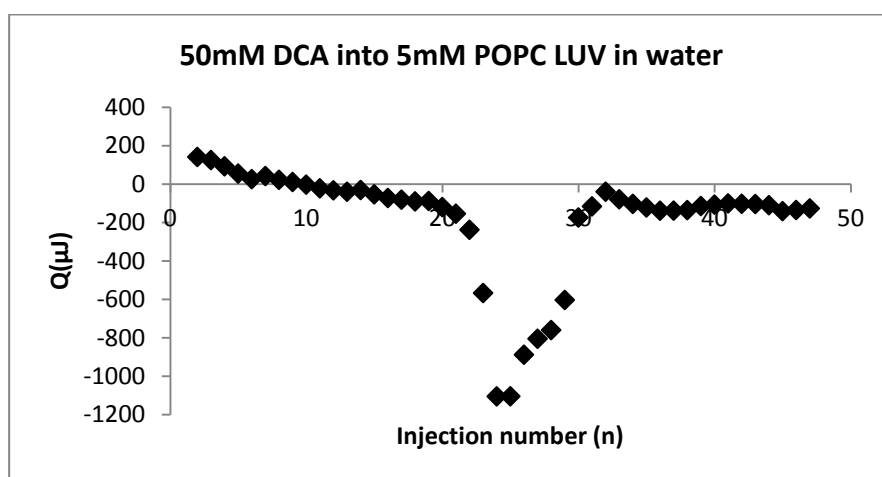
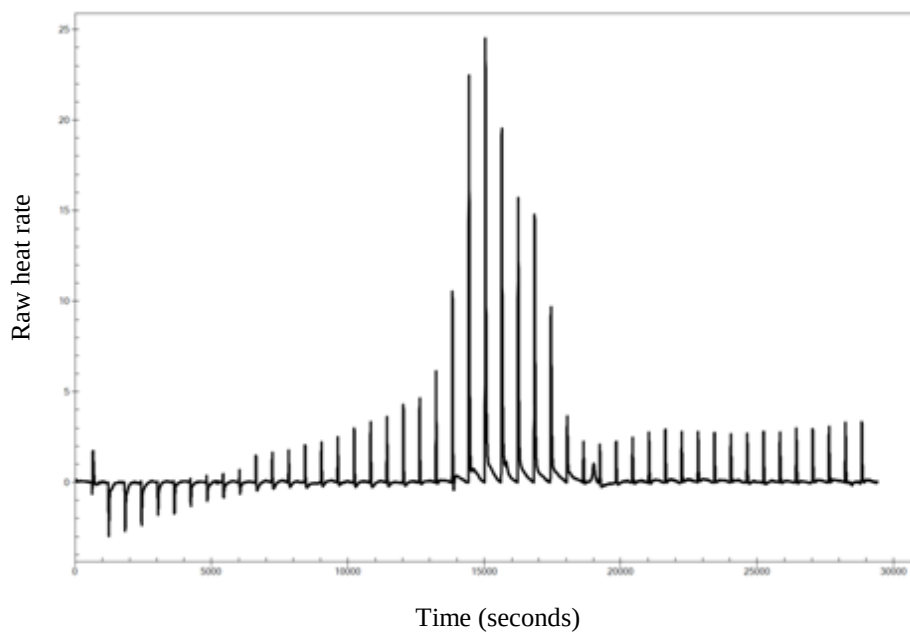
4.2.2.2 Phase transition diagrams

5 μ L aliquots of highly concentrated deoxycholic acid solution, which had been loaded in 250 μ L syringe, was titrated into lipid vesicles solution in order to construct the phase diagrams. For membrane solubilization experiments, four systems were performed that consist of deoxycholic acid as biosurfactant and POPC and DPPC lipid vesicles at different conditions. For the first experiment, titration of deoxycholic acid into POPC vesicle solution in the phosphate buffer pH 8.0 at 25°C was done, the another solubilization experiment was the titration of deoxycholic acid into POPC vesicle solution in the water pH 8.0 at 25°C. DPPC was also used as model lipid membrane for membrane solubilization experiments. DPPC and deoxycholic acid solutions were prepared only in phosphate buffer, but different temperatures were applied, 25°C and 55°C. Solubilization effect of deoxycholic acid was determined by ITC. Calorimetric traces were obtained with each injection of highly concentrated deoxycholic acid into lipid solutions at different concentrations for each systems. Then, the reaction heat (μ J) versus injection number curves were formed by integration of heat peaks of each injections. The first derivative of this curve provided to determine the deoxycholic concentration to saturate and solubilize the membranes, D_t^{sat} and D_t^{sol} , respectively. As a result, phase transition diagrams could be constructed by using values of D_t^{sat} and D_t^{sol} . Starting of saturation, solubilization of membranes by deoxycholic acid, and coexistence region of vesicles and micelles could be deduced from the curves of reaction heats versus injection number. Furthermore, the phase boundaries provided information about regions of mixed micelle, mixed vesicles and coexistence by presenting the characteristic bile acid/lipid ratios, R_e^{sat} and R_e^{sol} , and bile acid concentration in the aqueous phase, D_w^{sat} and D_w^{sol} , for saturation and solubilization of lipid membranes. The intercept points of phase boundaries in the y-axis have presented D_w^{sat} and D_w^{sol} , and the slopes of boundary lines have also shown R_e^{sat} and R_e^{sol} . Table 4.3 has indicated the parameters of solubilization of lipid membranes by deoxycholic acid.

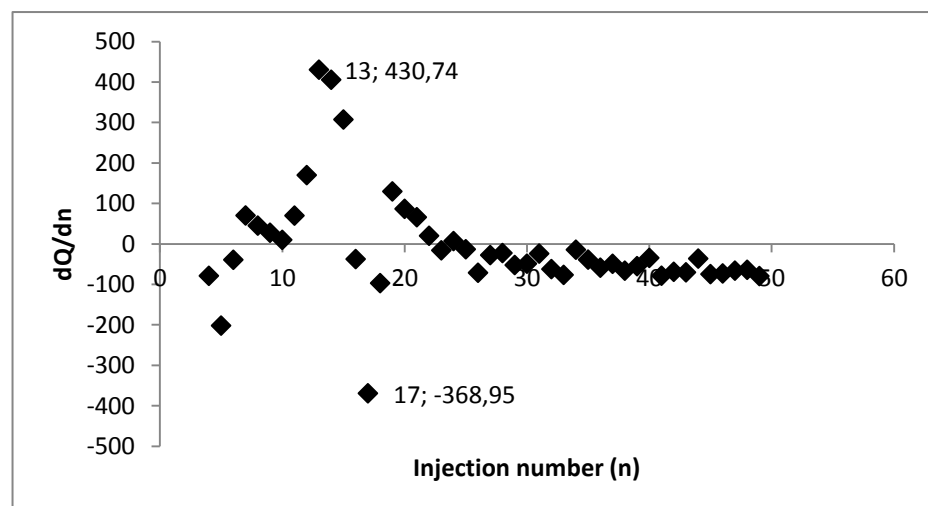
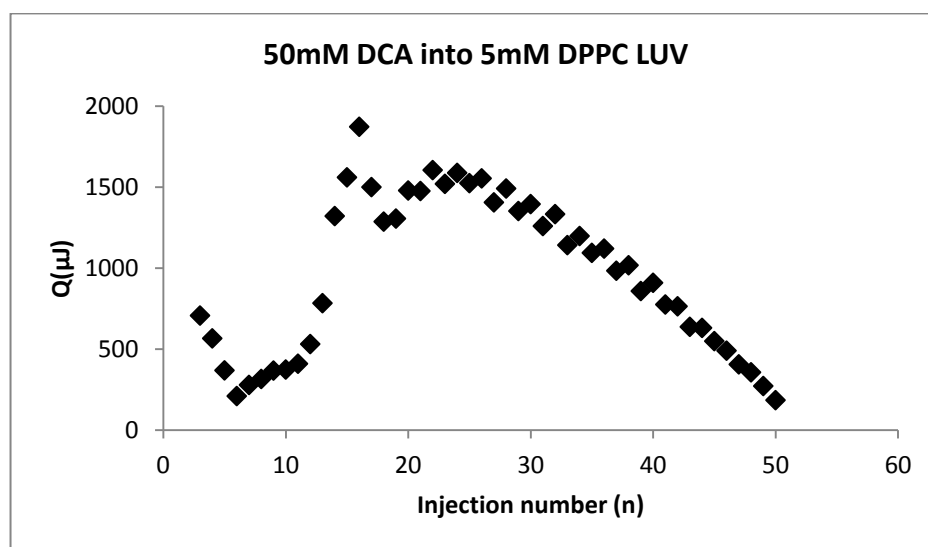
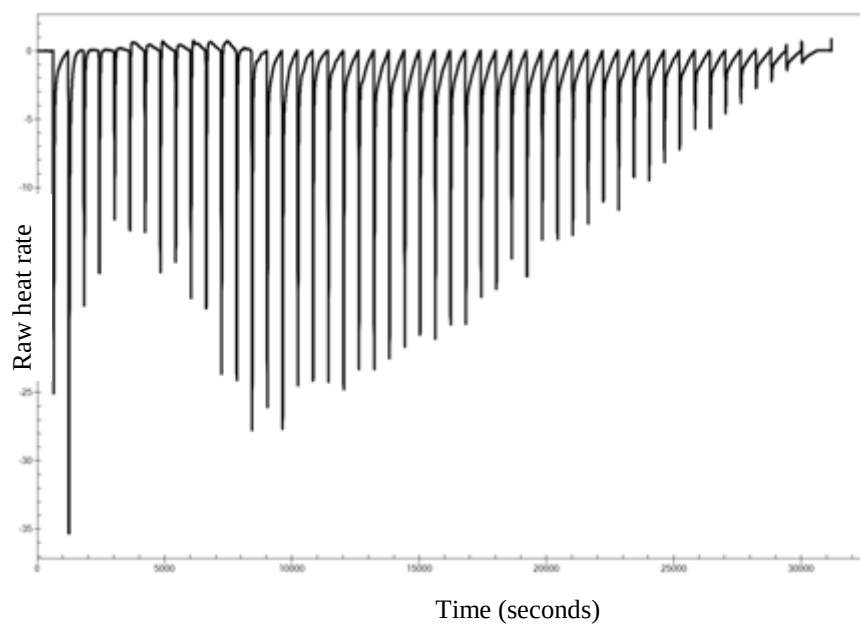
1. 50x5 μL aliquots of 50mM deoxycholic acid were injected into 1; 2; 5; 10mM POPC LUV solutions prepared with phosphate buffer pH 8.0 at 25°C.



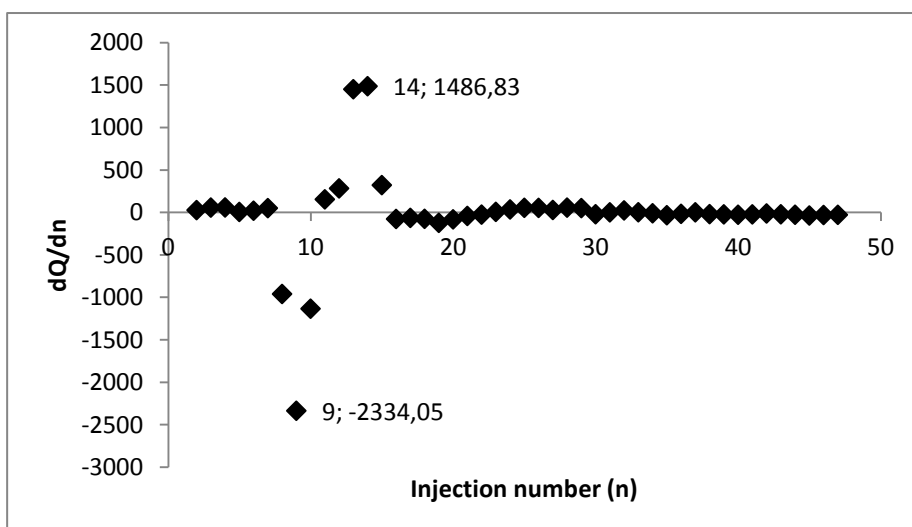
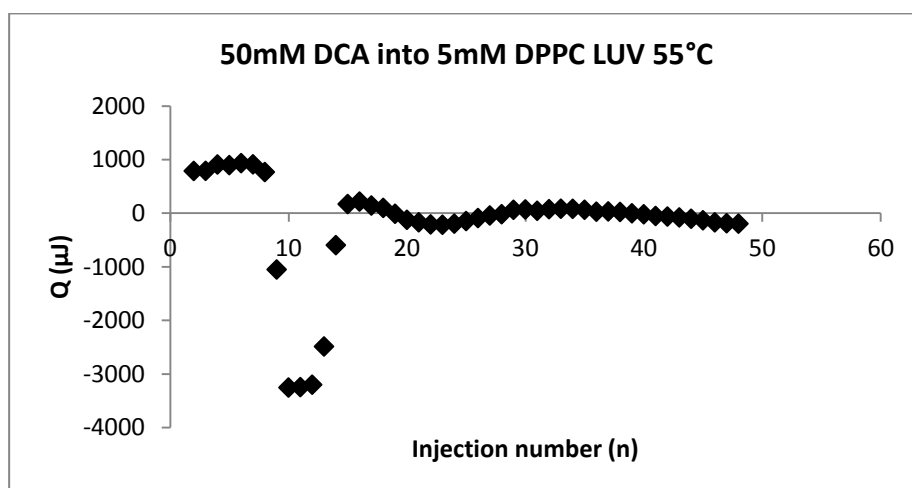
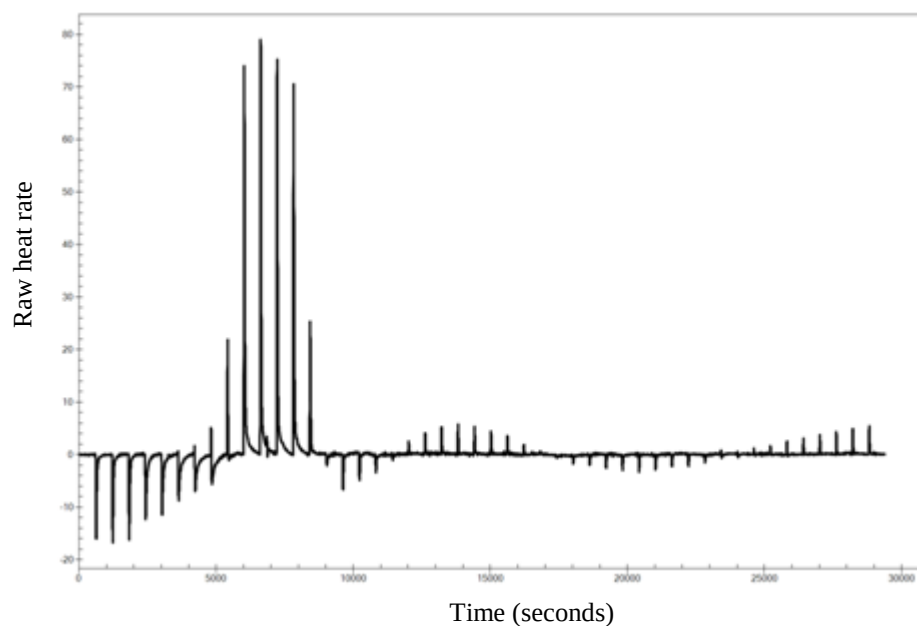
2. 50x5 μL aliquots of 50mM deoxycholic acid were injected into 2; 5; 10mM POPC LUV solutions prepared with water pH 8.0 at 25°C.



3. 50x5 μL injections of 50mM deoxycholic acid were titrated into 2; 5; 10mM DPPC LUV solutions prepared with phosphate buffer pH 8.0 at 25°C.

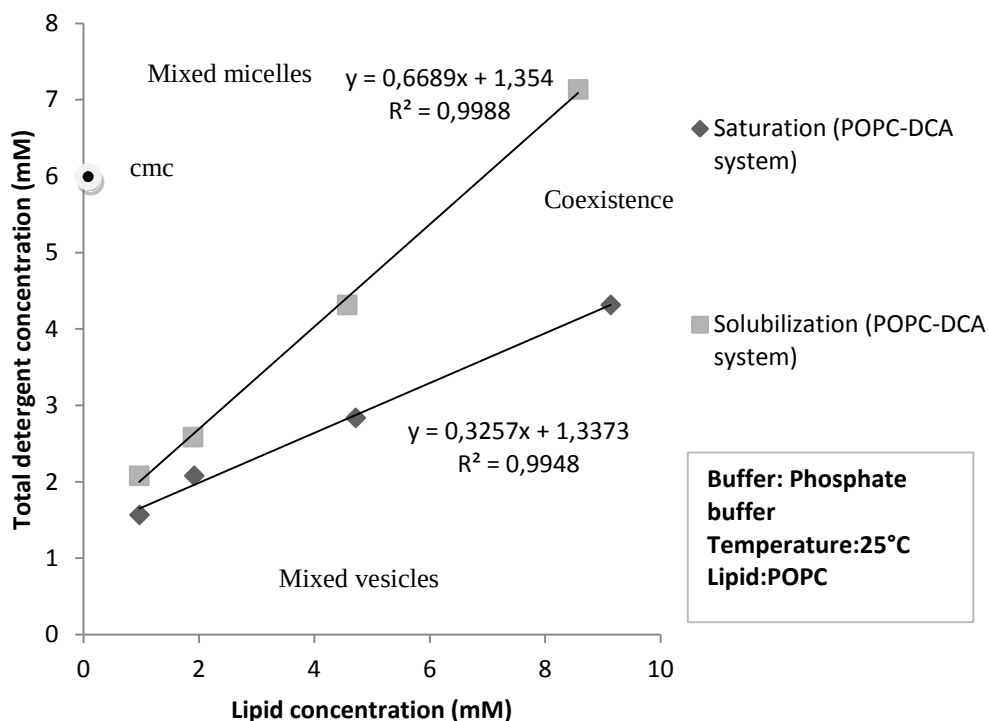


4. 50x5 μL injections of 50mM deoxycholic acid were titrated into 2; 5; 10mM liquid-crystalline phase DPPC LUV prepared with phosphate buffer pH 8.0 at 55°C.

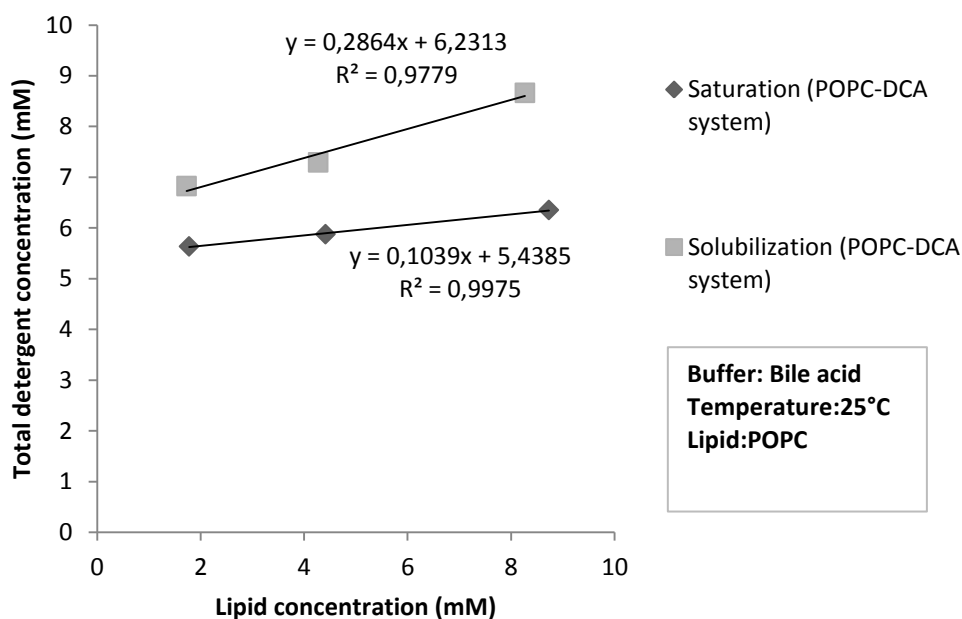


Vesicle to micelle transition diagrams:

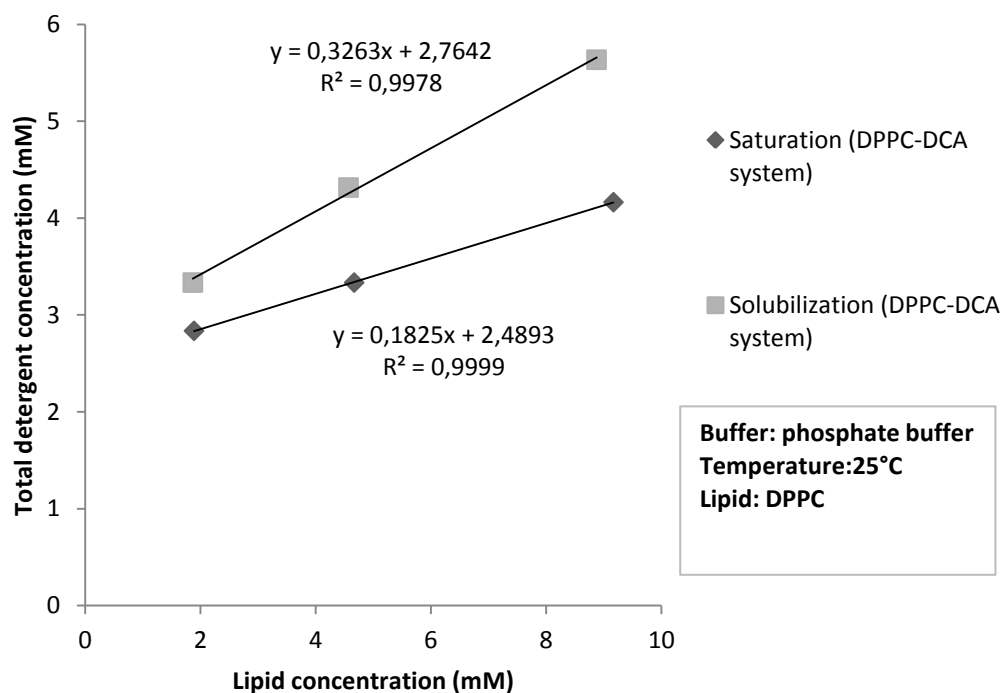
1. Phase transition diagram of POPC membrane by DCA in phosphate buffer, pH 8.0 at 25°C.



2. Phase transition diagram of POPC membrane by DCA in water, pH 8.0 at 25°C.



3. Phase transition diagram of DPPC membrane by DCA in phosphate buffer, pH 8.0 at 25°C.



4. Phase transition diagram of DPPC membrane by DCA in phosphate buffer, pH 8.0 at 55°C.

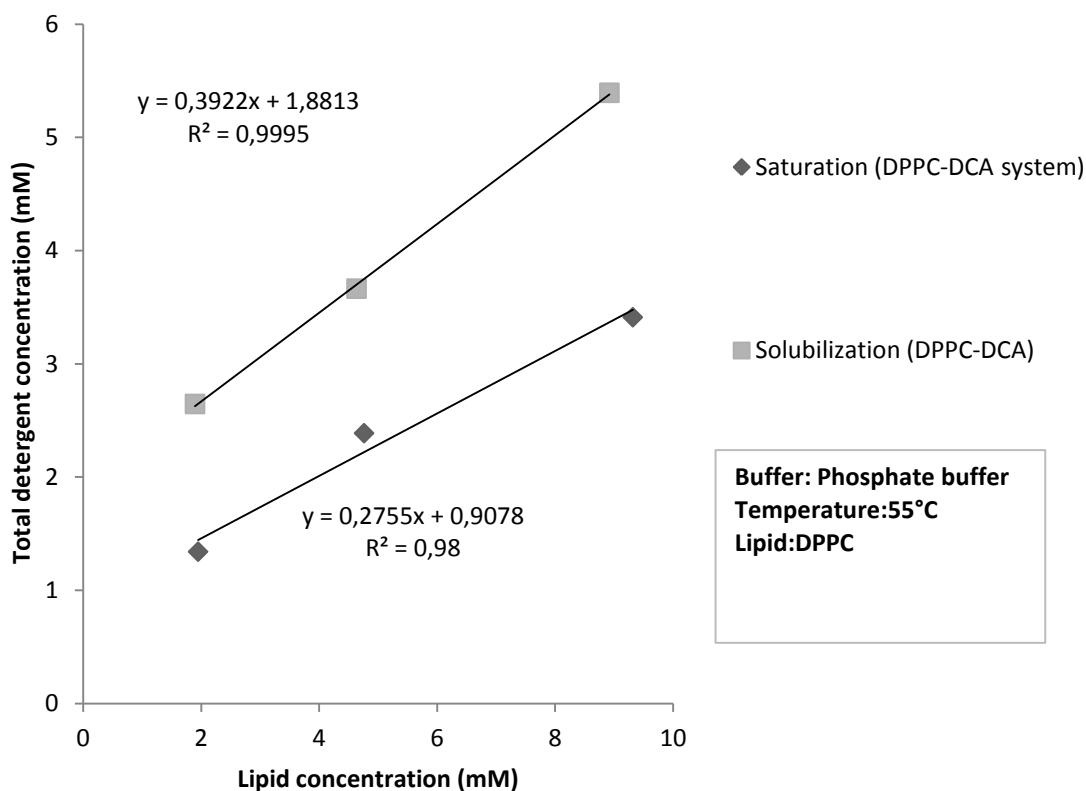


Table 4.3: Solubilization parameters of POPC and DPPC membrane by DCA at pH 8.0.

Bile Acid	Lipid	T(°C)	Buffer	R_e^{sat}	R_e^{sol}	D^{sat} (mM)	D^{sol} (mM)
DCA	POPC	25	Phosphate buffer	0,3257	0,6689	1,337	1,354
		25	Bile acid	0,1039	0,2864	5,4385	6,2313
	DPPC	25	Phosphate buffer	0,1825	0,3263	2,4893	2,7642
		55	Phosphate buffer	0,2755	0,3922	0,9078	1,8818

When the phase diagrams representing the different lipid-bile acid systems are compared to each other, the some results can be deduced that

- If the first and second phase diagrams of POPC membrane are compared, the effect of buffer can be understood. When phosphate buffer (pH 8.0) is used, it can be seen obviously that the values of D_w^{sat} and D_w^{sol} are smaller than those of using water as solvent. It means that more surfactant is required to saturate and solubilize the lipid membrane in the water. It might be related to surfactant micelle formation in different solvent system. Bile acids can form micelle in the higher concentrations in the water, so that more bile acid micelles is required to form mixed vesicle and mixed micelles. Potassium ions in the buffer have shielding effect on the micelle formation, so micellization can be achieved in the lower concentration of negatively charged bile acids in the phosphate buffer. Furthermore, the value of CMC of deoxycholic acid is $\sim 6\text{mM}$, and it can be seen that this concentration is higher than $\sim 1,3$ (D_w^{sat} and D_w^{sol} of POPC-Deoxycholic acid systems) in the first phase diagrams. Lower surfactant concentration is required to saturate and solubilize the lipid membranes beacuse of ionic strenght of solutions prepared with potassium hydrogen phosphate (K_2HPO_4), and potassium dihydrogen phosphate (KH_2PO_4).

However, water can be used as a solvent because bile acids behave as a buffer in the system and pH becomes constant.

- When third and fourth diagrams are compared, the effect of phase state of lipid on the membrane solubilization process can be interpreted. DPPC is in the liquid-crstalline state above the transition temperature, 41°C . Therefore, the solubilization experiments were performed at 25°C and 55°C . Membranes are not enough permeable to surfactant molecules in the gel state

that D_w^{sat} and D_w^{sol} are higher in the gel state of lipid. Also, the studying with lipid vesicles in the gel phase is difficult because of slow reaction kinetics that can be observed from the curve of reaction heat versus injection number regarding to DCA/DPPC system, was obtained from experiment carried out at 25°C.

- If the first phase diagram is compared with fourth phase diagram, it can be inferred how the degree of saturation of lipids affect the solubilization of membrane. Both of lipids have existed in liquid-crystalline state, but DPPC is saturated lipid whereas POPC is unsaturated lipids. The curvature of lipid can affect the micelle formation (saturated lipids can form micelle easily). It can be seen in the graphics of reaction heat versus injection number that the coexistence region of solubilization of POPC is wider than the solubilization of DPPC. Therefore, the surfactant concentrations for saturation and solubilization (D_t^{sat} and D_t^{sol}) have shifted to higher concentrations for the liposomes composed of POPC. This has shown that membrane packing properties of lipid molecules related with formation of curved structure is effective to determine surfactant concentration for membrane solubilization. Also, there is a temperature difference between this two bile acid-phospholipid systems. However, this difference may not be significant to form micelles.
- When the first and third systems, which consist of POPC as lipid prepared in phosphate buffer and consist of DPPC as lipid in phosphate buffer at the same temperature, are compared, the effects of lipid geometry and thermodynamic state of lipids can be determined. In gel-state, lipids may be more resistant to insertion of bile acids into membrane and solubilize the membrane so that DPPC can be preferred to delay the release of active molecule encapsulated in the lipid membrane because of slow saturation and solubilization process (R_e^{sat} and R_e^{sol} lower for DPPC system). Saturated and unsaturated structure of lipid may have effect on packing constraints of membranes so that phase boundaries for saturation and solubilization and the width between boundaries are different.
- In addition, it can be seen in the phase diagrams that D_w^{sat} and D_w^{sol} have not intersected in the same point and the values are different from each others. Roth et al. defined new model by considering the finite size of mixed

micelles and end-caps free energies of micelles while determining of the phase boundaries in the dilute aqueous solutions in their study with octylglucoside nonionic surfactant (2000). Also, it can be observed in the four phase diagrams presented in this study that the nature of lipid and solvents affect the difference between these values and composition of mixed vesicles and micelles in the coexistence region. Furthermore, it should be emphasized that negatively charged bile acids differ from ordinary surfactants in terms of structure and aggregation properties.

4.3 Determination of the Particle Sizes of Lipid Vesicles and Micelles by DLS

Dynamic light scattering was used to determine the particle size of different structures, from lipid riched mixed vesicles to surfactant riched mixed micelles, which are consisted of different ratio bile acid/phospholipid. Solubilization of POPC LUV was performed by determination of size distribution of particles in the lipid-bile acid solution at 25°C. The mean diameters of particles were obtained by measuring 90° scattered light. The measurements were performed as 5 runs and in 5 minutes totally. Surface area-weighted differential size distribution was used to determine the mean diameters. The results of solubilization of POPC by DLS were given in Table 4.4.

Multimodel size distribution uses Non-negatively constrained least squares algorithm to fit the data. According to multimodel size distribution (MSD),

Mean diameter:

$$d_{mean} = \frac{\sum dG(d)}{\sum G(d)} \quad (4.1)$$

Relative variance: variance/(mean diameter)²

$$variance = \frac{\sum (d - d_{mean})^2 G(d)}{\sum G(d)} \quad (4.2)$$

It can be observed that the mean diameters, which were determined by surface area-weighted size distribution, decrease with increasing of deoxycholic acid concentration. Also, increase of deoxycholic acid in POPC vesicles solution causes to increase polydispersity of particles.

The concentration ratio DCA /POPC in accordance with phases showed in vesicle-to-micelle phase diagram of DCA-POPC system in phosphate buffer at 25°C has proven

the phases in terms of size of particles and polydispersity. The size of mixed vesicles has changed depending on the ratio between bile acids and lipids. Also, the shape of micellar forms has altered in the phases. Finally, polydispersity has become more than 0,1 in the mixed micellar phase. The mixed vesicles and mixed micelles can form in the different shape such as rod-like, dynamic light scattering sometimes can not measure the exact size of particles.

Table 4.4: The results of measurement of size distribution of solubilization of POPC LUV by deoxycholic acid.

The ratio of DCA to POPC concentration	Mean diameter (nm)	Relative variance
0 (1mM POPC)	125,0	0,038
0.5	94,6	0,131
1	56,4	0,405
2	58,4	0,373
4	9,5	29,459
6	7,2	0,224

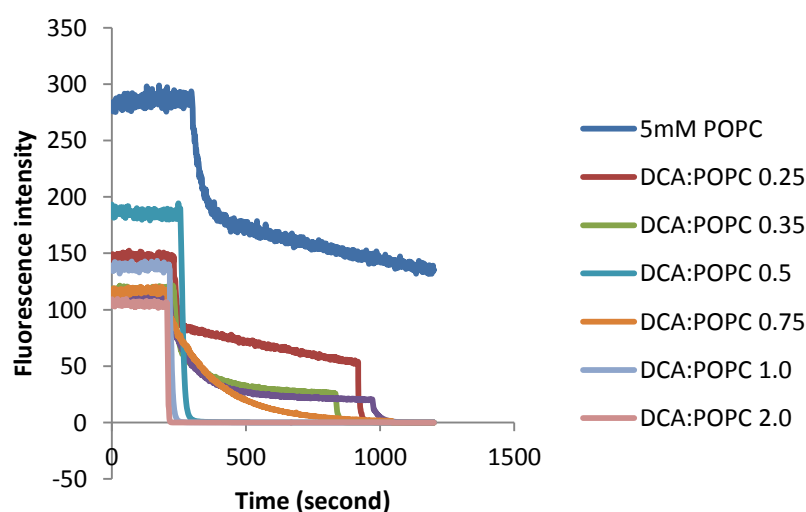
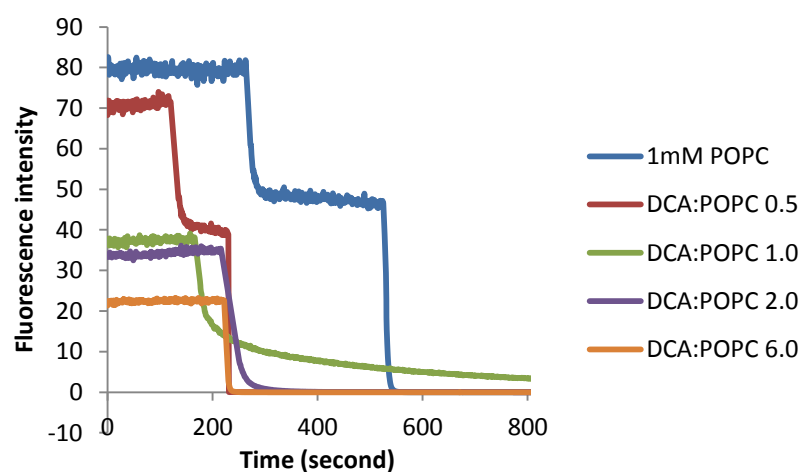
4.4 Determination of the Structure of Lipid Vesicles and Micelles by Fluorescence Spectroscopy

Fluorescence spectroscopy was used to determine the lamellarity of mixed lipid vesicles and mixed micelles to confirm the membrane solubilization results obtained by ITC. Membrane solubilization experiments were also performed at 25°C by means of fluorescence spectrophotometer. The decrease of fluorescence was observed after addition of fluorescence quencher, dithionite. Then, 10 % Triton-X 100 solution was added to sample cuvette in order to disrupt the lipid membrane. The results of fluorescence intensity were given as a sample/reference ratio versus time. The samples depending on the ratio of deoxycholic acid to lipid vesicles has shown different fluorescence intensity because of the structure of lipid-bile acid mixed systems such as mixed vesicles, mixed micelles or coexistence of vesicles and micelles. Therefore, the structure or phase of lipid vesicles including bile acid can be determined by fluorescence spectroscopy.

4.4.1 Solubilization of POPC

Solubilization of 1 mM, 5mM, and 10mM POPC vesicles, containing NBD-PE fluorescent lipids, by deoxycholic acid was investigated by fluorescence spectrofluorimeter while preparing the mixtures that are formed by different deoxycholic acid/lipid ratios which were chosen by considering the calorimetric experiments. In Figure 4.8, the graphics of fluorescence intensity versus time has been presented.

The lines in the graphics show the rapid decline of fluorescence intensity by addition of dithionite and surfactant 10 % Triton-X 100 solution, respectively. NBD is reversibly quenched by reducing agent dithionite and fluorescence intensity decreases because of formation nonfluorescent amine. Also, Triton-X 100 solution causes the disruption of liposomes that intensity goes down the zero level.



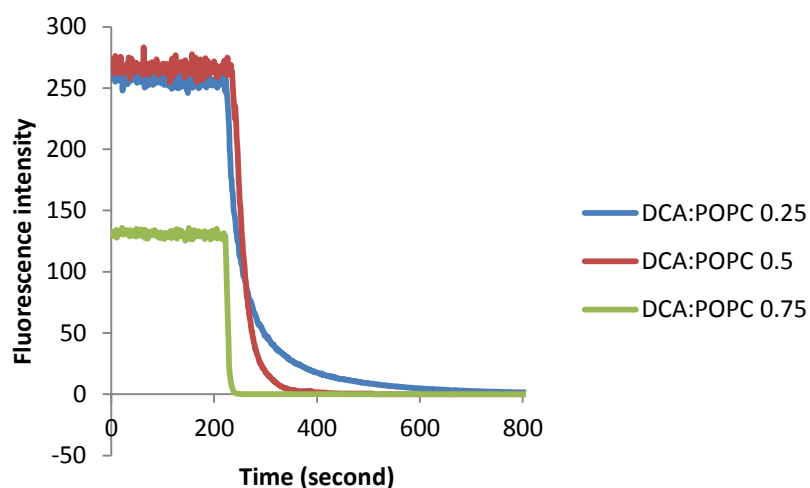
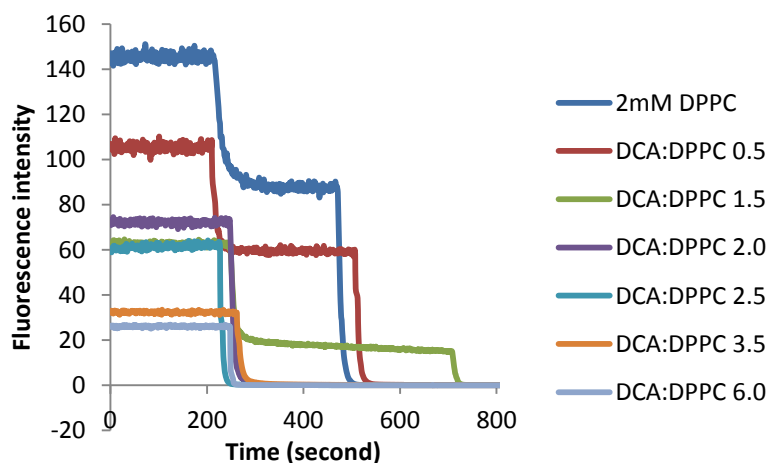


Figure 4.8: Normalized fluorescence intensity (as sample/reference ratio) versus time curves of solubilization of POPC membranes by deoxycholic acid.

4.4.2 Solubilization of DPPC

Solubilization of 2 mM, 5mM, and 10mM DPPC vesicles, containing NBD-PE fluorescent lipids, by deoxycholic acid was investigated by fluorescence spectrofluorimeter following the same procedure with determination of phases of POPC vesicles (Figure 4.9).

DPPC exists in the gel state at the experiment temperature, 25°C, so fluorescence quenching by dithionite may not be effective, especially in a high lipid concentrations as it can be seen in the third graphic in Figure 4.9. The lamellarity of lipid vesicles were determined by fluorescence spectroscopy to confirm the membrane solubilization parameters obtained by ITC. The solubilization values determined by ITC and fluorescence spectroscopy are given in the Table 4.5.



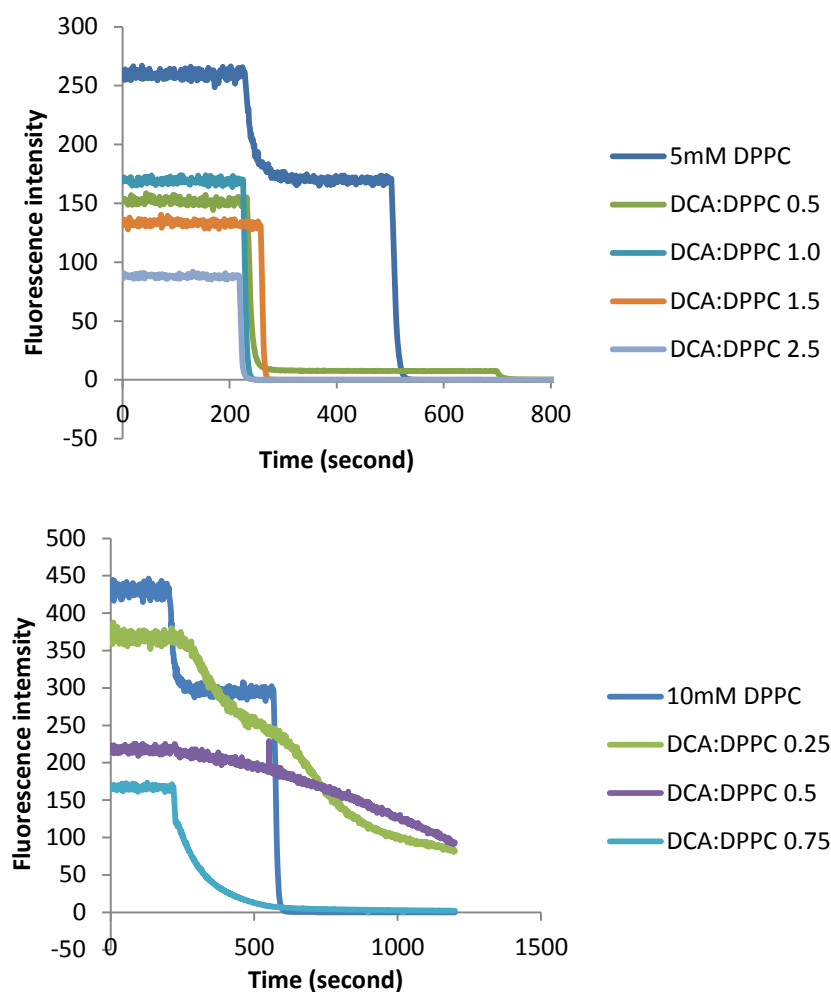


Figure 4.9: Normalized fluorescence intensity (as sample/reference ratio) versus time curves of solubilization of DPPC membranes by deoxycholic acid.

Table 4.5: The comparison of the surfactant to lipid ratios (C_D/C_L) by calorimetric and spectroscopic methods (25°C).

Lipid	Lipid Concentration (mM)	C_D/C_L	C_D/C_L
		by ITC (sat.- sol.)	by Fluorescence Spectroscopy
POPC	1	1.6-2.1	1.0-2.0
	5	0.6-1.0	0.75-1.0
	10	0.47-0.83	0.25-0.5
DPPC	2	1.5-1.8	1.5-2.0
	5	0.7-0.95	0.5-1.0
	10	0.45-0.64	0.25-0.75

It is indicated in Table 4.5 that the interval of the concentration of surfactant to lipid between saturation and solubilization can be obtained by isothermal titration

calorimetry in a narrow range accurately from vesicle to micelle phase diagrams. Some results show difference between ITC and fluorescence spectroscopy. However, it can be accepted because the different methods are utilized to determine the surfactant to lipid ratios for membrane solubilization. Also, the results are approximately close to each others. The use of fluorescent probe molecule is a drawback of measurement of fluorescence. For example, DPPC exists in the gel state at the experiment temperature, 25°C and fluorescence quenching by dithionite may not be effective, especially in a high lipid concentrations. Different transition forms between mixed vesicles and mixed micelles can be formed, for instance in a coexistence region of lipid-surfactant mixtures. This can be another reason of different results obtained by two different methods. Visualization of vesicles at different surfactant/lipid ratios may be performed by electron microscopy techniques to get accurate results.

5. CONCLUSION AND RECOMMENDATIONS

Partitioning of bile acids between water and model lipid membranes and solubilization of lipid membranes by bile acids were investigated in this study to understand the interactions of biosurfactant bile acids, which are synthesized from cholesterol in the liver, with lipid membranes to create bile acid-phospholipid mixed micellar systems for use as delivery systems of pharmaceutical or nutraceutical molecules.

The thermodynamic profile of interactions can be determined directly by isothermal titration calorimetry in one experiment. Electrostatic interactions between negatively charged bile acids and neutral phospholipid vesicles, including negatively charged bile acids, took into consideration while partitioning of bile acids into lipid membrane was studying by calculation of partition coefficients of bile acids. Partitioning coefficients, obtained for partitioning of chenodeoxycholic acid into DPPC lipid membrane were higher ($146816955 \pm 0,206$) than the partitioning coefficients of deoxycholic acid into DPPC lipid membrane ($3545520 \pm 0,354$). Also, partitioning coefficients ($774056 \pm 0,708$; $652400 \pm 0,684$, respectively) of both of bile acids, chenodeoxycholic and deoxycholic acid, into POPC lipid membrane were lower than those for DPPC lipid membrane partitioning that it means the affinity of bile acids to partition between aqueous phase and DPPC is favorable than POPC. The difference between packing of saturated and unsaturated lipids (geometrical and structural difference) can cause the difference in strength of partitioning of bile acids. Moreover, partitioning coefficients of chenodeoxycholic acid into POPC lipid membrane were higher than the partitioning coefficients of deoxycholic acid into POPC lipid membrane. Although the both of bile acids are dihydroxybile acids, the position of one of the hydroxyl group bound to steroidal backbone of chenodeoxycholic acid (at C7) is different than the position in the deoxycholic acid (at C12). It can be concluded that hydroxyl groups on the bile acid structure have important role on the partitioning of these molecules into membranes.

The concentration of bile acids below their CMC value provide the partitioning of bile acid monomers into lipid membrane, hence forming mixed lipid vesicles in

different sizes and shapes. Increase the concentration of bile acids induces the transformation of mixed vesicles into mixed micelles which is called as membrane solubilization. As a result of the studies on lipid membrane solubilization by deoxycholic acid, important solubilization parameters of POPC and DPPC lipids which are critical bile acid/ lipid ratios (R_e^{sat} and R_e^{sol}) at saturation and solubilization conditions of membranes, surfactant concentrations in aqueous phase for saturation and solubilization (D_w^{sol} , D_w^{sat}) were determined from phase diagrams which are constructed by using lipid concentrations and total detergent concentration in the ITC sample cell. These values provide to elucidate the effects of geometry and state of lipids, temperature, and buffer on the formation of different structures and phases of lipid-bile acid systems. Therefore, vesicle-to-micelle transitions can be determined by phase diagrams depending on the experiment conditions. According to these phase diagrams, potassium ions have a shielding effect on bile acid micelles that causes the shift of surfactant concentration to lower concentrations to solubilize the lipid membranes. Also, solubilization of POPC, unsaturated lipid, require more surfactants and wider coexistence region occurs for solubilization of POPC that saturation degree of lipids is another parameter for activity of bile acids on lipid membranes and geometry of lipids effects the formation of lipid curvature. Furthermore, DPPC lipids in the gel-state require higher concentration of bile acids to saturate and solubilize the lipids and solubilization process of membranes occurs slowly.

Solubilizations of POPC and DPPC membranes by deoxycholic acid in the phosphate buffer at 25°C were performed by fluorescence spectroscopy by determination lamellerity of lipid vesicles, including certain ratio of deoxycholic acid. The results from fluorescence spectroscopy have shown same tendency with the results of titration calorimetric experiments eventhough they have technical differences in the methods. The concentration ratios of deoxycholic acid to lipids are almost similar with ratios, determined from the vesicle-to-micelle transition boundaries. However, ITC can provide more accurate concentration ranges because the results were obtained from reaction heats of membrane solubilization by bile acid that there are not external effects to impact the results. ITC is a widely-used efficient method to characterize the interactions between surfactant molecules and lipid macromolecules.

Furthermore, the measurements of particle size were performed by DLS during the preparation of liposomes to control the quality of obtained liposomes. Also, the results of POPC membrane solubilization by deoxycholic acid were confirmed by means of DLS by determination of particle size and polydispersity which can provide information about the structures and phases of mixed vesicles and micelles. For DCA-POPC systems, the changes of effective diameter and polydispersity have confirmed the different kind of phases in which bile acid-lipid systems occur. DSC, which can inform about the structural change, hence change of thermodynamic state, was used to study the effect of deoxycholic acid on transition temperature of DPPC.

To sum up, it has been known that bile acids have different structure, hence different aggregation properties than ordinary surfactants, such as forming small sized aggregates. Therefore, they are strong biological surfactants. Mimicking of gastrointestinal system with bile acids and phospholipids provides the understanding of effects of digestion of liposomes as delivery systems. In this study, it has been shown that DPPC is more stable than POPC to natural surfactant, deoxycholic acid, because of presence in gel-state at physiological temperature. Also, ionic strength of solutions is effective for solubilization process by surfactants. Conditions such as nature of lipid, temperature, properties of solutions can be chosen regarding to aim of research. It has been thought that the knowledge about critical values of partitioning of bile acids into membrane and solubilization of membranes will assist to formulate appropriate liposome systems. Colloidal delivery systems, consisting of proper ratio of bile acid and phospholipids can also result in formation of mixed micelles when they are started to digest in the gastrointestinal system that it makes them convenient vehicles for delivery of lipophilic bioactive compounds.

In this study, isothermal titration calorimetry, differential scanning calorimetry, dynamic light scattering and fluorescence spectroscopy have been utilized to investigate the interactions between bile acids and model lipid membranes. The principles of these techniques and effect of surfactants on lipid systems were reviewed before experimental part of study. Because colloidal systems, which are consisting of surfactants and lipids, have been suggested to use as a delivery tool for pharmaceuticals and nutraceuticals, biological relevant materials of these compounds can be thought as a better biocompatible vehicles. Also, the interactions between these compounds can be accepted analogous of interactions between drugs and cell

membranes. The results presented in this study can confirm the results in the literature and provide understanding the insight of pharmaceutical applications.

Different environmental conditions and lipid combinations can be studied to obtain the convenience colloidal systems as further researches. Also, other methods such as electron microscopy can be applied to investigate the mixed phospholipid- bile acid structures. As a result of understanding interactions, application of these phospholipid-bile acid mixed micellar structures in order to enhance bioavailability of nutraceutical compounds can be considered as next step of study. Following the developments in drug delivery science would provide to form of colloidal delivery systems for food ingredients by means of manipulation of solubility, improving better absorption and cellular uptake.

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APPENDICES

APPENDIX A: Equations for membrane partitioning and results

APPENDIX B: Membrane solubilization results and graphics

APPENDIX A

Equations of Partitioning of Charged Molecules into Lipid Membranes

$$\Delta G_{total} = \Delta G_{ins} + \Delta G_E \quad (\text{A.1.1})$$

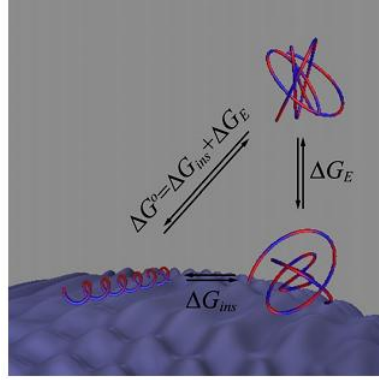


Figure A.1: Illustration of membrane partitioning process (Etzerodt et al.,2011).

$$\Delta G_E = -zF\varphi_s \quad (\text{A.1.2})$$

where z is a charge, F is a Faraday constant (96485.3365 C/mol) and φ_s is a membrane surface potential.

$$\Delta G_{total} = \Delta G_{ins} - zF\varphi_s \text{ (for overall partitioning process)} \quad (\text{A.1.3})$$

Chemical potentials

For water phase;

$$\mu_w = \mu_w^\theta + RT \ln a_w \quad (\text{A.1.4})$$

where a_w is a molar fraction of molecule in the water phase,

$$a_w = x_w = \frac{c_m}{c_{water} + c_m} \sim \frac{c_m}{c_{water}} \text{ and } c_{water} \text{ is } 55.5 \text{ M.}$$

For lipid phase;

$$\mu_m = \mu_m^\theta - zF\varphi_s + RT \ln a_m \quad (\text{A.1.5})$$

where a_m is a molar fraction of molecule in the lipid membrane phase, $a_m = x_m = \frac{c_m}{c_{lipid} + c_m} \sim \frac{c_m}{c_{lipid}} = r$

Free energy:

$$F = N_w \mu_w + N_m \mu_m \quad (\text{A.1.6})$$

$$N = N_w + N_m \quad (\text{A.1.7})$$

$$dF = dN_w \mu_w + dN_m \mu_m \quad (\text{A.1.8})$$

$$dN = dN_w + dN_m = 0 \text{ (at minimum energy, derivative must be equal to zero)} \quad (\text{A.1.9})$$

$$dF = -dN_m \mu_w + dN_m \mu_m \quad (\text{A.1.10})$$

$$dF = (\mu_m - \mu_w) dN_m = 0 \text{ (when free energy becomes minimum)} \quad (\text{A.1.11})$$

$$\mu_m = \mu_w \quad (\text{A.1.12})$$

$$\mu_m^\theta - zF\phi_s + RT \ln a_m = \mu_w^\theta + RT \ln a_w \quad (\text{A.1.13})$$

$$zF\phi_s = RT \ln \frac{a_m}{a_w} \quad (\text{A.1.14})$$

$$\frac{x_m}{x_w} = e^{\frac{zF\phi_s}{RT}} \quad (\text{A.1.15})$$

$$\frac{x_m}{x_w} = e^{\left(\frac{-\Delta G_{ins}}{RT}\right)} e^{\left(\frac{-zF\phi_s}{RT}\right)} \quad (\text{A.1.16})$$

$$\frac{x_m}{x_w} = K_{ins} e^{\left(\frac{-zF\phi_s}{RT}\right)} = K_{eff} \quad (\text{A.1.17})$$

$$K_{eff} = e^{\frac{-\Delta G_{total}}{RT}} \quad (\text{A.1.18})$$

Surface charge density, $\sigma = \frac{e}{A}$

The standard free energy and the partitioning coefficient are functions of the membrane surface potential, ϕ_s . Also, it is depending on the r , the ratio of bounded surfactant concentration in the membrane to lipid concentration (C_m/C_{lipid}).

According to Gouy-Chapman equation,

$$\phi_s = \frac{2RT}{F} \sinh^{-1} \left(\frac{\sigma}{\sqrt{8000 RTC_0 \epsilon_0 \epsilon_r}} \right) \quad (\text{A.1.19})$$

where C_0 is a concentration of electrolyte, ϵ_0 and ϵ_r are vacuum permittivity and relative permittivity, respectively.

F is a Faraday constant, $F = eN_A$ (and $k_B = R/N_A$).

In this situation, the surface charge density, σ ,

$$\sigma = ze_0 \frac{r/A_{lipid}}{1 + r(A_{surfactant}/A_{lipid})} \quad (\text{A.1.20})$$

Where A_{lipid} is a cross-sectional area of lipid (A_{lipid} is assumed as $70 \text{ \AA}^2$)

Q_i is obtained from membrane partitioning experiments by means of isothermal titration calorimeter that gives reaction heat as a result of injection of highly concentrated lipid into surfactant monomer solution in ITC cell.

$$Q_i = \Delta n \Delta H + Q_{dil} \quad (\text{A.1.21})$$

where Q_{dil} is the heat of lipid dilution, ΔH is a molar enthalpy, and Δn is the number of molecule. C_{lipid} and C_m can be calculated, when z , ΔG_{ins} , C_{lipid} , and $C_{surfactant}$ are known.

Membrane Partitioning Results

- **Partitioning of bile acids into DPPC liposome**

filename : 50mMDPPCinto200uMDCA_C25.txt

Fitting range [2;25]

zpep = 1.82671qe

%charge = 0 (%PG)

tK = 298K

alip = 70Å

$\wedge^2$

alpha = -14.0449kbT (-34799.6J/mol)

Geff_low = 10.854kbT Geff_high = 14.0449kbT

H = 4.94593e+10Cal/mol (2.06938e+11J/mol)

q = -5.63503e+07uCal

Removed injection 8, from the Chi2 fit

Chi2 = 8.371 (based on 22 injections)

filename : 50mMDPPCinto200uMDCA_C25_2.txt

Fitting range [2;25]

zpep = 2.58181qe

%charge = 0 (%PG)

tK = 298K

alip = 70Å

$\wedge^2$

alpha = -14.7163kbT (-36463.3J/mol)

Geff_low = 10.2409kbT Geff_high = 14.7163kbT

H = 6.1516e+10Cal/mol (2.57383e+11J/mol)

q = -1.27913e+08uCal

Removed injection 9, from the Chi2 fit

Chi2 = 2.46633 (based on 22 injections)

filename : 50mMDPPCinto200uMDCA_C25_3.txt

Fitting range [2;25]

zpep = 3.13919qe

%charge = 0 (%PG)

tK = 298K

alip = 70Å

$\wedge^2$

alpha = -16.5045kbT (-40894J/mol)

Geff_low = 10.222kbT Geff_high = 16.5045kbT

H = 6.62973e+10Cal/mol (2.77388e+11J/mol)

q = -2.08001e+08uCal

Removed injection 2,5, from the Chi2 fit

Chi2 = 4.85325 (based on 21 injections)

filename : 50mMDPPCintoCDCA.txt

Fitting range [2;25]

zpep = 4.08634qe

%charge = 0 (%PG)

tK = 298K

alip = 70Å
^2
alpha = -18.7638kbT (-46492J/mol)
Geff_low = 9.97667kbT Geff_high = 18.7638kbT
H = 6.28288e+10Cal/mol (2.62875e+11J/mol)
q = -2.43374e+08uCal
Removed injection 24, from the Chi2 fit
Chi2 = 14.3808 (based on 22 injections)

filename : 50mMDPPCinto200uMCDCA_C25_2.txt
Fitting range [2;25]
zpep = 4.08362qe
%charge = 0 (%PG)
tK = 298K
alip = 70Å
^2
alpha = -20.7731kbT (-51470.4J/mol)
Geff_low = 10.3427kbT Geff_high = 20.7731kbT
H = 4.22133e+10Cal/mol (1.7662e+11J/mol)
q = -1.94468e+08uCal
Removed injection 24, from the Chi2 fit
Chi2 = 24.5678 (based on 22 injections)

filename : 50mMDPPCinto200uMCDCA_C25_3.txt
Fitting range [2;25]
zpep = 3.21037qe
%charge = 0 (%PG)
tK = 298K
alip = 70Å
^2
alpha = -16.9047kbT (-41885.7J/mol)
Geff_low = 10.2568kbT Geff_high = 16.9047kbT
H = 3.21666e+10Cal/mol (1.34585e+11J/mol)
q = -1.49229e+08uCal
Removed injection from the Chi2 fit
Chi2 = 68.8956 (based on 23 injections)

filename : 50mMDPPCinto200uMTCA_C25.txt
Fitting range [2;25]
zpep = 0qe
%charge = 0 (%PG)
tK = 298K
alip = 70Å
^2
alpha = -19.0833kbT (-47283.5J/mol)
Geff_low = 19.0833kbT Geff_high = 19.0833kbT
H = -2.25084e+11Cal/mol (-9.4175e+11J/mol)
q = -1.94385e+08uCal
Removed injection 15, from the Chi2 fit
Chi2 = 119.881 (based on 22 injections)

filename : 50mMDPPCinto200uMGCA_C25.txt
 Fitting range [2;25]
 zpep = 0qe
 %charge = 0 (%PG)
 tK = 298K
 alip = 70Å
 ^2
 alpha = -18.0098kbT (-44623.8J/mol)
 Geff_low = 18.0098kbT Geff_high = 18.0098kbT
 H = -3.87127e+11Cal/mol (-1.61974e+12J/mol)
 q = -2.34924e+08uCal
 Removed injection 13, from the Chi2 fit
 Chi2 = 46.7527 (based on 22 injections)

- **Partitioning of bile acids into POPC liposome**

filename : 50mMPOPCinto200uMDCA_C25.txt
 Fitting range [2;25]
 zpep = 2qe
 %charge = 0 (%PG)
 tK = 298K
 alip = 70Å
 ^2
 alpha = -13.211kbT (-32733.4J/mol)
 Geff_low = 10.3067kbT Geff_high = 13.211kbT
 H = -3.80854e+10Cal/mol (-1.59349e+11J/mol)
 q = -4.00037e+07uCal
 Removed injection 16, from the Chi2 fit
 Chi2 = 173.841 (based on 22 injections)

filename : 50mMPOPCinto200uMDCA_C25_2.txt
 Fitting range [2;25]
 zpep = 2qe
 %charge = 0 (%PG)
 tK = 298K
 alip = 70Å
 ^2
 alpha = -13.9247kbT (-34501.9J/mol)
 Geff_low = 10.5835kbT Geff_high = 13.9247kbT
 H = -3.39244e+10Cal/mol (-1.4194e+11J/mol)
 q = -4.00926e+07uCal
 Removed injection 11, from the Chi2 fit
 Chi2 = 107.199 (based on 22 injections)

filename : 50mMPOPCinto200uMDCA_C25_3.txt
 Fitting range [2;25]
 zpep = 2qe
 %charge = 0 (%PG)
 tK = 298K

alip = 70Å
^2
alpha = -13.0492kbT (-32332.6J/mol)
Geff_low = 10.244kbT Geff_high = 13.0492kbT
H = -2.93212e+10Cal/mol (-1.2268e+11J/mol)
q = -1.0215e+07uCal
Removed injection from the Chi2 fit
Chi2 = 703.569 (based on 23 injections)

filename : 50mMPOPCinto200uMCDCA_C25.txt
Fitting range [2;25]
zpep = 2qe
%charge = 0 (%PG)
tK = 298K
alip = 70Å
^2
alpha = -13.3553kbT (-33091J/mol)
Geff_low = 10.3625kbT Geff_high = 13.3553kbT
H = -6.64865e+10Cal/mol (-2.7818e+11J/mol)
q = -1.68835e+08uCal
Removed injection from the Chi2 fit
Chi2 = 11.6836 (based on 23 injections)

filename : 50mMPOPCinto200uMCDCA_C25_2.txt
Fitting range [2;25]
zpep = 2qe
%charge = 0 (%PG)
tK = 298K
alip = 70Å
^2
alpha = -14.0518kbT (-34816.8J/mol)
Geff_low = 10.6332kbT Geff_high = 14.0518kbT
H = -4.71246e+10Cal/mol (-1.97169e+11J/mol)
q = -3.38904e+07uCal
Removed injection 13, from the Chi2 fit
Chi2 = 176.394 (based on 22 injections)

filename : 50mMPOPCinto200uMCDCA_C25_3.txt
Fitting range [2;25]
zpep = 2qe
%charge = 0 (%PG)
tK = 298K
alip = 70Å
^2
alpha = -13.291kbT (-32931.7J/mol)
Geff_low = 10.3376kbT Geff_high = 13.291kbT
H = -4.10482e+10Cal/mol (-1.71746e+11J/mol)
q = -2.61103e+07uCal
Removed injection from the Chi2 fit
Chi2 = 128.048 (based on 23 injections)

filename : 50mMPOPCinto200uMGCA_C25.txt
Fitting range [2;25]
zpep = 2qe
%charge = 0 (%PG)
tK = 298K
alip = 70Å
^2
alpha = -10.8678kbT (-26927.8J/mol)
Geff_low = 9.34523kbT Geff_high = 10.8678kbT
H = -4.07819e+10Cal/mol (-1.70631e+11J/mol)
q = -6.67967e+07uCal
Removed injection 4,7, from the Chi2 fit
Chi2 = 5.09839 (based on 21 injections)

APPENDIX B

Membrane solubilization results and graphics

injection	mean Q (μJ)	dQ/dn	inj volume (μL)	moles titrant (moles) (DCA)	moles titrate (moles) (POPC)	moles titrant / moles titrate	conc.of det(mM)	total volume (μL)
1	-53,74727056	-12,3253	5	2,50E-07	9,39E-07	0,266240682	0,264830508	944
2	-12,06840833	65,81198	5	4,99E-07	9,34E-07	0,533899045	0,528258313	944
3	51,355775	35,82416	5	7,46E-07	9,29E-07	0,80298264	0,790290843	944
4	51,08065833	-12,7692	5	9,92E-07	9,24E-07	1,073499054	1,050935489	944
5	21,55535	-29,3303	5	1,24E-06	9,19E-07	1,345455918	1,310199602	944
6	-97,242	-516,156	5	1,48E-06	9,14E-07	1,618860902	1,568090494	944
7	-783,2621333	-60,6889	5	1,72E-06	9,10E-07	1,893721716	1,824615438	944
8	-193,5812083	503,3173	5	1,96E-06	9,05E-07	2,170046113	2,07978167	944
9	-20,891625	38,88911	5	2,20E-06	9,00E-07	2,447841885	2,333596386	944
10	-38,08496667	-7,65595	5	2,44E-06	8,95E-07	2,727116869	2,586066744	944
11	-5,961533333	34,28257	5	2,68E-06	8,90E-07	3,007878939	2,837199865	944
12	17,7309	9,004283	5	2,91E-06	8,86E-07	3,290136016	3,087002832	944
13	14,244475	-1,60034	5	3,15E-06	8,81E-07	3,573896058	3,33548269	944
14	15,51170833	-8,69075	5	3,38E-06	8,76E-07	3,85916707	3,582646447	944
15	-4,511016667	-23,4996	5	3,61E-06	8,72E-07	4,145957097	3,828501074	944
16	-28,02405833	-23,9824	5	3,84E-06	8,67E-07	4,434274228	4,073053504	944
17	-52,04618333	-24,7802	5	4,07E-06	8,63E-07	4,724126593	4,316310636	944
18	-76,98119167	-23,194	5	4,30E-06	8,58E-07	5,015522369	4,55827933	944
19	-98,806025	-23,7011	5	4,53E-06	8,53E-07	5,308469772	4,79896641	944
20	-123,7742917	-23,0818	5	4,76E-06	8,49E-07	5,602977066	5,038378664	944
21	-141,9782417	-9,14349	5	4,98E-06	8,44E-07	5,899052556	5,276522845	944
22	-145,377	-10,1132	5	5,20E-06	8,40E-07	6,196704593	5,513405669	944
23	-161,9058417	-7,97825	5	5,43E-06	8,35E-07	6,495941572	5,749033816	944
24	-161,8370583	-8,43573	5	5,65E-06	8,31E-07	6,796771932	5,983413934	944
25	-177,9197167	-5,91195	5	5,87E-06	8,27E-07	7,099204157	6,216552631	944
26	-172,2592583	3,293453	5	6,09E-06	8,22E-07	7,403246778	6,448456484	944

27	-174,34005	-0,49693	5	6,31E-06	8,18E-07	7,708908369	6,679132032	944
28	-172,7211583	-1,32005	5	6,52E-06	8,14E-07	8,016197551	6,908585782	944
29	-175,651725	3,302834	5	6,74E-06	8,09E-07	8,32512299	7,136824205	944
30	-166,9120667	4,74529	5	6,95E-06	8,05E-07	8,6356934	7,363853738	944
31	-167,501325	0,661733	5	7,16E-06	8,01E-07	8,94791754	7,589680784	944
32	-164,4614333	1,651595	5	7,38E-06	7,96E-07	9,261804214	7,814311712	944
33	-163,98745	2,948466	5	7,59E-06	7,92E-07	9,577362277	8,037752857	944
34	-158,6202083	4,125333	5	7,80E-06	7,88E-07	9,894600628	8,260010522	944
35	-156,1531167	3,362998	5	8,01E-06	7,84E-07	10,21352821	8,481090975	944
36	-151,2907667	5,306183	5	8,21E-06	7,80E-07	10,53415403	8,701000451	944
37	-145,7078917	7,249683	5	8,42E-06	7,76E-07	10,85648712	8,919745152	944
38	-138,7326083	0,363256	5	8,63E-06	7,71E-07	11,18053657	9,137331247	944
39	-142,6840417	3,475042	5	8,83E-06	7,67E-07	11,50631153	9,353764874	944
40	-131,4590417	7,634239	5	9,03E-06	7,63E-07	11,83382117	9,569052136	944
41	-129,2198667	4,412047	5	9,24E-06	7,59E-07	12,16307475	9,783199106	944
42	-122,630075	0,967535	5	9,44E-06	7,55E-07	12,49408153	9,996211823	944
43	-124,996875	7,135269	5	9,64E-06	7,51E-07	12,82685087	10,20809629	944
44	-109,285525	9,408103	5	9,84E-06	7,47E-07	13,16139214	10,4188585	944
45	-108,0866917	4,098585	5	1,00E-05	7,43E-07	13,49771478	10,62850437	944
46	-100,2458417	2,979053	5	1,02E-05	7,39E-07	13,83582828	10,83703984	944
47	-101,862425	2,40204	5	1,04E-05	7,35E-07	14,17574217	11,04447077	944
48	-95,24003333	2,411149	5	1,06E-05	7,32E-07	14,51746604	11,25080302	944
49	-96,86470833	-4,67312	5	1,08E-05	7,28E-07	14,86100952	11,45604241	944
50	-89,19789167	58,34126	5	1,10E-05	7,24E-07	15,20638231	11,66019473	944
51	2,552183333	51,3932	5	1,12E-05	7,20E-07	15,55359414	11,86326573	944

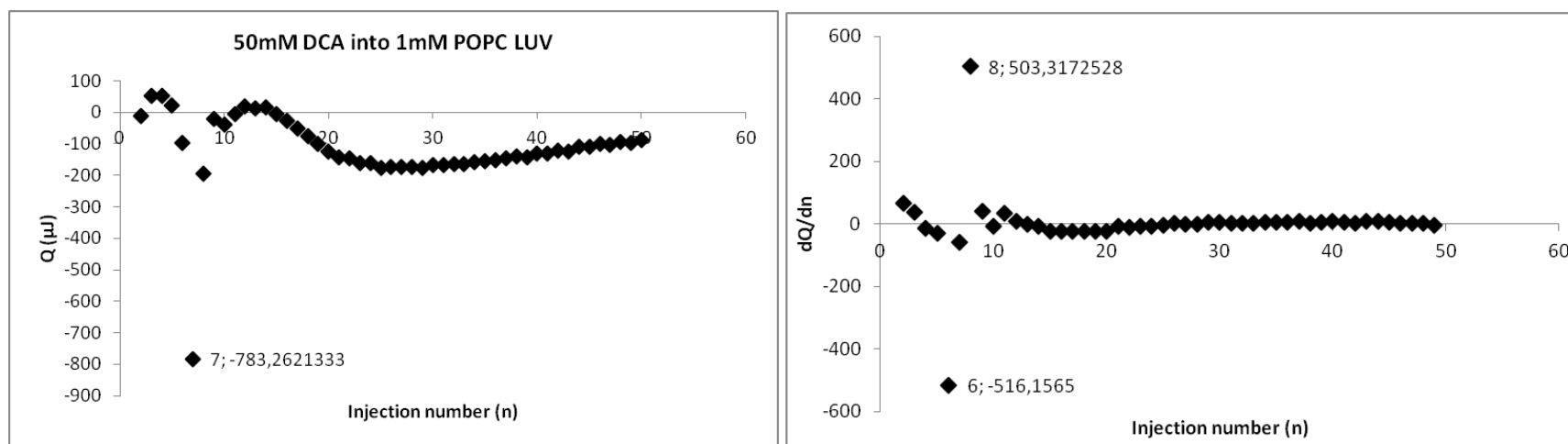


Figure B. 2: Titration of 50 mM DCA into 1mM POPC LUVs solutions, in phosphate buffer, pH 8,0.

injection	Mean Q (μJ)	dQ/dn	inj volume (μL)	moles titrant (moles)(DCA)	moles titrate (moles)(POPC)	moles titrant / moles titrate	conc.of det. (mM)	total volume (μL)
1	-149,3294342	-90,14602917	5	2,50E-07	1,88E-06	0,133120341	0,264830508	944
2	-147,535275	36,92472143	5	4,99E-07	1,87E-06	0,266949523	0,528258313	944
3	-98,52985	65,49299923	5	7,46E-07	1,86E-06	0,40149132	0,790290843	944
4	-36,69998333	26,2819375	5	9,92E-07	1,85E-06	0,536749527	1,050935489	944
5	-48,56309167	-14,41578403	5	1,24E-06	1,84E-06	0,672727959	1,310199602	944
6	-63,18445833	-14,18347847	5	1,48E-06	1,83E-06	0,809430451	1,568090494	944
7	-137,4162417	-270,4689104	5	1,72E-06	1,82E-06	0,946860858	1,824615438	944
8	-577,3234417	-449,6700583	5	1,96E-06	1,81E-06	1,085023056	2,07978167	944
9	-916,0480333	-219,0875021	5	2,20E-06	1,80E-06	1,223920943	2,333596386	944
10	-896,1980917	529,1762528	5	2,44E-06	1,79E-06	1,363558434	2,586066744	944
11	-59,36341667	480,4576799	5	2,68E-06	1,78E-06	1,50393947	2,837199865	944
12	-73,96154167	-93,85692778	5	2,91E-06	1,77E-06	1,645068008	3,087002832	944
13	-103,6477917	-29,71484167	5	3,15E-06	1,76E-06	1,786948029	3,33548269	944
14	-124,1899583	0,204050694	5	3,38E-06	1,75E-06	1,929583535	3,582646447	944
15	-104,61265	22,16750347	5	3,61E-06	1,74E-06	2,072978549	3,828501074	944
16	-84,12901667	29,40905833	5	3,84E-06	1,73E-06	2,217137114	4,073053504	944
17	-49,1703	26,68987222	5	4,07E-06	1,73E-06	2,362063297	4,316310636	944
18	-33,55985833	14,24487847	5	4,30E-06	1,72E-06	2,507761184	4,55827933	944
19	-20,33785	5,297304861	5	4,53E-06	1,71E-06	2,654234886	4,79896641	944
20	-24,40795833	-9,715373611	5	4,76E-06	1,70E-06	2,801488533	5,038378664	944
21	-39,52275833	-27,42547083	5	4,98E-06	1,69E-06	2,949526278	5,276522845	944
22	-70,45464167	-8,83800625	5	5,20E-06	1,68E-06	3,098352297	5,513405669	944
23	-59,60566667	-0,91119375	5	5,43E-06	1,67E-06	3,247970786	5,749033816	944
24	-79,01515	-21,50924028	5	5,65E-06	1,66E-06	3,398385966	5,983413934	944
25	-97,0725	-16,42976458	5	5,87E-06	1,65E-06	3,549602079	6,216552631	944
26	-112,078425	-15,02934444	5	6,09E-06	1,64E-06	3,701623389	6,448456484	944

27	-126,9546917	-12,85353403	5	6,31E-06	1,64E-06	3,854454184	6,679132032	944
28	-137,72055	-10,46690694	5	6,52E-06	1,63E-06	4,008098775	6,908585782	944
29	-147,9670917	-9,117915972	5	6,74E-06	1,62E-06	4,162561495	7,136824205	944
30	-154,5747417	-0,721768056	5	6,95E-06	1,61E-06	4,3178467	7,363853738	944
31	-152,3732333	-6,126206944	5	7,16E-06	1,60E-06	4,47395877	7,589680784	944
32	-164,3084667	0,869943056	5	7,38E-06	1,59E-06	4,630902107	7,814311712	944
33	-152,3224083	0,367877083	5	7,59E-06	1,58E-06	4,788681139	8,037752857	944
34	-164,6074583	-3,584026389	5	7,80E-06	1,58E-06	4,947300314	8,260010522	944
35	-159,1796917	-5,672957639	5	8,01E-06	1,57E-06	5,106764107	8,481090975	944
36	-176,1584167	-10,72011181	5	8,21E-06	1,56E-06	5,267077015	8,701000451	944
37	-176,6545833	1,582098611	5	8,42E-06	1,55E-06	5,428243559	8,919745152	944
38	-175,76525	2,740642361	5	8,63E-06	1,54E-06	5,590268285	9,137331247	944
39	-175,0195417	-14,51104375	5	8,83E-06	1,53E-06	5,753155763	9,353764874	944
40	-195,9657917	7,962534722	5	9,03E-06	1,53E-06	5,916910586	9,569052136	944
41	-164,1263917	10,11701042	5	9,24E-06	1,52E-06	6,081537373	9,783199106	944
42	-184,1704667	-27,43158889	5	9,44E-06	1,51E-06	6,247040767	9,996211823	944
43	-202,0610667	11,23416181	5	9,64E-06	1,50E-06	6,413425435	10,20809629	944
44	-170,264125	10,60546319	5	9,84E-06	1,49E-06	6,580696071	10,4188585	944
45	-187,6856	-17,74009931	5	1,00E-05	1,49E-06	6,748857392	10,62850437	944
46	-196,4322917	-5,253570833	5	1,02E-05	1,48E-06	6,917914141	10,83703984	944
47	-198,5252083	2,121609722	5	1,04E-05	1,47E-06	7,087871085	11,04447077	944
48	-193,9381417	2,151353472	5	1,06E-05	1,46E-06	7,258733018	11,25080302	944
49	-193,1917167	-7,820938194	5	1,08E-05	1,46E-06	7,43050476	11,45604241	944
50	-179,5806	119,4238882	5	1,10E-05	1,45E-06	7,603191154	11,66019473	944
51	10,18638333	103,6210903	5	1,12E-05	1,44E-06	7,77679707	11,86326573	944

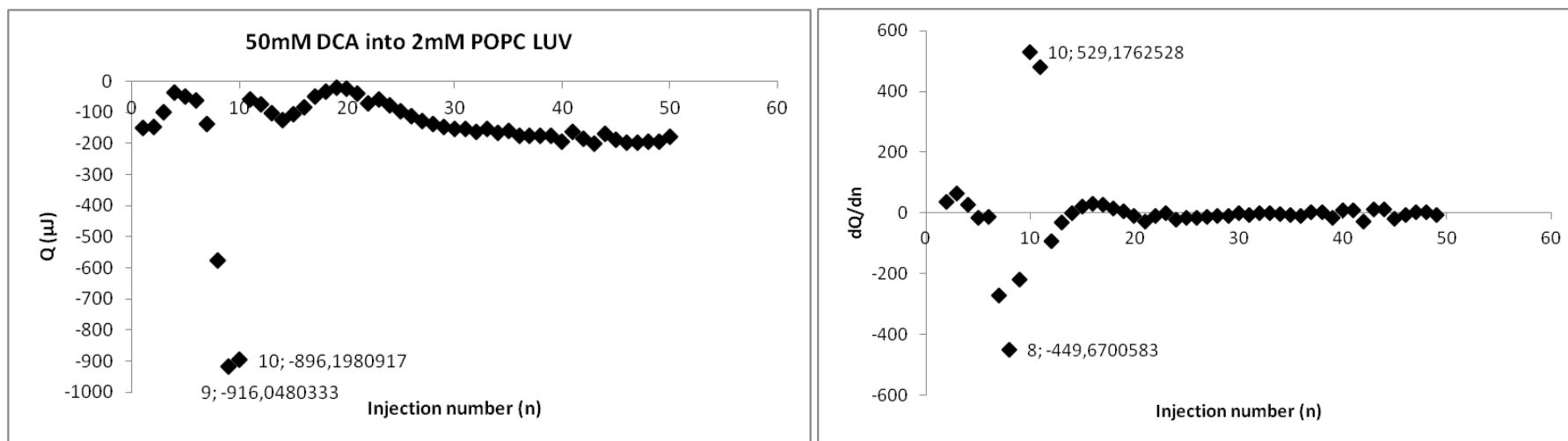


Figure B.3: Titration of 50 mM DCA into 2 mM POPC LUVs solutions, in phosphate buffer, pH 8,0.

injection	Mean Q (μJ)	dQ/dn	inj volume (μL)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	conc. of det. (mM)	total volume (μL)
1	-467,6282227	-297,754	5	2,50E-07	4,70E-06	0,053248136	0,264830508	944
2	-501,686075	47,56654	5	4,99E-07	4,67E-06	0,106779809	0,528258313	944
3	-440,4385833	79,83928	5	7,46E-07	4,65E-06	0,160596528	0,790290843	944
4	-353,2814	114,5017	5	9,92E-07	4,62E-06	0,214699811	1,050935489	944
5	-238,4622083	40,63779	5	1,24E-06	4,60E-06	0,269091184	1,310199602	944
6	-259,8957917	25,40416	5	1,48E-06	4,57E-06	0,32377218	1,568090494	944
7	-181,0072	38,19892	5	1,72E-06	4,55E-06	0,378744343	1,824615438	944
8	-198,491275	-19,4472	5	1,96E-06	4,52E-06	0,434009223	2,07978167	944
9	-205,6131417	5,492265	5	2,20E-06	4,50E-06	0,489568377	2,333596386	944
10	-223,3774417	-106,42	5	2,44E-06	4,48E-06	0,545423374	2,586066744	944
11	-446,0037083	-349,652	5	2,68E-06	4,45E-06	0,601575788	2,837199865	944
12	-844,574875	-289,51	5	2,91E-06	4,43E-06	0,658027203	3,087002832	944
13	-979,3690833	-68,5113	5	3,15E-06	4,41E-06	0,714779212	3,33548269	944
14	-1016,184725	2,128848	5	3,38E-06	4,38E-06	0,771833414	3,582646447	944
15	-996,7473583	5,594806	5	3,61E-06	4,36E-06	0,829191419	3,828501074	944
16	-1009,14725	-36,0203	5	3,84E-06	4,34E-06	0,886854846	4,073053504	944
17	-990,20695	247,2574	5	4,07E-06	4,31E-06	0,944825319	4,316310636	944
18	-531,6178333	491,7876	5	4,30E-06	4,29E-06	1,003104474	4,55827933	944
19	-143,601175	193,5489	5	4,53E-06	4,27E-06	1,061693954	4,79896641	944
20	-137,752525	-40,8333	5	4,76E-06	4,24E-06	1,120595413	5,038378664	944
21	-161,8716667	-27,6065	5	4,98E-06	4,22E-06	1,179810511	5,276522845	944
22	-187,7816167	-27,7532	5	5,20E-06	4,20E-06	1,239340919	5,513405669	944
23	-212,5562833	-9,30642	5	5,43E-06	4,18E-06	1,299188314	5,749033816	944
24	-210,1907417	-9,04826	5	5,65E-06	4,16E-06	1,359354386	5,983413934	944
25	-229,4676583	-1,95319	5	5,87E-06	4,13E-06	1,419840831	6,216552631	944
26	-214,4935167	3,333294	5	6,09E-06	4,11E-06	1,480649356	6,448456484	944
27	-223,54015	4,815915	5	6,31E-06	4,09E-06	1,541781674	6,679132032	944

28	-202,7702	16,45968	5	6,52E-06	4,07E-06	1,60323951	6,908585782	944
29	-193,4721	16,24752	5	6,74E-06	4,05E-06	1,665024598	7,136824205	944
30	-171,4652917	12,4334	5	6,95E-06	4,02E-06	1,72713868	7,363853738	944
31	-168,0711417	10,81056	5	7,16E-06	4,00E-06	1,789583508	7,589680784	944
32	-148,7633583	14,71797	5	7,38E-06	3,98E-06	1,852360843	7,814311712	944
33	-141,5833167	5,371372	5	7,59E-06	3,96E-06	1,915472455	8,037752857	944
34	-136,1783917	5,186374	5	7,80E-06	3,94E-06	1,978920126	8,260010522	944
35	-131,8478667	1,30042	5	8,01E-06	3,92E-06	2,042705643	8,481090975	944
36	-133,11625	-0,1325	5	8,21E-06	3,90E-06	2,106830806	8,701000451	944
37	-132,691225	-4,62197	5	8,42E-06	3,88E-06	2,171297424	8,919745152	944
38	-141,3353167	-5,34971	5	8,63E-06	3,86E-06	2,236107314	9,137331247	944
39	-142,1367667	-0,89134	5	8,83E-06	3,84E-06	2,301262305	9,353764874	944
40	-144,4840167	-2,2812	5	9,03E-06	3,82E-06	2,366764234	9,569052136	944
41	-147,1847417	-4,90089	5	9,24E-06	3,80E-06	2,432614949	9,783199106	944
42	-154,3447417	-8,30724	5	9,44E-06	3,78E-06	2,498816307	9,996211823	944
43	-162,2119333	-4,86965	5	9,64E-06	3,76E-06	2,565370174	10,20809629	944
44	-165,0146333	-6,14957	5	9,84E-06	3,74E-06	2,632278429	10,4188585	944
45	-174,108075	-5,14354	5	1,00E-05	3,72E-06	2,699542957	10,62850437	944
46	-175,7190167	-6,81525	5	1,02E-05	3,70E-06	2,767165656	10,83703984	944
47	-186,1244917	-0,87267	5	1,04E-05	3,68E-06	2,835148434	11,04447077	944
48	-179,362925	-3,34325	5	1,06E-05	3,66E-06	2,903493207	11,25080302	944
49	-192,7872833	-22,3483	5	1,08E-05	3,64E-06	2,972201904	11,45604241	944
50	-188,9024	117,404	5	1,10E-05	3,62E-06	3,041276461	11,66019473	944
51	5,73915	109,8693	5	1,12E-05	3,60E-06	3,110718828	11,86326573	944

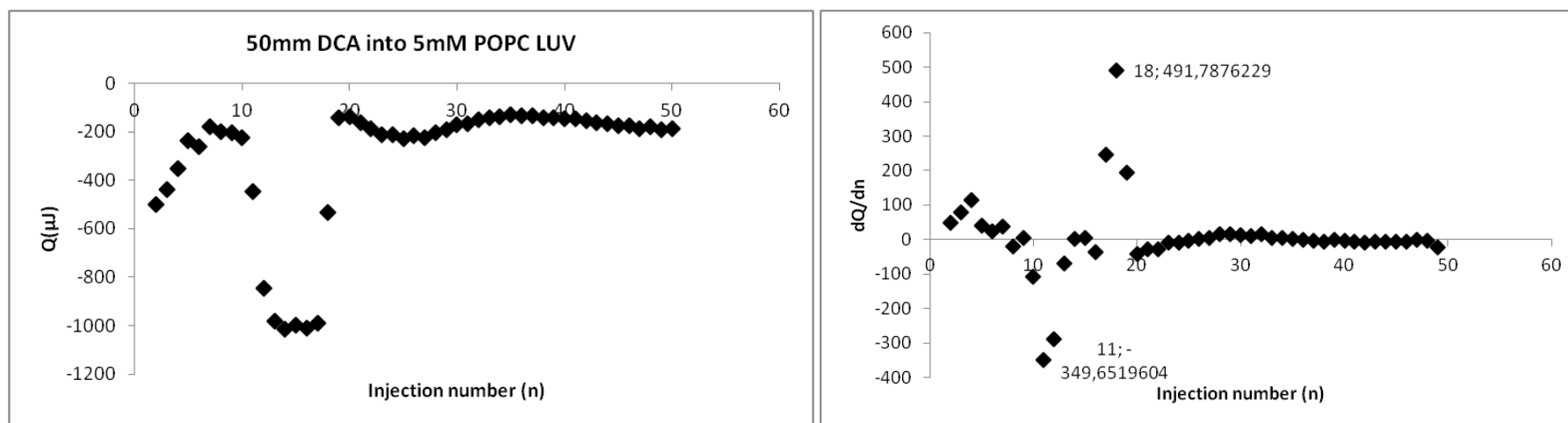


Figure B.4: Titration of 50 mM DCA into 5 mM POPC LUVs solutions, in phosphate buffer, pH 8,0.

injection	mean Q (μJ)	dQ/dn	inj volume (μL)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	conc. of det. (mM)	total volume (μL)
1	-425,0255778	-407,701	5	2,50E-07	9,39E-06	0,026624068	0,264830508	944
2	-687,4579917	-74,7782	5	4,99E-07	9,34E-06	0,053389905	0,528258313	944
3	-607,2521083	92,87703	5	7,46E-07	9,29E-06	0,080298264	0,790290843	944
4	-560,474225	36,48589	5	9,92E-07	9,24E-06	0,107349905	1,050935489	944
5	-523,6798583	52,47661	5	1,24E-06	9,19E-06	0,134545592	1,310199602	944
6	-456,71065	46,75931	5	1,48E-06	9,14E-06	0,16188609	1,568090494	944
7	-406,862875	169,065	5	1,72E-06	9,10E-06	0,189372172	1,824615438	944
8	-187,0500833	-6,70124	5	1,96E-06	9,05E-06	0,217004611	2,07978167	944
9	-395,1758417	-73,9397	5	2,20E-06	9,00E-06	0,244784189	2,333596386	944
10	-282,7994583	82,20225	5	2,44E-06	8,95E-06	0,272711687	2,586066744	944
11	-285,582075	-19,1506	5	2,68E-06	8,90E-06	0,300787894	2,837199865	944
12	-296,7269	6,137708	5	2,91E-06	8,86E-06	0,329013602	3,087002832	944
13	-276,788325	7,313665	5	3,15E-06	8,81E-06	0,357389606	3,33548269	944
14	-286,10195	0,143735	5	3,38E-06	8,76E-06	0,385916707	3,582646447	944
15	-288,3464583	-30,3987	5	3,61E-06	8,72E-06	0,41459571	3,828501074	944
16	-390,9167917	-251,154	5	3,84E-06	8,67E-06	0,443427423	4,073053504	944
17	-750,5231167	-320,793	5	4,07E-06	8,63E-06	0,472412659	4,316310636	944
18	-969,670075	-156,418	5	4,30E-06	8,58E-06	0,501552237	4,55827933	944
19	-1068,855617	-32,7608	5	4,53E-06	8,53E-06	0,530846977	4,79896641	944
20	-1060,555625	-4,49547	5	4,76E-06	8,49E-06	0,560297707	5,038378664	944
21	-1084,477683	16,22877	5	4,98E-06	8,44E-06	0,589905256	5,276522845	944
22	-1040,70095	-12,8451	5	5,20E-06	8,40E-06	0,619670459	5,513405669	944
23	-1104,763492	-14,4002	5	5,43E-06	8,35E-06	0,649594157	5,749033816	944
24	-1068,700692	-17,4414	5	5,65E-06	8,31E-06	0,679677193	5,983413934	944
25	-1135,673783	-6,32965	5	5,87E-06	8,27E-06	0,709920416	6,216552631	944
26	-1078,686975	10,6183	5	6,09E-06	8,22E-06	0,740324678	6,448456484	944
27	-1108,698017	13,57468	5	6,31E-06	8,18E-06	0,770890837	6,679132032	944

28	-980,3142083	328,4673	5	6,52E-06	8,14E-06	0,801619755	6,908585782	944
29	-511,5878667	410,8954	5	6,74E-06	8,09E-06	0,832512299	7,136824205	944
30	-243,4139417	174,6221	5	6,95E-06	8,05E-06	0,86356934	7,363853738	944
31	-144,240875	40,58956	5	7,16E-06	8,01E-06	0,894791754	7,589680784	944
32	-137,0031917	-8,70521	5	7,38E-06	7,96E-06	0,926180421	7,814311712	944
33	-147,3765917	-15,1573	5	7,59E-06	7,92E-06	0,957736228	8,037752857	944
34	-164,0371667	-15,2384	5	7,80E-06	7,88E-06	0,989460063	8,260010522	944
35	-178,6254	-20,6949	5	8,01E-06	7,84E-06	1,021352821	8,481090975	944
36	-204,1327667	-22,1379	5	8,21E-06	7,80E-06	1,053415403	8,701000451	944
37	-219,8030167	-10,7248	5	8,42E-06	7,76E-06	1,085648712	8,919745152	944
38	-227,8035667	-8,78653	5	8,63E-06	7,71E-06	1,118053657	9,137331247	944
39	-239,2946083	-14,9512	5	8,83E-06	7,67E-06	1,150631153	9,353764874	944
40	-254,6271667	-7,84322	5	9,03E-06	7,63E-06	1,183382117	9,569052136	944
41	-254,9773917	-0,78166	5	9,24E-06	7,59E-06	1,216307475	9,783199106	944
42	-259,1471833	-7,07487	5	9,44E-06	7,55E-06	1,249408153	9,996211823	944
43	-266,074825	1,566015	5	9,64E-06	7,51E-06	1,282685087	10,20809629	944
44	-258,5081833	-2,46228	5	9,84E-06	7,47E-06	1,316139214	10,4188585	944
45	-268,6575667	4,889385	5	1,00E-05	7,43E-06	1,349771478	10,62850437	944
46	-250,2618167	5,366203	5	1,02E-05	7,39E-06	1,383582828	10,83703984	944
47	-258,7765167	1,598164	5	1,04E-05	7,35E-06	1,417574217	11,04447077	944
48	-243,8542167	12,60667	5	1,06E-05	7,32E-06	1,451746604	11,25080302	944
49	-236,5747333	-8,52298	5	1,08E-05	7,28E-06	1,486100952	11,45604241	944
50	-223,9276333	139,3365	5	1,10E-05	7,24E-06	1,520638231	11,66019473	944
51	2,9118625	129,5705	5	1,12E-05	7,20E-06	1,555359414	11,86326573	944

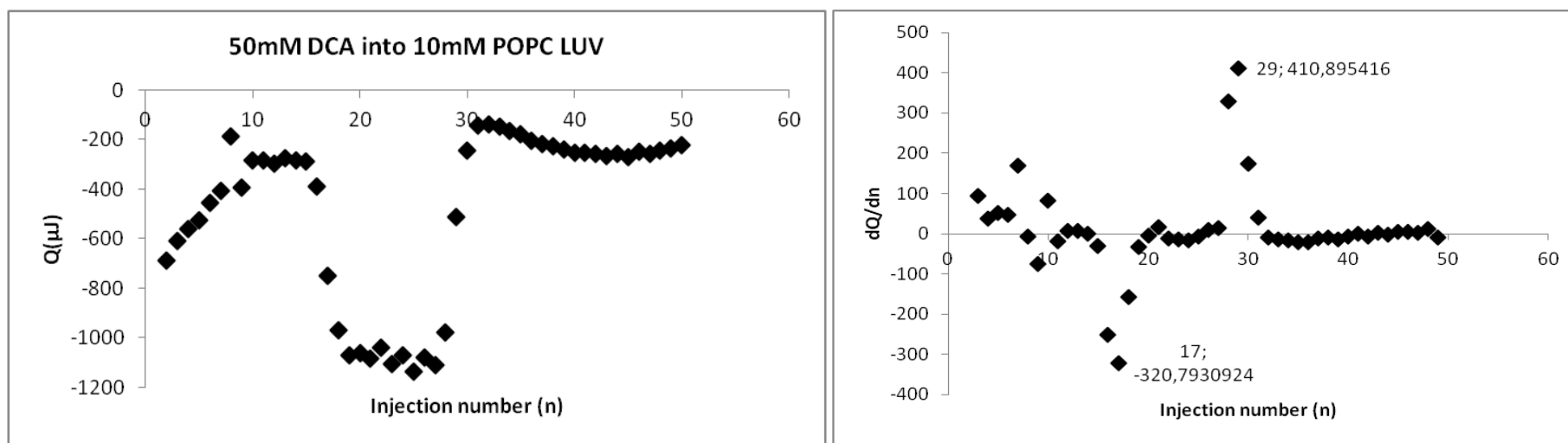


Figure B.5: Titration of 50 mM DCA into 10 mM POPC LUVs solutions, in phosphate buffer, pH 8,0.

injection	Mean Q	dQ/dn	inj volume (μ L)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	conc. of det. (mM)	total volume (μ L)
1	21,50356	67,22086	5,15	2,58E-07	1,89E-06	0,136265016	0,271052632	950
2	111,2548	33,88029	5,15	5,14E-07	1,88E-06	0,273272757	0,540635873	950
3	83,38817	-20,1614	5,15	7,68E-07	1,87E-06	0,411027274	0,808757689	950
4	88,51341	1,34686	5,15	1,02E-06	1,86E-06	0,549532635	1,075426002	950
5	81,5095	-5,44144	5,15	1,27E-06	1,85E-06	0,688792933	1,340648693	950
6	80,06309	2,033119	5,15	1,52E-06	1,84E-06	0,828812284	1,604433597	950
7	81,08285	-10,9168	5,15	1,77E-06	1,83E-06	0,969594825	1,86678851	950
8	60,70279	-14,1264	5,15	2,02E-06	1,82E-06	1,111144714	2,127721183	950
9	57,62888	2,346064	5,15	2,27E-06	1,81E-06	1,253466136	2,387239326	950
10	61,94793	4,276518	5,15	2,51E-06	1,80E-06	1,396563295	2,645350607	950
11	62,89119	-7,084	5,15	2,76E-06	1,79E-06	1,540440419	2,902062654	950
12	51,48308	-2,63674	5,15	3,00E-06	1,78E-06	1,68510176	3,157383051	950
13	58,91803	7,03767	5,15	3,24E-06	1,77E-06	1,830551593	3,411319343	950
14	61,80346	3,059014	5,15	3,48E-06	1,76E-06	1,976794214	3,663879033	950
15	61,00225	-17,7664	5,15	3,72E-06	1,75E-06	2,123833945	3,915069583	950
16	31,44871	-17,4681	5,15	3,96E-06	1,74E-06	2,271675132	4,164898417	950
17	29,27735	-5,44095	5,15	4,19E-06	1,73E-06	2,420322141	4,413372915	950
18	17,62195	-7,79517	5,15	4,43E-06	1,72E-06	2,569779366	4,66050042	950
19	15,67954	0,176807	5,15	4,66E-06	1,71E-06	2,720051223	4,906288233	950
20	16,20824	3,587693	5,15	4,89E-06	1,70E-06	2,871142151	5,150743618	950
21	15,84596	-5,59068	5,15	5,12E-06	1,70E-06	3,023056616	5,393873797	950
22	-24,099	-142,337	5,15	5,35E-06	1,69E-06	3,175799106	5,635685955	950
23	-239,69	-166,998	5,15	5,58E-06	1,68E-06	3,329374134	5,876187237	950
24	-320,034	-35,7048	5,15	5,81E-06	1,67E-06	3,483786238	6,115384748	950
25	-347,654	-78,5395	5,15	6,04E-06	1,66E-06	3,639039982	6,353285557	950
26	-459,352	-71,1597	5,15	6,26E-06	1,65E-06	3,795139951	6,589896693	950
27	-411,763	291,2388	5,15	6,48E-06	1,64E-06	3,952090759	6,825225148	950

28	21,00624	236,9471	5,15	6,71E-06	1,63E-06	4,109897042	7,059277875	950
29	0,348575	-51,4536	5,15	6,93E-06	1,62E-06	4,268563465	7,292061789	950
30	-5,82272	-2,18249	5,15	7,15E-06	1,61E-06	4,428094716	7,52358377	950
31	-8,95138	-14,4373	5,15	7,37E-06	1,61E-06	4,588495507	7,753850658	950
32	-27,2035	6,796628	5,15	7,58E-06	1,60E-06	4,749770579	7,982869257	950
33	2,550037	19,37231	5,15	7,80E-06	1,59E-06	4,911924697	8,210646334	950
34	4,629038	4,600301	5,15	8,02E-06	1,58E-06	5,074962653	8,43718862	950
35	13,24096	-0,04404	5,15	8,23E-06	1,57E-06	5,238889263	8,662502808	950
36	3,120325	-13,5249	5,15	8,44E-06	1,56E-06	5,403709372	8,886595556	950
37	-8,99123	-4,81929	5,15	8,65E-06	1,55E-06	5,569427849	9,109473485	950
38	-10,93	-16,982	5,15	8,86E-06	1,55E-06	5,736049592	9,331143182	950
39	-41,33	-23,4567	5,15	9,07E-06	1,54E-06	5,903579523	9,551611195	950
40	-51,8057	-3,91303	5,15	9,28E-06	1,53E-06	6,072022592	9,77088404	950
41	-54,5165	-9,4808	5,15	9,49E-06	1,52E-06	6,241383778	9,988968194	950
42	-69,4661	-6,04785	5,15	9,70E-06	1,51E-06	6,411668084	10,2058701	950
43	-68,8439	-13,3889	5,15	9,90E-06	1,50E-06	6,582880542	10,42159618	950
44	-93,8505	-10,7993	5,15	1,01E-05	1,50E-06	6,75502621	10,63615279	950
45	-88,9249	0,565272	5,15	1,03E-05	1,49E-06	6,928110176	10,84954627	950
46	-100,522	-25,2497	5,15	1,05E-05	1,48E-06	7,102137553	11,06178294	950
47	-128,999	-14,6933	5,15	1,07E-05	1,47E-06	7,277113484	11,27286907	950
48	-111,446	77,62247	5,15	1,09E-05	1,46E-06	7,453043139	11,48281088	950

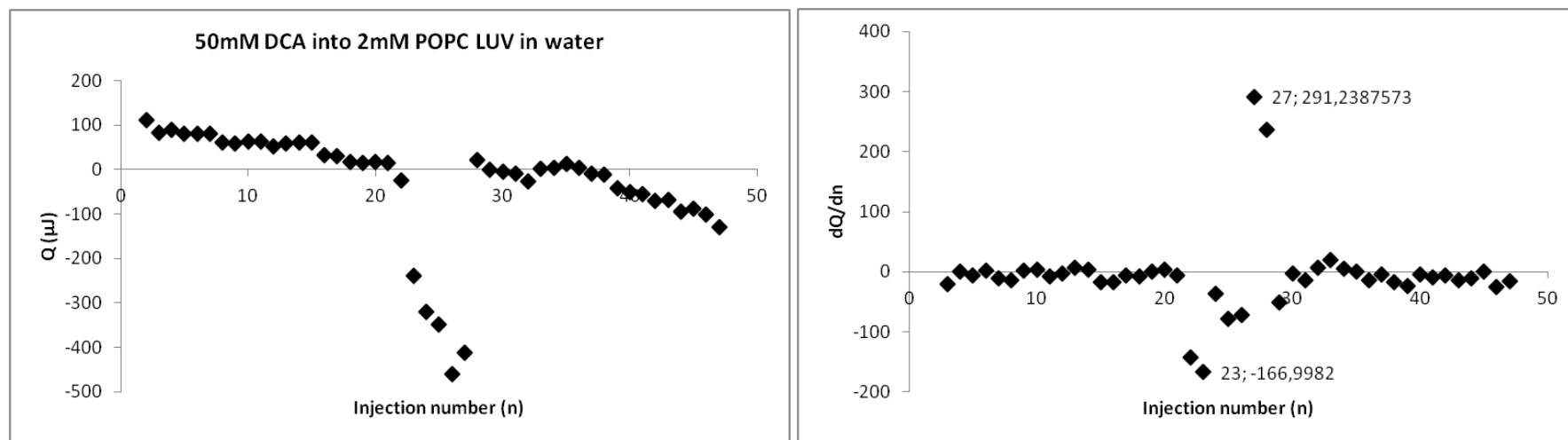


Figure B.6: Titration of 50 mM DCA into 2 mM POPC LUVs solutions, in water, pH 8,0.

injection	Mean Q	dQ/dn	inj volume (μ L)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	conc.of det(mM)	total volume (μ L)
1	-55,23474	83,62275	5,15	2,58E-07	4,72E-06	0,054506006	0,271052632	950
2	141,04605	112,3865	5,15	5,14E-07	4,70E-06	0,109309103	0,540635873	950
3	124,89545	-41,5847	5,15	7,68E-07	4,67E-06	0,164410909	0,808757689	950
4	92,404025	-37,2204	5,15	1,02E-06	4,65E-06	0,219813054	1,075426002	950
5	54,6457625	-37,5506	5,15	1,27E-06	4,62E-06	0,275517173	1,340648693	950
6	25,6934875	-2,69847	5,15	1,52E-06	4,60E-06	0,331524914	1,604433597	950
7	41,8184875	1,312356	5,15	1,77E-06	4,57E-06	0,38783793	1,86678851	950
8	22,1674375	-18,3772	5,15	2,02E-06	4,55E-06	0,444457886	2,127721183	950
9	10,6890875	-11,3229	5,15	2,27E-06	4,52E-06	0,501386454	2,387239326	950
10	-2,8151125	-17,3407	5,15	2,51E-06	4,50E-06	0,558625318	2,645350607	950
11	-22,1671625	-15,6457	5,15	2,76E-06	4,47E-06	0,616176168	2,902062654	950
12	-32,5943125	-9,41471	5,15	3,00E-06	4,45E-06	0,674040704	3,157383051	950
13	-39,7956125	3,752385	5,15	3,24E-06	4,43E-06	0,732220637	3,411319343	950
14	-30,866175	-5,73117	5,15	3,48E-06	4,40E-06	0,790717686	3,663879033	950
15	-53,3706875	-24,2575	5,15	3,72E-06	4,38E-06	0,849533578	3,915069583	950
16	-72,4208875	-13,5681	5,15	3,96E-06	4,35E-06	0,908670053	4,164898417	950
17	-81,1430125	-8,97492	5,15	4,19E-06	4,33E-06	0,968128856	4,413372915	950
18	-90,2270125	-0,7052	5,15	4,43E-06	4,31E-06	1,027911746	4,66050042	950
19	-88,12065	-13,6092	5,15	4,66E-06	4,28E-06	1,088020489	4,906288233	950
20	-119,7796375	-31,7919	5,15	4,89E-06	4,26E-06	1,14845686	5,150743618	950
21	-154,2538875	-38,78	5,15	5,12E-06	4,24E-06	1,209222646	5,393873797	950
22	-237,790175	-193,005	5,15	5,35E-06	4,21E-06	1,270319642	5,635685955	950
23	-566,845025	-498,618	5,15	5,58E-06	4,19E-06	1,331749654	5,876187237	950
24	-1104,4506	-304,019	5,15	5,81E-06	4,17E-06	1,393514495	6,115384748	950
25	-1104,12175	164,272	5,15	6,04E-06	4,15E-06	1,455615993	6,353285557	950
26	-887,7732875	170,8758	5,15	6,26E-06	4,12E-06	1,51805598	6,589896693	950
27	-804,6905875	43,74742	5,15	6,48E-06	4,10E-06	1,580836303	6,825225148	950

28	-759,5114375	74,98542	5,15	6,71E-06	4,08E-06	1,643958817	7,059277875	950
29	-602,99605	332,9227	5,15	6,93E-06	4,06E-06	1,707425386	7,292061789	950
30	-174,042075	264,6733	5,15	7,15E-06	4,04E-06	1,771237886	7,52358377	950
31	-116,0082625	45,90521	5,15	7,37E-06	4,01E-06	1,835398203	7,753850658	950
32	-39,6890875	18,73255	5,15	7,58E-06	3,99E-06	1,899908232	7,982869257	950
33	-79,034725	-41,7537	5,15	7,80E-06	3,97E-06	1,964769879	8,210646334	950
34	-103,044375	-20,2773	5,15	8,02E-06	3,95E-06	2,029985061	8,43718862	950
35	-121,80585	-18,7307	5,15	8,23E-06	3,93E-06	2,095555705	8,662502808	950
36	-138,53045	-8,18698	5,15	8,44E-06	3,91E-06	2,161483749	8,886595556	950
37	-138,1552	1,334281	5,15	8,65E-06	3,88E-06	2,22777114	9,109473485	950
38	-135,5953625	13,24702	5,15	8,86E-06	3,86E-06	2,294419837	9,331143182	950
39	-114,336525	15,98075	5,15	9,07E-06	3,84E-06	2,361431809	9,551611195	950
40	-106,9452625	6,333412	5,15	9,28E-06	3,82E-06	2,428809037	9,77088404	950
41	-100,7234375	1,949419	5,15	9,49E-06	3,80E-06	2,496553511	9,988968194	950
42	-102,6916125	-1,74662	5,15	9,70E-06	3,78E-06	2,564667234	10,2058701	950
43	-103,70035	-1,39759	5,15	9,90E-06	3,76E-06	2,633152217	10,42159618	950
44	-109,801125	-22,0585	5,15	1,01E-05	3,74E-06	2,702010484	10,63615279	950
45	-140,8285125	-14,8999	5,15	1,03E-05	3,72E-06	2,77124407	10,84954627	950
46	-135,0148	12,75756	5,15	1,05E-05	3,70E-06	2,840855021	11,06178294	950
47	-126,6113125	-21,162	5,15	1,07E-05	3,68E-06	2,910845394	11,27286907	950
48	-149,1543	73,15631	5,15	1,09E-05	3,66E-06	2,981217256	11,48281088	950

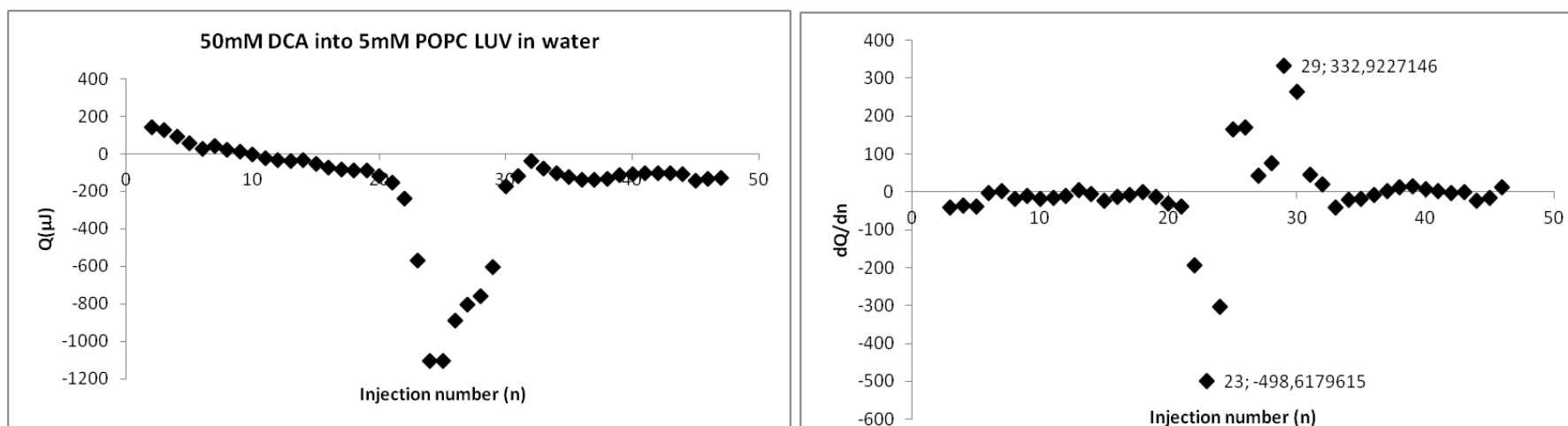


Figure B.7: Titration of 50 mM DCA into 5 mM POPC LUVs solutions, in water, pH 8,0.

injection	Mean Q	dQ/dn	inj volume (μ L)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	total volume (μ L)	conc.of det(mM)
1	-149,6390473	166,8857	5,15	2,58E-07	9,45E-06	0,027253003	950	0,271052632
2	282,953525	257,7608	5,15	5,14E-07	9,40E-06	0,054654551	950	0,540635873
3	260,999925	-82,1741	5,15	7,68E-07	9,35E-06	0,082205455	950	0,808757689
4	191,981725	-84,9391	5,15	1,02E-06	9,30E-06	0,109906527	950	1,075426002
5	108,676225	-52,8126	5,15	1,27E-06	9,25E-06	0,137758587	950	1,340648693
7	83,633375	-35,1317	5,15	1,77E-06	9,15E-06	0,193918965	950	1,86678851
8	27,964775	-63,701	5,15	2,02E-06	9,10E-06	0,222228943	950	2,127721183
10	-32,12915	-42,2664	5,15	2,51E-06	9,00E-06	0,279312659	950	2,645350607
11	-53,012525	-6,87274	5,15	2,76E-06	8,95E-06	0,308088084	950	2,902062654
12	-56,987825	-17,4829	5,15	3,00E-06	8,90E-06	0,337020352	950	3,157383051
13	-88,4317	-25,9774	5,15	3,24E-06	8,85E-06	0,366110319	950	3,411319343
14	-105,68835	-21,5661	5,15	3,48E-06	8,80E-06	0,395358843	950	3,663879033
15	-130,8885	-18,2334	5,15	3,72E-06	8,76E-06	0,424766789	950	3,915069583
16	-137,849475	7,141952	5,15	3,96E-06	8,71E-06	0,454335026	950	4,164898417
17	-126,92015	-7,59384	5,15	4,19E-06	8,66E-06	0,484064428	950	4,413372915
18	-159,644975	-51,8493	5,15	4,43E-06	8,61E-06	0,513955873	950	4,66050042
19	-214,12645	-25,719	5,15	4,66E-06	8,57E-06	0,544010245	950	4,906288233
20	-213,3079	-13,0479	5,15	4,89E-06	8,52E-06	0,57422843	950	5,150743618
21	-247,59495	-26,4012	5,15	5,12E-06	8,48E-06	0,604611323	950	5,393873797
22	-270,817975	-51,4088	5,15	5,35E-06	8,43E-06	0,635159821	950	5,635685955
23	-357,392625	-87,0138	5,15	5,58E-06	8,38E-06	0,665874827	950	5,876187237
24	-474,783575	-245,008	5,15	5,81E-06	8,34E-06	0,696757248	950	6,115384748
25	-835,153725	-377,671	5,15	6,04E-06	8,29E-06	0,727807996	950	6,353285557
26	-1152,80815	-205,902	5,15	6,26E-06	8,25E-06	0,75902799	950	6,589896693
27	-1249,534475	-72,0048	5,15	6,48E-06	8,20E-06	0,790418152	950	6,825225148
28	-1319,002325	-20,7557	5,15	6,71E-06	8,16E-06	0,821979408	950	7,059277875
29	-1300,649725	1,182754	5,15	6,93E-06	8,11E-06	0,853712693	950	7,292061789

30	-1312,661875	29,44277	5,15	7,15E-06	8,07E-06	0,885618943	950	7,52358377
31	-1213,003925	191,4732	5,15	7,37E-06	8,03E-06	0,917699101	950	7,753850658
32	-971,149175	191,1817	5,15	7,58E-06	7,98E-06	0,949954116	950	7,982869257
33	-866,226675	43,10751	5,15	7,80E-06	7,94E-06	0,982384939	950	8,210646334
34	-832,62455	115,5254	5,15	8,02E-06	7,90E-06	1,014992531	950	8,43718862
35	-622,097	232,5482	5,15	8,23E-06	7,85E-06	1,047777853	950	8,662502808
36	-404,4169	203,9333	5,15	8,44E-06	7,81E-06	1,080741874	950	8,886595556
37	-231,14345	134,2837	5,15	8,65E-06	7,77E-06	1,11388557	950	9,109473485
38	-152,195725	-5,18476	5,15	8,86E-06	7,73E-06	1,147209918	950	9,331143182
39	-215,7321	-49,1945	5,15	9,07E-06	7,69E-06	1,180715905	950	9,551611195
40	-218,90895	28,61975	5,15	9,28E-06	7,64E-06	1,214404518	950	9,77088404
41	-174,5156	35,31135	5,15	9,49E-06	7,60E-06	1,248276756	950	9,988968194
42	-165,900775	-27,7335	5,15	9,70E-06	7,56E-06	1,282333617	950	10,2058701
43	-215,40285	-31,7755	5,15	9,90E-06	7,52E-06	1,316576108	950	10,42159618
44	-213,20475	32,09385	5,15	1,01E-05	7,48E-06	1,351005242	950	10,63615279
45	-171,6409	9,702008	5,15	1,03E-05	7,44E-06	1,385622035	950	10,84954627
46	-200,93135	-38,0302	5,15	1,05E-05	7,40E-06	1,420427511	950	11,06178294
47	-233,63975	-48,9043	5,15	1,07E-05	7,36E-06	1,455422697	950	11,27286907
48	-252,83265	139,0156	5,15	1,09E-05	7,32E-06	1,490608628	950	11,48281088

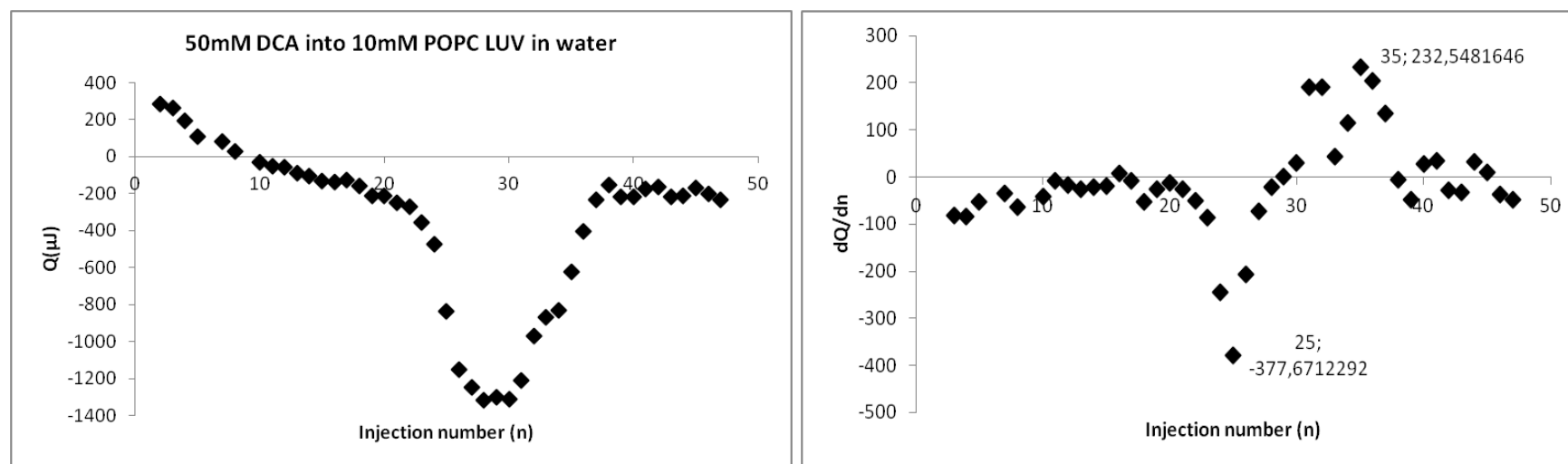


Figure B.8: Titration of 50 mM DCA into 10 mM POPC LUVs solutions, in water, pH 8,0.

injection	Q	dQ/dn	inj volume (μL)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	conc.of det(mM)	total volume (μL)
1	683,4790088	491,0233	5	2,50E-07	1,88E-06	0,133120341	0,264830508	944
2	789,4105	-199,785	5	4,99E-07	1,87E-06	0,266949523	0,528258313	944
3	423,0045	-283,177	5	7,46E-07	1,86E-06	0,40149132	0,790290843	944
4	313,62815	-53,9967	5	9,92E-07	1,85E-06	0,536749527	1,050935489	944
5	275,348325	-32,4927	5	1,24E-06	1,84E-06	0,672727959	1,310199602	944
6	256,121025	48,35656	5	1,48E-06	1,83E-06	0,809430451	1,568090494	944
7	352,860375	54,20543	5	1,72E-06	1,82E-06	0,946860858	1,824615438	944
8	353,44585	12,66747	5	1,96E-06	1,81E-06	1,085023056	2,07978167	944
9	403,481725	77,00184	5	2,20E-06	1,80E-06	1,223920943	2,333596386	944
10	509,08215	135,2361	5	2,44E-06	1,79E-06	1,363558434	2,586066744	944
11	673,9287	207,2961	5	2,68E-06	1,78E-06	1,50393947	2,837199865	944
12	894,188975	185,8849	5	2,91E-06	1,77E-06	1,645068008	3,087002832	944
13	996,782725	-55,7603	5	3,15E-06	1,76E-06	1,786948029	3,33548269	944
14	861,29605	32,27216	5	3,38E-06	1,75E-06	1,929583535	3,582646447	944
15	1079,90915	180,6045	5	3,61E-06	1,74E-06	2,072978549	3,828501074	944
16	1171,9345	124,9722	5	3,84E-06	1,73E-06	2,217137114	4,073053504	944
17	1314,635775	29,62749	5	4,07E-06	1,73E-06	2,362063297	4,316310636	944
18	1239,4425	-28,113	5	4,30E-06	1,72E-06	2,507761184	4,55827933	944
19	1264,4433	-75,0155	5	4,53E-06	1,71E-06	2,654234886	4,79896641	944
20	1107,750875	-47,7262	5	4,76E-06	1,70E-06	2,801488533	5,038378664	944
21	1161,2886	-57,4269	5	4,98E-06	1,69E-06	2,949526278	5,276522845	944
22	986,919175	-93,2091	5	5,20E-06	1,68E-06	3,098352297	5,513405669	944
23	986,913	-73,8145	5	5,43E-06	1,67E-06	3,247970786	5,749033816	944
24	831,254775	-93,1667	5	5,65E-06	1,66E-06	3,398385966	5,983413934	944
25	801,747225	-108,504	5	5,87E-06	1,65E-06	3,549602079	6,216552631	944
26	623,59285	-85,9701	5	6,09E-06	1,64E-06	3,701623389	6,448456484	944
27	627,66915	-68,0453	5	6,31E-06	1,64E-06	3,854454184	6,679132032	944

28	470,2709	-127,322	5	6,52E-06	1,63E-06	4,008098775	6,908585782	944
29	391,715175	-99,0639	5	6,74E-06	1,62E-06	4,162561495	7,136824205	944
30	263,8199	-116,966	5	6,95E-06	1,61E-06	4,3178467	7,363853738	944
31	164,8281	-100,991	5	7,16E-06	1,60E-06	4,47395877	7,589680784	944
32	58,77215	-104,668	5	7,38E-06	1,59E-06	4,630902107	7,814311712	944
33	-36,77595	-77,9063	5	7,59E-06	1,58E-06	4,788681139	8,037752857	944
34	-92,99975	-36,9395	5	7,80E-06	1,58E-06	4,947300314	8,260010522	944
35	-114,471825	-9,43027	5	8,01E-06	1,57E-06	5,106764107	8,481090975	944
36	-119,521025	-10,4889	5	8,21E-06	1,56E-06	5,267077015	8,701000451	944
37	-135,782925	-9,60486	5	8,42E-06	1,55E-06	5,428243559	8,919745152	944
38	-137,622325	-3,25313	5	8,63E-06	1,54E-06	5,590268285	9,137331247	944
39	-144,02385	-4,86261	5	8,83E-06	1,53E-06	5,753155763	9,353764874	944
40	-146,4109	-1,58404	5	9,03E-06	1,53E-06	5,916910586	9,569052136	944
41	-147,74025	-0,24109	5	9,24E-06	1,52E-06	6,081537373	9,783199106	944
42	-148,3451	-5,66252	5	9,44E-06	1,51E-06	6,247040767	9,996211823	944
43	-156,604325	0,597308	5	9,64E-06	1,50E-06	6,413425435	10,20809629	944
44	-149,3732	-4,44614	5	9,84E-06	1,49E-06	6,580696071	10,4188585	944
45	-163,13275	1,674177	5	1,00E-05	1,49E-06	6,748857392	10,62850437	944
46	-147,218825	2,473142	5	1,02E-05	1,48E-06	6,917914141	10,83703984	944
47	-159,45945	-2,47964	5	1,04E-05	1,47E-06	7,087871085	11,04447077	944
48	-149,6645	4,348942	5	1,06E-05	1,46E-06	7,258733018	11,25080302	944
49	-152,942525	-11,7479	5	1,08E-05	1,46E-06	7,43050476	11,45604241	944
50	-147,270725	89,93352	5	1,10E-05	1,45E-06	7,603191154	11,66019473	944
51	0,665825	85,43527	5	1,12E-05	1,44E-06	7,77679707	11,86326573	944

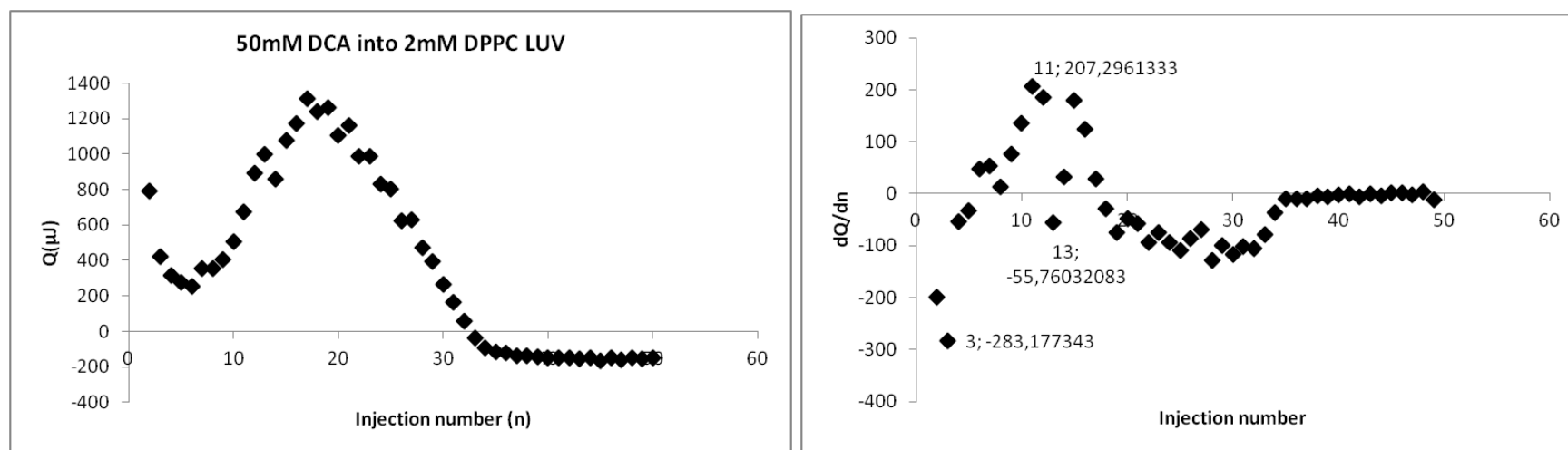


Figure B.9: Titration of 50 mM DCA into 2 mM DPPC LUVs solutions, in phosphate buffer , pH 8,0.

injection	Q	dQ/dn	inj volume (μL)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	total volume (μL)	conc.of det(mM)
1	1506,012753	1256,899	5	2,50E-07	4,70E-06	0,053248136	944	0,264830508
2	1973,8142	-579,434	5	4,99E-07	4,67E-06	0,106779809	944	0,528258313
3	707,726525	-843,208	5	7,46E-07	4,65E-06	0,160596528	944	0,790290843
4	566,921925	-78,6607	5	9,92E-07	4,62E-06	0,214699811	944	1,050935489
5	369,375875	-201,645	5	1,24E-06	4,60E-06	0,269091184	944	1,310199602
6	210,937075	-38,9922	5	1,48E-06	4,57E-06	0,32377218	944	1,568090494
7	279,5875	70,4859	5	1,72E-06	4,55E-06	0,378744343	944	1,824615438
8	316,521175	45,44925	5	1,96E-06	4,52E-06	0,434009223	944	2,07978167
9	368,21785	27,80475	5	2,20E-06	4,50E-06	0,489568377	944	2,333596386
10	374,58885	10,22465	5	2,44E-06	4,48E-06	0,545423374	944	2,586066744
11	410,471925	70,18323	5	2,68E-06	4,45E-06	0,601575788	944	2,837199865
12	531,857975	170,2038	5	2,91E-06	4,43E-06	0,658027203	944	3,087002832
13	784,17205	430,7468	5	3,15E-06	4,41E-06	0,714779212	944	3,33548269
14	1321,7441	405,8793	5	3,38E-06	4,38E-06	0,771833414	944	3,582646447
15	1560,599925	307,6336	5	3,61E-06	4,36E-06	0,829191419	944	3,828501074
16	1872,7292	-37,2461	5	3,84E-06	4,34E-06	0,886854846	944	4,073053504
17	1500,449625	-368,958	5	4,07E-06	4,31E-06	0,944825319	944	4,316310636
18	1287,495325	-96,7018	5	4,30E-06	4,29E-06	1,003104474	944	4,55827933
19	1306,22995	129,9438	5	4,53E-06	4,27E-06	1,061693954	944	4,79896641
20	1479,393	86,88979	5	4,76E-06	4,24E-06	1,120595413	944	5,038378664
21	1476,305325	66,17222	5	4,98E-06	4,22E-06	1,179810511	944	5,276522845
22	1605,420825	20,33189	5	5,20E-06	4,20E-06	1,239340919	944	5,513405669
23	1520,3859	-15,6174	5	5,43E-06	4,18E-06	1,299188314	944	5,749033816
24	1588,054975	7,191746	5	5,65E-06	4,16E-06	1,359354386	944	5,983413934
25	1524,7869	-12,9667	5	5,87E-06	4,13E-06	1,419840831	944	6,216552631
26	1554,327875	-71,054	5	6,09E-06	4,11E-06	1,480649356	944	6,448456484
27	1406,169675	-27,3781	5	6,31E-06	4,09E-06	1,541781674	944	6,679132032

28	1491,76545	-22,3353	5	6,52E-06	4,07E-06	1,60323951	944	6,908585782
29	1352,8249	-51,944	5	6,74E-06	4,05E-06	1,665024598	944	7,136824205
30	1395,593475	-48,6319	5	6,95E-06	4,02E-06	1,72713868	944	7,363853738
31	1260,1213	-23,7193	5	7,16E-06	4,00E-06	1,789583508	944	7,589680784
32	1333,719475	-62,0226	5	7,38E-06	3,98E-06	1,852360843	944	7,814311712
33	1142,463925	-76,2936	5	7,59E-06	3,96E-06	1,915472455	944	8,037752857
34	1198,606	-14,0884	5	7,80E-06	3,94E-06	1,978920126	944	8,260010522
35	1094,737225	-38,5909	5	8,01E-06	3,92E-06	2,042705643	944	8,481090975
36	1120,966575	-58,4957	5	8,21E-06	3,90E-06	2,106830806	944	8,701000451
37	984,4399	-48,8975	5	8,42E-06	3,88E-06	2,171297424	944	8,919745152
38	1018,1761	-65,9228	5	8,63E-06	3,86E-06	2,236107314	944	9,137331247
39	859,183075	-54,7564	5	8,83E-06	3,84E-06	2,301262305	944	9,353764874
40	909,9858	-34,3478	5	9,03E-06	3,82E-06	2,366764234	944	9,569052136
41	775,993975	-78,3482	5	9,24E-06	3,80E-06	2,432614949	944	9,783199106
42	764,836975	-68,6806	5	9,44E-06	3,78E-06	2,498816307	944	9,996211823
43	638,1704	-70,0075	5	9,64E-06	3,76E-06	2,565370174	944	10,20809629
44	631,564825	-36,0187	5	9,84E-06	3,74E-06	2,632278429	944	10,4188585
45	549,90665	-74,441	5	1,00E-05	3,72E-06	2,699542957	944	10,62850437
46	490,9508	-72,7347	5	1,02E-05	3,70E-06	2,767165656	944	10,83703984
47	406,55025	-65,8569	5	1,04E-05	3,68E-06	2,835148434	944	11,04447077
48	357,53015	-63,706	5	1,06E-05	3,66E-06	2,903493207	944	11,25080302
49	272,8246	-79,2529	5	1,08E-05	3,64E-06	2,972201904	944	11,45604241
50	185,617825	-163,898	5	1,10E-05	3,62E-06	3,041276461	944	11,66019473
51	-17,714075	-101,01	5	1,12E-05	3,60E-06	3,110718828	944	11,86326573

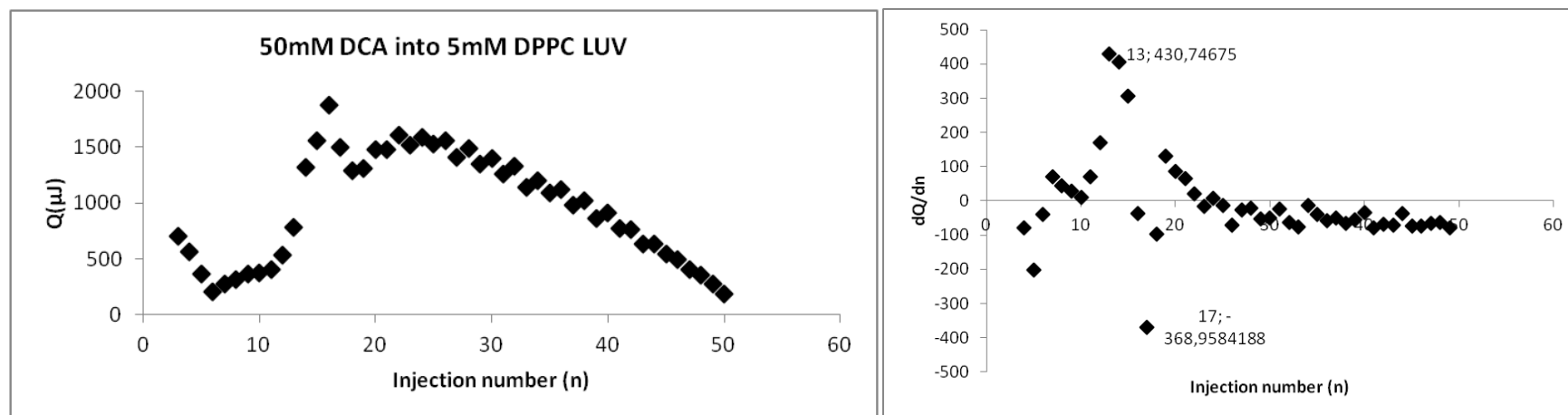


Figure B.10: Titration of 50 mM DCA into 5 mM DPPC LUVs solutions, in phosphate buffer , pH 8,0.

injection	Q	dQ/dn	inj volume (μL)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	total volume (μL)	conc.of det(mM)
1	1486,735284	2230,595	5,15	2,58E-07	9,45E-06	0,027253003	950	0,271052632
2	3552,414725	35,67779	5,15	5,14E-07	9,40E-06	0,054654551	950	0,540635873
3	1652,176575	-1706,7	5,15	7,68E-07	9,35E-06	0,082205455	950	0,808757689
4	895,3969	-362,104	5,15	1,02E-06	9,30E-06	0,109906527	950	1,075426002
5	711,046775	-219,846	5,15	1,27E-06	9,25E-06	0,137758587	950	1,340648693
6	368,618525	-358,141	5,15	1,52E-06	9,20E-06	0,165762457	950	1,604433597
7	76,10565	-123,14	5,15	1,77E-06	9,15E-06	0,193918965	950	1,86678851
8	113,558825	59,03289	5,15	2,02E-06	9,10E-06	0,222228943	950	2,127721183
9	148,2481	72,55389	5,15	2,27E-06	9,05E-06	0,250693227	950	2,387239326
10	237,3635	19,01226	5,15	2,51E-06	9,00E-06	0,279312659	950	2,645350607
11	195,89635	3,362375	5,15	2,76E-06	8,95E-06	0,308088084	950	2,902062654
12	266,597725	89,54952	5,15	3,00E-06	8,90E-06	0,337020352	950	3,157383051
13	341,7734	11,73283	5,15	3,24E-06	8,85E-06	0,366110319	950	3,411319343
14	329,785675	68,39256	5,15	3,48E-06	8,80E-06	0,395358843	950	3,663879033
15	560,605975	465,3043	5,15	3,72E-06	8,76E-06	0,424766789	950	3,915069583
16	1196,54765	604,9979	5,15	3,96E-06	8,71E-06	0,454335026	950	4,164898417
17	1692,217225	467,2337	5,15	4,19E-06	8,66E-06	0,484064428	950	4,413372915
18	2122,7006	338,1967	5,15	4,43E-06	8,61E-06	0,513955873	950	4,66050042
19	2363,0246	187,389	5,15	4,66E-06	8,57E-06	0,544010245	950	4,906288233
20	2504,645725	74,36372	5,15	4,89E-06	8,52E-06	0,57422843	950	5,150743618
21	2499,11065	-90,895	5,15	5,12E-06	8,48E-06	0,604611323	950	5,393873797
22	2319,024375	-309,918	5,15	5,35E-06	8,43E-06	0,635159821	950	5,635685955
23	1968,793825	-176,602	5,15	5,58E-06	8,38E-06	0,665874827	950	5,876187237
24	1981,122925	-15,1156	5,15	5,81E-06	8,34E-06	0,696757248	950	6,115384748
25	1915,121775	56,58372	5,15	6,04E-06	8,29E-06	0,727807996	950	6,353285557
26	2071,03575	53,45042	5,15	6,26E-06	8,25E-06	0,75902799	950	6,589896693
27	2009,091775	-0,26714	5,15	6,48E-06	8,20E-06	0,790418152	950	6,825225148

28	2091,4779	41,99603	5,15	6,71E-06	8,16E-06	0,821979408	950	7,059277875
29	2081,8646	33,27328	5,15	6,93E-06	8,11E-06	0,853712693	950	7,292061789
30	2149,266	-10,3843	5,15	7,15E-06	8,07E-06	0,885618943	950	7,52358377
31	2072,11725	-5,22306	5,15	7,37E-06	8,03E-06	0,917699101	950	7,753850658
32	2138,110425	-11,9221	5,15	7,58E-06	7,98E-06	0,949954116	950	7,982869257
33	2055,296675	13,92783	5,15	7,80E-06	7,94E-06	0,982384939	950	8,210646334
34	2157,767125	8,208156	5,15	8,02E-06	7,90E-06	1,014992531	950	8,43718862
35	2062,23685	-41,1144	5,15	8,23E-06	7,85E-06	1,047777853	950	8,662502808
36	2095,13395	-5,59884	5,15	8,44E-06	7,81E-06	1,080741874	950	8,886595556
37	2047,603825	16,65908	5,15	8,65E-06	7,77E-06	1,11388557	950	9,109473485
38	2107,889	-52,894	5,15	8,86E-06	7,73E-06	1,147209918	950	9,331143182
39	1964,368325	-21,1554	5,15	9,07E-06	7,69E-06	1,180715905	950	9,551611195
40	2063,97775	-1,99482	5,15	9,28E-06	7,64E-06	1,214404518	950	9,77088404
41	1950,178375	-33,3847	5,15	9,49E-06	7,60E-06	1,248276756	950	9,988968194
42	2018,3072	38,5115	5,15	9,70E-06	7,56E-06	1,282333617	950	10,2058701
43	1999,6208	-7,28511	5,15	9,90E-06	7,52E-06	1,316576108	950	10,42159618
44	1997,3792	-75,0204	5,15	1,01E-05	7,48E-06	1,351005242	950	10,63615279
45	1870,1757	90,37608	5,15	1,03E-05	7,44E-06	1,385622035	950	10,84954627
46	1882,990725	-1080,34	5,15	1,05E-05	7,40E-06	1,420427511	950	11,06178294

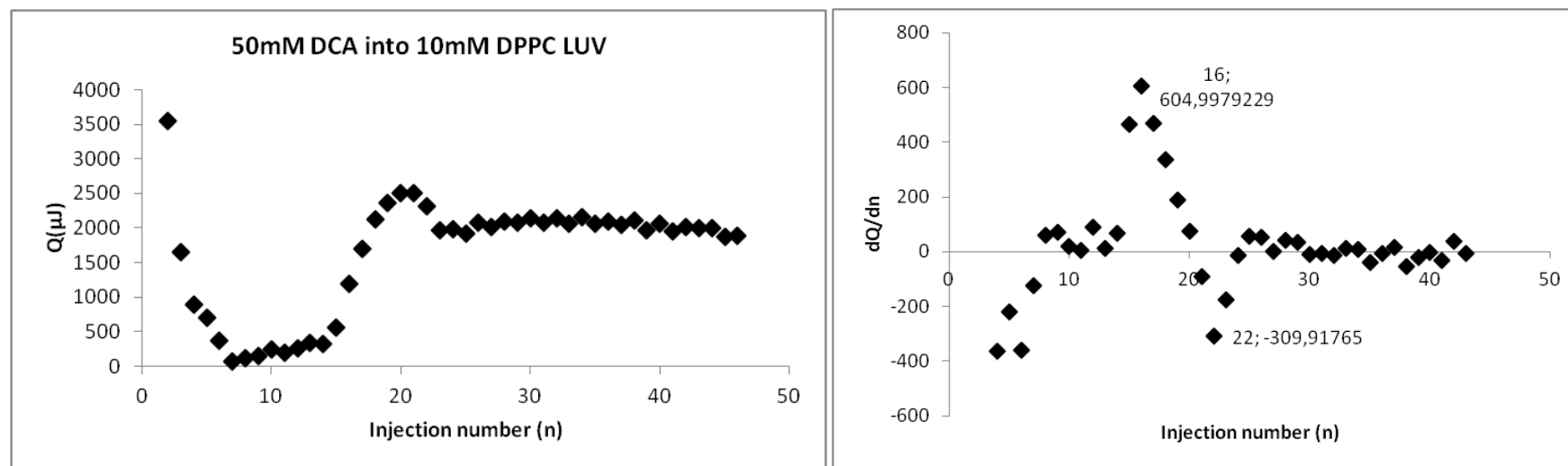


Figure B.11: Titration of 50 mM DCA into 10 mM DPPC LUVs solutions, in phosphate buffer, pH 8,0.

injection	Q Mean	dQ/dn	inj volume (μ L)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	total volume (μ L)	conc.of det(mM)
1	835,4170977	800,4543	5,15	2,58E-07	1,89E-06	0,136265016	950	0,271052632
2	1369,9617	252,4095	5,15	5,14E-07	1,88E-06	0,273272757	950	0,540635873
3	1354,241742	-110,222	5,15	7,68E-07	1,87E-06	0,411027274	950	0,808757689
4	1121,68335	-662,935	5,15	1,02E-06	1,86E-06	0,549532635	950	1,075426002
5	171,859125	-714,141	5,15	1,27E-06	1,85E-06	0,688792933	950	1,340648693
6	-133,8847833	-72,8206	5,15	1,52E-06	1,84E-06	0,828812284	950	1,604433597
7	-120,6106667	-75,2764	5,15	1,77E-06	1,83E-06	0,969594825	950	1,86678851
8	-344,2281833	-333,799	5,15	2,02E-06	1,82E-06	1,111144714	950	2,127721183
9	-607,5712667	163,2919	5,15	2,27E-06	1,81E-06	1,253466136	950	2,387239326
10	-23,98561667	657,1322	5,15	2,51E-06	1,80E-06	1,396563295	950	2,645350607
11	481,827375	258,9517	5,15	2,76E-06	1,79E-06	1,540440419	950	2,902062654
12	485,3747667	-112,128	5,15	3,00E-06	1,78E-06	1,68510176	950	3,157383051
13	359,8911	-84,1901	5,15	3,24E-06	1,77E-06	1,830551593	950	3,411319343
14	346,058875	15,1123	5,15	3,48E-06	1,76E-06	1,976794214	950	3,663879033
15	377,5816833	53,32006	5,15	3,72E-06	1,75E-06	2,123833945	950	3,915069583
16	445,5518833	72,43219	5,15	3,96E-06	1,74E-06	2,271675132	950	4,164898417
17	515,9944667	76,80453	5,15	4,19E-06	1,73E-06	2,420322141	950	4,413372915
18	584,1748167	23,36135	5,15	4,43E-06	1,72E-06	2,569779366	950	4,66050042
19	564,9108167	-20,8028	5,15	4,66E-06	1,71E-06	2,720051223	950	4,906288233
20	556,5464917	-8,05692	5,15	4,89E-06	1,70E-06	2,871142151	950	5,150743618
21	544,601975	-18,0422	5,15	5,12E-06	1,70E-06	3,023056616	950	5,393873797
22	518,38715	-35,9546	5,15	5,35E-06	1,69E-06	3,175799106	950	5,635685955
23	476,1430833	-34,898	5,15	5,58E-06	1,68E-06	3,329374134	950	5,876187237
24	440,3308	-72,3815	5,15	5,81E-06	1,67E-06	3,483786238	950	6,115384748
25	338,9273	-80,9945	5,15	6,04E-06	1,66E-06	3,639039982	950	6,353285557
26	289,2391333	-45,4212	5,15	6,26E-06	1,65E-06	3,795139951	950	6,589896693
27	239,3432917	-49,983	5,15	6,48E-06	1,64E-06	3,952090759	950	6,825225148

28	188,7137167	-51,6681	5,15	6,71E-06	1,63E-06	4,109897042	950	7,059277875
29	134,5196083	-59,3807	5,15	6,93E-06	1,62E-06	4,268563465	950	7,292061789
30	70,66628333	-65,2349	5,15	7,15E-06	1,61E-06	4,428094716	950	7,52358377
31	7,5319	-61,6134	5,15	7,37E-06	1,61E-06	4,588495507	950	7,753850658
32	-44,369625	-24,4182	5,15	7,58E-06	1,60E-06	4,749770579	950	7,982869257
33	-46,40636667	-9,1172	5,15	7,80E-06	1,59E-06	4,911924697	950	8,210646334
34	-67,82100833	-12,6663	5,15	8,02E-06	1,58E-06	5,074962653	950	8,43718862
35	-70,67275	-4,97847	5,15	8,23E-06	1,57E-06	5,238889263	950	8,662502808
36	-86,5048	-32,5738	5,15	8,44E-06	1,56E-06	5,403709372	950	8,886595556
37	-136,13505	-67,4144	5,15	8,65E-06	1,55E-06	5,569427849	950	9,109473485
38	-200,6339667	-19,6967	5,15	8,86E-06	1,55E-06	5,736049592	950	9,331143182
39	-174,7337	27,98137	5,15	9,07E-06	1,54E-06	5,903579523	950	9,551611195
40	-158,9330583	21,02963	5,15	9,28E-06	1,53E-06	6,072022592	950	9,77088404
41	-138,3042	0,72116	5,15	9,49E-06	1,52E-06	6,241383778	950	9,988968194
42	-161,5535417	-41,021	5,15	9,70E-06	1,51E-06	6,411668084	950	10,2058701
43	-204,3514833	-19,8039	5,15	9,90E-06	1,50E-06	6,582880542	950	10,42159618
44	-195,0591583	23,37339	5,15	1,01E-05	1,50E-06	6,75502621	950	10,63615279
45	-168,7028833	22,37455	5,15	1,03E-05	1,49E-06	6,928110176	950	10,84954627
46	-156,84545	0,947573	5,15	1,05E-05	1,48E-06	7,102137553	950	11,06178294
47	-167,1363917	-38,7603	5,15	1,07E-05	1,47E-06	7,277113484	950	11,27286907
48	-193,8981	98,35381	5,15	1,09E-05	1,46E-06	7,453043139	950	11,48281088

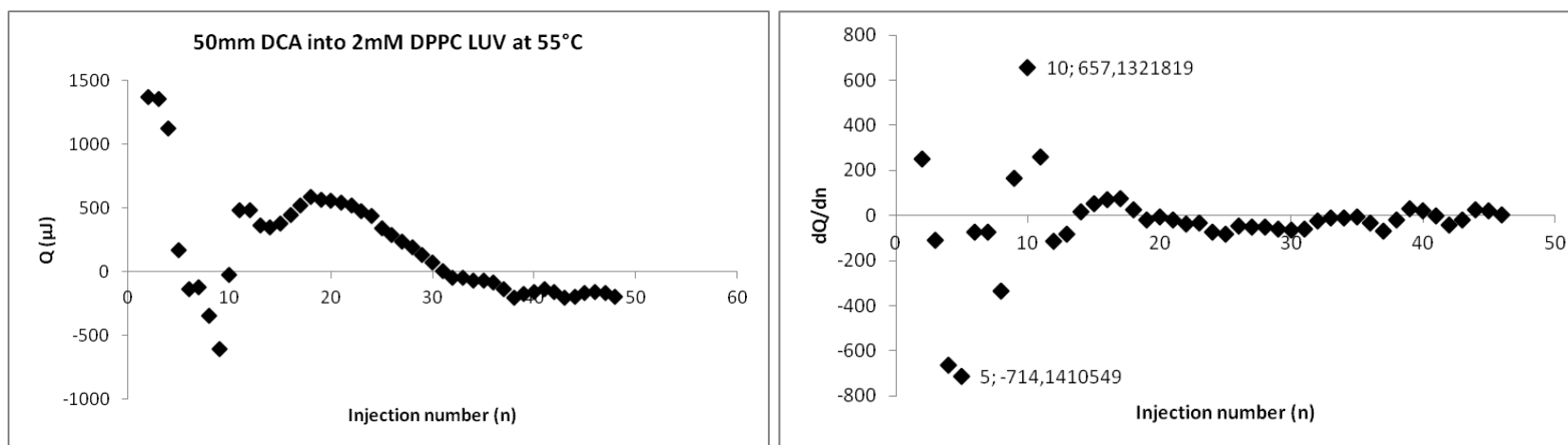


Figure B.12: Titration of 50 mM DCA into 2 mM DPPC LUVs solutions, in phosphate buffer pH 8.0, at 55°C.

injection	Q mean	dQ/dn	inj volume (μ L)	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	total volume (μ L)	conc. of det. (mM)
1	636,3392466	461,1799	5,15	2,58E-07	4,72E-06	0,054506006	950	0,271052632
2	790,872775	28,38916	5,15	5,14E-07	4,70E-06	0,109309103	950	0,540635873
3	792,8232167	58,24769	5,15	7,68E-07	4,67E-06	0,164410909	950	0,808757689
4	911,2017833	59,32114	5,15	1,02E-06	4,65E-06	0,219813054	950	1,075426002
5	899,999025	6,694124	5,15	1,27E-06	4,62E-06	0,275517173	950	1,340648693
6	936,4256083	21,25537	5,15	1,52E-06	4,60E-06	0,331524914	950	1,604433597
7	914,284325	51,65272	5,15	1,77E-06	4,57E-06	0,38783793	950	1,86678851
8	770,4197833	-959,218	5,15	2,02E-06	4,55E-06	0,444457886	950	2,127721183
9	-1047,880225	-2334,05	5,15	2,27E-06	4,52E-06	0,501386454	950	2,387239326
10	-3250,277067	-1132,56	5,15	2,51E-06	4,50E-06	0,558625318	950	2,645350607
11	-3242,687442	154,9968	5,15	2,76E-06	4,47E-06	0,616176168	950	2,902062654
12	-3197,336125	284,1747	5,15	3,00E-06	4,45E-06	0,674040704	950	3,157383051
13	-2484,313992	1451,326	5,15	3,24E-06	4,43E-06	0,732220637	950	3,411319343
14	-593,3855583	1486,836	5,15	3,48E-06	4,40E-06	0,790717686	950	3,663879033
15	173,002475	322,5198	5,15	3,72E-06	4,38E-06	0,849533578	950	3,915069583
16	219,1590167	-76,0212	5,15	3,96E-06	4,35E-06	0,908670053	950	4,164898417
17	145,8047917	-63,4958	5,15	4,19E-06	4,33E-06	0,968128856	950	4,413372915
18	101,2879417	-74,2478	5,15	4,43E-06	4,31E-06	1,027911746	950	4,66050042
19	-8,0169	-121,714	5,15	4,66E-06	4,28E-06	1,088020489	950	4,906288233
20	-120,4411083	-80,6275	5,15	4,89E-06	4,26E-06	1,14845686	950	5,150743618
21	-167,456575	-40,3926	5,15	5,12E-06	4,24E-06	1,209222646	950	5,393873797
22	-206,6996333	-24,607	5,15	5,35E-06	4,21E-06	1,270319642	950	5,635685955
23	-213,3733167	7,517681	5,15	5,58E-06	4,19E-06	1,331749654	950	5,876187237
24	-192,49085	36,32239	5,15	5,81E-06	4,17E-06	1,393514495	950	6,115384748
25	-143,998475	55,51606	5,15	6,04E-06	4,15E-06	1,455615993	950	6,353285557
26	-87,569575	54,5178	5,15	6,26E-06	4,12E-06	1,51805598	950	6,589896693
27	-40,19579167	30,00201	5,15	6,48E-06	4,10E-06	1,580836303	950	6,825225148

28	-16,28293333	57,65118	5,15	6,71E-06	4,08E-06	1,643958817	950	7,059277875
29	66,270525	51,77173	5,15	6,93E-06	4,06E-06	1,707425386	950	7,292061789
30	72,34675833	-20,2896	5,15	7,15E-06	4,04E-06	1,771237886	950	7,52358377
31	47,580925	1,927563	5,15	7,37E-06	4,01E-06	1,835398203	950	7,753850658
32	77,67584167	24,44627	5,15	7,58E-06	3,99E-06	1,899908232	950	7,982869257
33	85,77244167	2,999167	5,15	7,80E-06	3,97E-06	1,964769879	950	8,210646334
34	84,52360833	-9,00061	5,15	8,02E-06	3,95E-06	2,029985061	950	8,43718862
35	66,37305	-31,8364	5,15	8,23E-06	3,93E-06	2,095555705	950	8,662502808
36	30,48803333	-15,6565	5,15	8,44E-06	3,91E-06	2,161483749	950	8,886595556
37	35,52425	2,204635	5,15	8,65E-06	3,88E-06	2,22777114	950	9,109473485
38	25,61110833	-19,0655	5,15	8,86E-06	3,86E-06	2,294419837	950	9,331143182
39	0,902033333	-21,7998	5,15	9,07E-06	3,84E-06	2,361431809	950	9,551611195
40	-17,70349167	-26,3934	5,15	9,28E-06	3,82E-06	2,428809037	950	9,77088404
41	-49,39471667	-21,7693	5,15	9,49E-06	3,80E-06	2,496553511	950	9,988968194
42	-60,04225833	-11,1498	5,15	9,70E-06	3,78E-06	2,564667234	950	10,2058701
43	-76,57636667	-20,8665	5,15	9,90E-06	3,76E-06	2,633152217	950	10,42159618
44	-101,3596583	-26,3108	5,15	1,01E-05	3,74E-06	2,702010484	950	10,63615279
45	-129,5360167	-35,3882	5,15	1,03E-05	3,72E-06	2,77124407	950	10,84954627
46	-167,989875	-29,335	5,15	1,05E-05	3,70E-06	2,840855021	950	11,06178294
47	-184,9598917	-27,2881	5,15	1,07E-05	3,68E-06	2,910845394	950	11,27286907
48	-192,7300667	109,3074	5,15	1,09E-05	3,66E-06	2,981217256	950	11,48281088

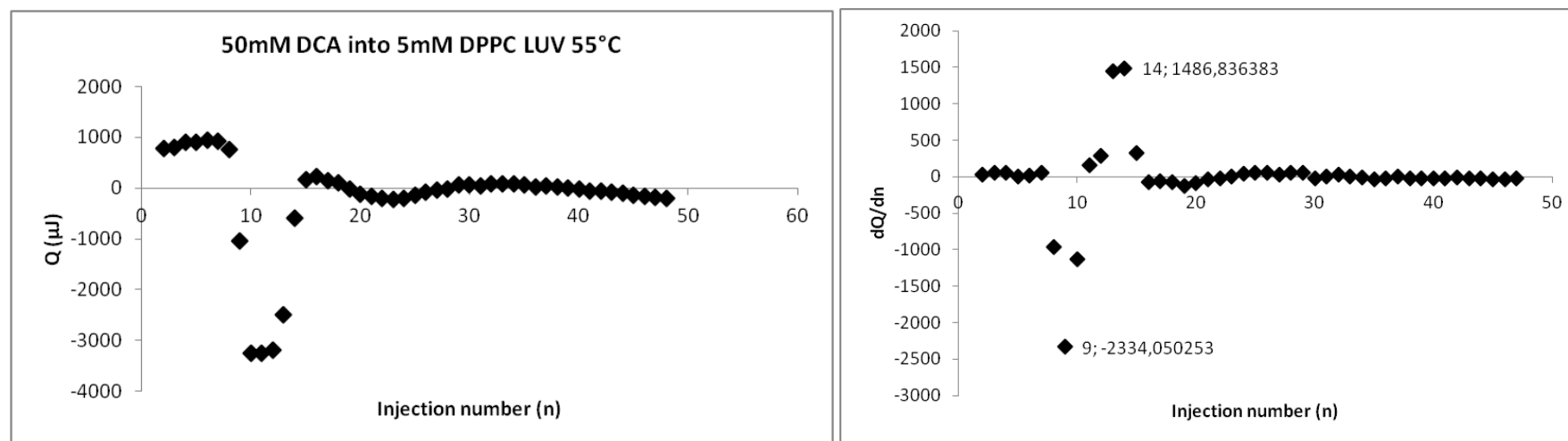


Figure B.13: Titration of 50 mM DCA into 5 mM DPPC LUVs solutions, in phosphate buffer pH 8.0, at 55°C.

injection	Q mean	dQ/dn	moles titrant (moles)	moles titrate (moles)	moles titrant / moles titrate	total volume (μ L)	conc of det. (mM)
1	263,6713922	214,5916	2,58E-07	9,45E-06	0,027253003	950	0,271052632
2	365,2823375	24,24316	5,14E-07	9,40E-06	0,054654551	950	0,540635873
3	347,1593	-1,45595	7,68E-07	9,35E-06	0,082205455	950	0,808757689
4	376,9853875	13,98499	1,02E-06	9,30E-06	0,109906527	950	1,075426002
5	374,76725	18,17367	1,27E-06	9,25E-06	0,137758587	950	1,340648693
6	418,3261	50,47788	1,52E-06	9,20E-06	0,165762457	950	1,604433597
7	459,8010125	18,9177	1,77E-06	9,15E-06	0,193918965	950	1,86678851
8	451,5209125	-28,7766	2,02E-06	9,10E-06	0,222228943	950	2,127721183
9	413,31335	-28,6039	2,27E-06	9,05E-06	0,250693227	950	2,387239326
10	391,7443375	-36,3999	2,51E-06	9,00E-06	0,279312659	950	2,645350607
11	324,8347	0,074177	2,76E-06	8,95E-06	0,308088084	950	2,902062654
12	180,4902875	-747,964	3,00E-06	8,90E-06	0,337020352	950	3,157383051
13	-1277,609175	-2117,41	3,24E-06	8,85E-06	0,366110319	950	3,411319343
14	-3452,243	-1067,02	3,48E-06	8,80E-06	0,395358843	950	3,663879033
15	-3328,07565	192,1631	3,72E-06	8,76E-06	0,424766789	950	3,915069583
16	-3419,057725	-5,57246	3,96E-06	8,71E-06	0,454335026	950	4,164898417
17	-3318,0845	66,59765	4,19E-06	8,66E-06	0,484064428	950	4,413372915
18	-3305,444275	48,56552	4,43E-06	8,61E-06	0,513955873	950	4,66050042
19	-3218,339825	-12,5332	4,66E-06	8,57E-06	0,544010245	950	4,906288233
20	-3203,886587	381,7992	4,89E-06	8,52E-06	0,57422843	950	5,150743618
21	-2355,224663	1237,629	5,12E-06	8,48E-06	0,604611323	950	5,393873797
22	-982,113025	1106,155	5,35E-06	8,43E-06	0,635159821	950	5,635685955
23	-295,694025	457,8869	5,58E-06	8,38E-06	0,665874827	950	5,876187237
24	-1,503	114,2741	5,81E-06	8,34E-06	0,696757248	950	6,115384748
25	-4,98725	-35,6511	6,04E-06	8,29E-06	0,727807996	950	6,353285557
26	-27,7477625	-36,7355	6,26E-06	8,25E-06	0,75902799	950	6,589896693
27	-77,838975	-60,4375	6,48E-06	8,20E-06	0,790418152	950	6,825225148

28	-143,49115	-64,8272	6,71E-06	8,16E-06	0,821979408	950	7,059277875
29	-205,684075	-65,2693	6,93E-06	8,11E-06	0,853712693	950	7,292061789
30	-272,5825375	-61,9065	7,15E-06	8,07E-06	0,885618943	950	7,52358377
31	-327,3381375	-50,0941	7,37E-06	8,03E-06	0,917699101	950	7,753850658
32	-373,8454375	-42,2239	7,58E-06	7,98E-06	0,949954116	950	7,982869257
33	-414,6583375	-49,4924	7,80E-06	7,94E-06	0,982384939	950	8,210646334
34	-464,457925	-19,3594	8,02E-06	7,90E-06	1,014992531	950	8,43718862
35	-458,3289	-12,7416	8,23E-06	7,85E-06	1,047777853	950	8,662502808
36	-490,8968125	-7,53606	8,44E-06	7,81E-06	1,080741874	950	8,886595556
37	-473,270425	-0,44249	8,65E-06	7,77E-06	1,11388557	950	9,109473485
38	-493,557375	-1,53835	8,86E-06	7,73E-06	1,147209918	950	9,331143182
39	-474,303475	5,719995	9,07E-06	7,69E-06	1,180715905	950	9,551611195
40	-480,7009625	18,56853	9,28E-06	7,64E-06	1,214404518	950	9,77088404
41	-439,0590625	25,42531	9,49E-06	7,60E-06	1,248276756	950	9,988968194
42	-434,4244	12,21162	9,70E-06	7,56E-06	1,282333617	950	10,2058701
43	-409,1947125	28,63237	9,90E-06	7,52E-06	1,316576108	950	10,42159618
44	-388,3256375	2,030609	1,01E-05	7,48E-06	1,351005242	950	10,63615279
45	-413,8574625	-76,3618	1,03E-05	7,44E-06	1,385622035	950	10,84954627
46	-496,0937125	15,93186	1,05E-05	7,40E-06	1,420427511	950	11,06178294
47	-354,997675	223,8212	1,07E-05	7,36E-06	1,455422697	950	11,27286907
48	-108,6297125	195,324	1,09E-05	7,32E-06	1,490608628	950	11,48281088

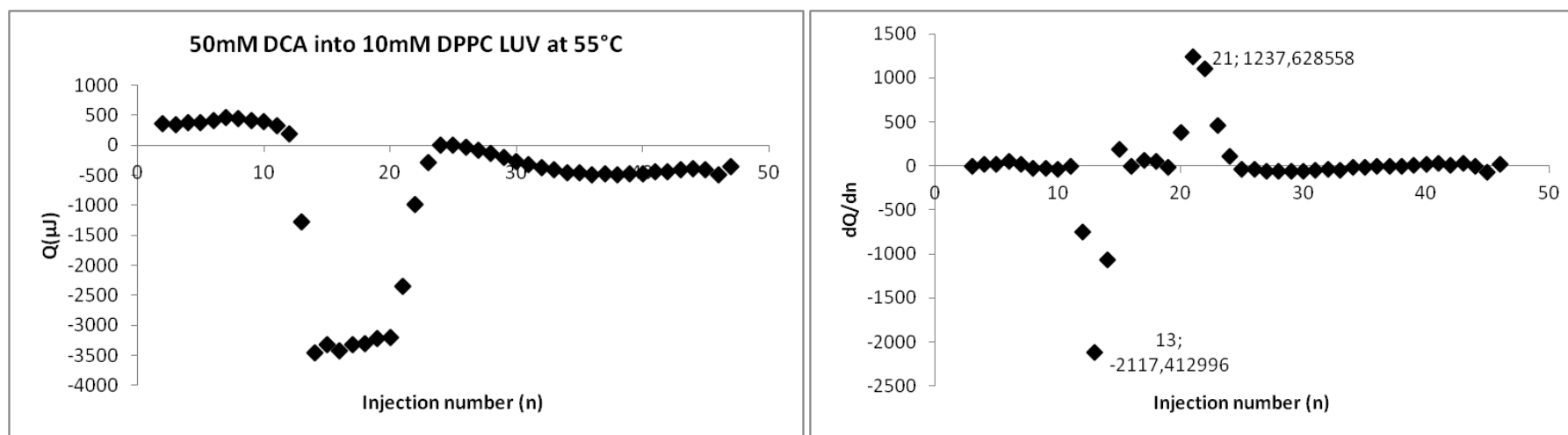


Figure B.14: Titration of 50 mM DCA into 10 mM DPPC LUVs solutions, in phosphate buffer pH 8.0, at 55°C.

Injection	Q (μJ)	inj volume (μL)	moles titrant (moles)	total volume (μL)	Bile		dQ/dC	kJ/mol
					concentratio n (M)	mM		
2	233,5985	5	4,99E-07	944	0,000528258	0,528258	11,15508	4,68E-01
3	232,41175	5	7,46E-07	944	0,000790291	0,790291	7,42594	3,12E-01
4	251,41615	5	9,92E-07	944	0,001050935	1,050935	3,898954	2,53E-01
5	233,60815	5	1,24E-06	944	0,0013102	1,3102	0,571123	1,89E-01
6	255,23455	5	1,48E-06	944	0,00156809	1,56809	-2,56051	1,72E-01
7	237,2478	5	1,72E-06	944	0,001824615	1,824615	-5,49888	1,38E-01
8	247,3759	5	1,96E-06	944	0,002079782	2,079782	-8,24687	1,26E-01
9	232,624075	5	2,20E-06	944	0,002333596	2,333596	-10,8073	1,06E-01
10	244,8632	5	2,44E-06	944	0,002586067	2,586067	-13,1831	1,00E-01
11	237,6404	5	2,68E-06	944	0,0028372	2,8372	-15,3769	8,87E-02
12	251,593575	5	2,91E-06	944	0,003087003	3,087003	-17,3916	8,63E-02
13	232,86935	5	3,15E-06	944	0,003335483	3,335483	-19,2299	7,40E-02
14	235,335825	5	3,38E-06	944	0,003582646	3,582646	-20,8943	6,96E-02
15	219,307	5	3,61E-06	944	0,003828501	3,828501	-22,3877	6,07E-02
16	220,8597	5	3,84E-06	944	0,004073054	4,073054	-23,7126	5,74E-02
17	201,032475	5	4,07E-06	944	0,004316311	4,316311	-24,8716	4,93E-02
18	209,48395	5	4,30E-06	944	0,004558279	4,558279	-25,8673	4,87E-02
19	184,2386	5	4,53E-06	944	0,004798966	4,798966	-26,7021	4,07E-02
20	183,505275	5	4,76E-06	944	0,005038379	5,038379	-27,3786	3,86E-02
21	159,308825	5	4,98E-06	944	0,005276523	5,276523	-27,8993	3,20E-02
22	160,830725	5	5,20E-06	944	0,005513406	5,513406	-28,2665	3,09E-02
23	148,47415	5	5,43E-06	944	0,005749034	5,749034	-28,4828	2,74E-02
24	149,959125	5	5,65E-06	944	0,005983414	5,983414	-28,5504	2,65E-02
25	136,922025	5	5,87E-06	944	0,006216553	6,216553	-28,4717	2,33E-02

26	140,4538	5	6,09E-06	944	0,006448456	6,448456	-28,249	2,31E-02
27	121,589275	5	6,31E-06	944	0,006679132	6,679132	-27,8847	1,93E-02
28	126,39525	5	6,52E-06	944	0,006908586	6,908586	-27,3809	1,94E-02
29	115,47945	5	6,74E-06	944	0,007136824	7,136824	-26,7399	1,71E-02
30	111,207125	5	6,95E-06	944	0,007363854	7,363854	-25,9639	1,60E-02
31	100,7774	5	7,16E-06	944	0,007589681	7,589681	-25,0551	1,41E-02
32	115,56275	5	7,38E-06	944	0,007814312	7,814312	-24,0157	1,57E-02
33	106,987	5	7,59E-06	944	0,008037753	8,037753	-22,8477	1,41E-02
34	105,629925	5	7,80E-06	944	0,008260011	8,260011	-21,5532	1,35E-02
35	94,20895	5	8,01E-06	944	0,008481091	8,481091	-20,1344	1,18E-02
36	100,8466	5	8,21E-06	944	0,008701	8,701	-18,5933	1,23E-02
37	89,88825	5	8,42E-06	944	0,008919745	8,919745	-16,9318	1,07E-02
38	93,57355	5	8,63E-06	944	0,009137331	9,137331	-15,152	1,08E-02
39	83,080075	5	8,83E-06	944	0,009353765	9,353765	-13,2559	9,41E-03
40	90,8696	5	9,03E-06	944	0,009569052	9,569052	-11,2454	1,01E-02
41	84,754025	5	9,24E-06	944	0,009783199	9,783199	-9,1224	9,18E-03
42	77,56155	5	9,44E-06	944	0,009996212	9,996212	-6,88882	8,22E-03
43	68,617625	5	9,64E-06	944	0,010208096	10,2081	-4,54653	7,12E-03
44	74,615225	5	9,84E-06	944	0,010418858	10,41886	-2,09739	7,59E-03
45	67,294775	5	1,00E-05	944	0,010628504	10,6285	0,456795	6,71E-03
46	72,9864	5	1,02E-05	944	0,01083704	10,83704	3,114208	7,13E-03
47	63,6033	5	1,04E-05	944	0,011044471	11,04447	5,873071	6,10E-03
48	72,52855	5	1,06E-05	944	0,011250803	11,2508	8,731627	6,83E-03
49	66,2894	5	1,08E-05	944	0,011456042	11,45604	11,68814	6,13E-03
50	65,45325	5	1,10E-05	944	0,011660195	11,66019	14,7409	5,95E-03

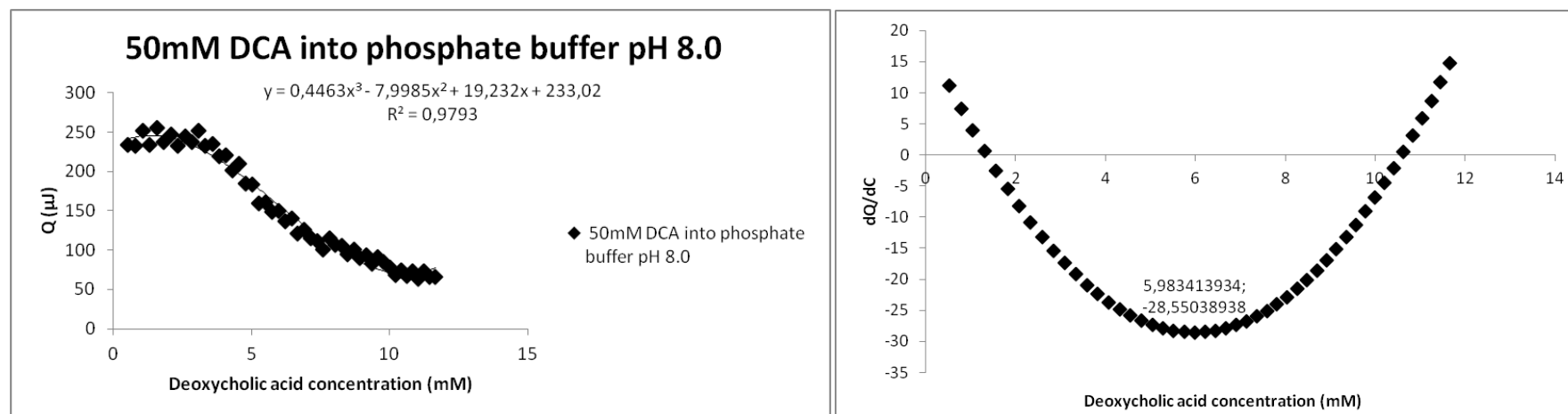


Figure B.15: Titration of 50 mM DCA into phosphate buffer, pH 8,0.



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List of Publications and Patents:

Ozcelik, B., **Engudar, G.**, Giz, H., Coruhli, T., Demir, N. *Monitoring tea catechins transport and release processes from alginate beads by continuous system.* In 103rd AOCS Annual Meeting & Expo. 2012, California, USA.

Ozcelik, B., **Engudar, G.**, Demir, N., Coruhli, T. *Encapsulation of catechin, characterization of beads and in vitro release studies.* In 18th International Symposium on Microencapsulation. 2011, Antalya, Turkey.

PUBLICATIONS/PRESENTATIONS ON THE THESIS

- **Engudar, G.**, Andresen, T., Ozcelik, B., 2012. *Interactions of Bile Salts with Model Lipid Membranes.* In 10th Euro Fed Lipid Congress, 2012, Cracow, Poland.

