Pharmaceutical Applications of Gemini Surfactants

by

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Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Gemini surfactants are an intriguing class of surface active agents that are comprised of two surfactant monomers chemically linked at or near the headgroups by a rigid or flexible spacer. In comparison to their corresponding monomer counterparts, gemini surfactants are more efficient at reducing surface tension, have better wetting properties, and typically have critical micelle concentration values that are one to two orders of magnitude lower. These intriguing properties characteristic of gemini surfactants make them of special interest for pharmaceutical applications.

Within this work, two different projects were carried out to assess the pharmaceutical applications of gemini surfactants. The aim of the first project was to assess the applications of gemini surfactants as transfection agents for non-viral gene delivery by evaluating the physical stability characteristics of gemini surfactant-based lipoplex systems. Prior to this investigation, an evaluation of the interaction properties between gemini surfactants and DNA, and between gemini surfactants and the neutral helper lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine was carried out using a combination of isothermal titration calorimetry, particle size, zeta potential and surface tension measurements. Following these evaluations, the physical stability of the gemini surfactant-based DNA delivery systems was assessed by examining the particle size distribution and membrane integrity characteristics of the lipoplexes. The results from this analysis revealed that the physical stability of these systems is limited by the membrane integrity characteristics of the lipoplex structure.

The second project carried out was an evaluation of the interactions between gemini surfactants and a series of Tween surfactants commonly found in pharmaceutical formulations. The results from this analysis were analyzed using Clint's, Rubingh's, Motomura's and Maeda's

theories for mixed micelle formation, where it was observed that there is a general synergistic mixing interaction present between gemini and Tween surfactants. The strength of synergism was found to be dependent upon the chain length and saturation of the Tween alkyl tail.

Acknowledgements

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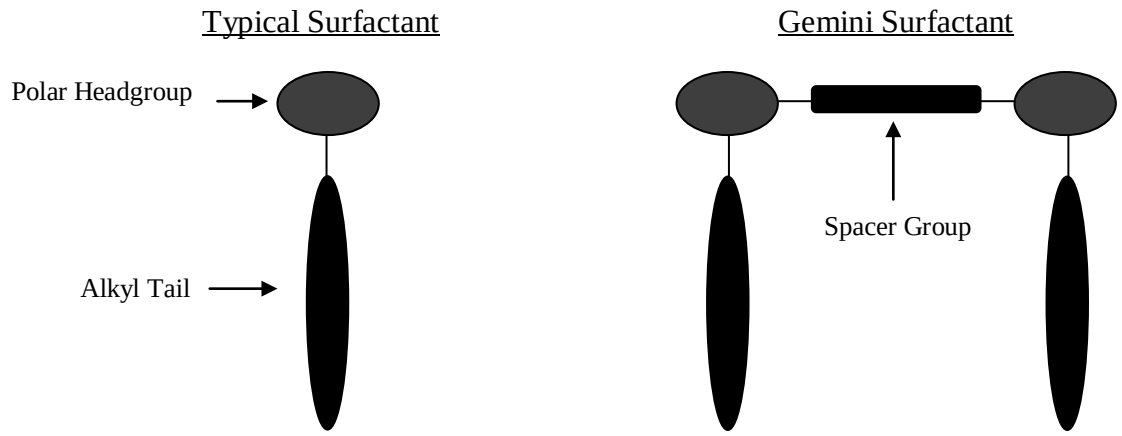
AFM	atomic force microscopy
CAC	critical aggregation concentration
CMC	critical micelle concentration
CMC*	critical micelle concentration in the presence of DNA
DLS	dynamic light scattering
DLVO	Derjaguin-Landau-Verwey-Overbeek
DNA	deoxyribonucleic acid
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTIM	1-[2-(9-(Z)-octadecenoyloxy)ethyl]-2-(8-(Z)-heptadecenyl)-3-(hydroxyethyl)imidazolinium chloride
FDA	food and drug administration
ITC	isothermal titration calorimetry
NMR	nuclear magnetic resonance
NNLS	non-negative least squares
TEM	transmission electron microscopy
X_1	mole fraction of surfactant 1 in the mixed micelle
X_{ideal}	ideal mole fraction of surfactant 1 in the mixed micelle
α_i	solution mole fraction of component *i*
β	interaction parameter
γ_1	activity coefficient of surfactant 1 in the mixed micelle
γ_2	activity coefficient of surfactant 2 in the mixed micelle

Chapter 1

Introduction

1.1 Introduction to Gemini Surfactants

Gemini surfactants represent an intriguing class of surface active agents that have been extensively studied by various research groups since the late 1980s. Their unique structure, which consists of two typical surfactant monomers that are covalently linked by either a rigid or flexible spacer group (see scheme 1.1), results in a number of interesting observations regarding their properties in aqueous solution.[1] In comparison to their corresponding monomer counterparts, gemini surfactants are more efficient at reducing surface tension, have better wetting properties, and typically have critical micelle concentration (CMC) values that are one to two orders of magnitude lower.[1-3] Furthermore, gemini surfactants have been shown to form a rich array of aggregate morphologies in solution, through alteration of their chemical structure.[4,5] The combination of the unique properties of these surfactants along with the increasing demand for high performance surfactants is currently driving research into their potential applications.



Scheme 1.1 Comparison of the structure of a typical surfactant with a gemini surfactant.

The prospective applications of gemini surfactants are multi-fold. These include their potential use in cleaning agents and detergents; cosmetics and personal care products;

preparative chemistry; pharmaceutical and biological applications; enhanced oil recovery etc.[6] One particular application that is currently heavily investigated, in particular by the Wettig research group, is the use of gemini surfactants in drug delivery.

The overall aim of this thesis is to provide an evaluation of the pharmaceutical applications of gemini surfactants. This was carried out in a series of projects which, i) evaluated the applications of gemini surfactants as transfection agents for non-viral DNA delivery, and ii) investigated the interactions of gemini surfactants with a series of Tween surfactants commonly found in pharmaceutical formulations. The details and rational of these two objectives are discussed below in sections 1.2 and 1.3, respectively.

1.2 Gemini Surfactants as Transfection Agents for Non-Viral Gene Therapy

1.2.1 Introduction to Gene Therapy

Gene therapy represents an intriguing therapeutic strategy for the treatment of both genetic and acquired diseases.[7] The main objective behind this technology is to insert genetic material into a patient's cells to help correct defective genes that may be responsible for disease development.[7] Through intervention at the genetic level, specific cellular functions can be restored, modified or enhanced.[7] As of June 2010, there are 1644 ongoing gene therapy clinical trials worldwide, where the majority of trials are oriented towards the treatment of cancer (1060), cardiovascular (143), monogenic (134) and infectious diseases (131).[8] The major gene types utilized in these trials include antigen (325), cytokine (302) and tumor suppressor (173) strategies.[8]

Currently, the most efficient method to deliver DNA into the cell nucleus is through the incorporation of therapeutic DNA into engineered viruses.[9,10] This method, entitled viral gene

therapy, has been found to show promising transfection results as this vector enables the integrated DNA to efficiently cross various physiological barriers upon administration.[11] As a result, the incorporated DNA can effectively be introduced into the cell nucleus and ultimately expressed.[11]

In light of the high transfection efficiencies characteristic to viral vectors, there are however various concerns that hinder their practicality as a suitable system for DNA delivery.[12] Viral delivery systems generally present safety and toxicity concerns as they can trigger severe immune responses, which have been observed in several human and animal clinical trials.[13] This was severely demonstrated during a gene therapy clinical trial conducted at the University of Pennsylvania in 1999, which resulted in the death of Jesse Gelsinger who received treatment from an adenoviral vector to correct partial ornithine transcarbamylase deficiency; Gelsinger's death was a result of multiple organ failure due to an immune response to the adenoviral vector.[12] Another setback was experienced in 2002, when two patients participating in a clinical trial for the treatment of severe combined immunodeficiency developed leukemia, which appeared to be linked to the activation of an oncogene adjacent to the site of vector insertion.[14] These two examples, among others, clearly exemplify the safety concerns associated with viral strategies. Viral vectors additionally suffer from the disadvantages of having limitations with respect to the size of the plasmid that can be incorporated,[15] long-term storage concerns, and difficult preparation and purification procedures.[16] As a result, there has been a shift into the research and development of non-viral vectors, which have been shown to counteract many of these disadvantages.

Non-viral gene therapy approaches offer numerous advantages in comparison to conventional viral vectors for several reasons. Most significantly, non-viral vectors are generally

non-toxic and non-immunogenic.[17] They additionally provide the advantages of being relatively cheap and easy to produce, and are not limited (from a practical standpoint) in the size of the plasmid that can be incorporated.[17,18] As well, they allow for specialized delivery options such as targeted delivery, time-dependent release, and enhanced circulation times.[17,18] Despite these numerous advantages, it is important to note that non-viral vectors suffer from low transfection efficiencies *in vivo*, as compared to viral based systems.[16]

Non-viral approaches range from a variety of different physical (direct needle injection of naked DNA, gene gun, electroporation etc.) and chemical (cationic lipids, surfactants, polymers etc.) gene transfer methods.[19] Since the first description of successful *in vitro* transfection using cationic lipids in 1987,[20] lipofection is regarded as one of the more popular studied strategies for non-viral gene delivery. Lipofection essentially involves the use of cationic lipids to form liposome/DNA complexes (termed a "lipoplex"), which can be utilized as a delivery vector for therapeutic DNA.[19] As of June 2010, there are 109 ongoing clinical trials worldwide utilizing lipofection-based strategies.[8]

1.2.2 Gemini Surfactants as Transfection Agents for Non-Viral Gene Therapy

As previously introduced, one of the potential applications of gemini surfactants is their use as transfection agents for non-viral gene delivery. More specifically, gemini surfactants can be used as building blocks for the construction of liposome/DNA complexes. The unique structural and solution properties of gemini surfactants make them intriguing candidates for use in such systems for a variety of reasons. For one, their multi-cationic nature allows for the effective binding and thus compaction of DNA.[21] In addition, gemini surfactants can be synthesized fairly easily and at low cost, therefore making them advantageous from a

pharmaceutical industrial manufacturing and economical perspective. As well, their unique solution properties allow them to readily associate in aqueous environments, thus making them efficient at low concentrations which subsequently reduces their concentration requirements during complex formation. This is advantageous from a toxicity perspective, where the first and simplest step to optimize the safety profile of any foreign complex is to reduce its concentration *in vivo*.

Overall, gemini surfactants are viewed as effective potential transfection agents for non-viral gene therapy. *In vitro* experiments have shown that gemini surfactant-based lipoplex systems are capable of providing comparable transfection efficiencies to commercially available transfection agents, one example of which is Lipofectamine Plus (Invitrogen).[16] Despite these positive results, further work is still needed to

i) improve the transfection efficiencies of these delivery systems, ideally to comparable levels of their viral counterparts

ii) assess the stability properties of these lipoplexes.

An evaluation of the physical stability of gemini surfactant-based DNA delivery systems will form the basis of the first objective of this thesis.

1.2.3 Stability of Gemini Surfactant-based Lipoplexes

In the literature, the majority of studies on lipoplex delivery systems have focused on characterizing and improving their transfection efficiencies, while evaluations on their stability characteristics has received little attention.[22] As will be discussed in more detail in Chapter 2.3, the stability properties of these systems is of great importance because they carry significant

implications with respect to the safety and efficacy characteristics of the lipoplex within the human body. If these lipoplex delivery systems are found to be relatively unstable during storage, their pharmaceutical applications become limited in that they must be prepared prior to administration, by specifically trained individuals. Without argument, it can be stated that the stability characteristics of non-viral vectors is of great significance when assessing their overall viable potential towards gene therapy applications.

In order to effectively evaluate the stability properties of gemini surfactant-based DNA delivery systems, an overall understanding of the physicochemical properties of the lipoplex structure is crucial. This requires an understanding of the main interactions involved between the components of these systems, which are composed of the gemini surfactant, the plasmid DNA, and the neutral helper lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). The helper lipid DOPE is included in the formulation to provide flexibility to the lipoplex structure, so that multiple conformations can be adopted.[16,23] This ability to adopt multiple conformations is believed to aid in membrane fusion and destabilization of the endosomal membrane, thus enhancing the entry of the incorporated DNA into the cell.[23] Previously reported results have shown that the inclusion of DOPE in gemini surfactant-based lipoplexes significantly increases the transfection efficiency.[16]

The two main interactions within gemini surfactant-based lipoplex systems that are of great significance and interest are, i) the interaction between gemini surfactants and DNA, and ii) the interaction between gemini surfactants and DOPE. The interaction between gemini surfactants and DNA is of great importance because the main driving force of complexation between the liposome structure and DNA is from an electrostatic interaction between the cationic gemini headgroups and the negatively charged DNA backbone. The interaction between gemini

surfactants and DOPE is of importance because the liposome structure is a mixed aggregate of these two components; therefore the overall properties of the liposome structure are dependent on the mixing behaviour of gemini surfactants and DOPE. Ultimately, a better understanding of these two key interactions will lay the foundation for an understanding and rationalization of the physical stability characteristics of gemini surfactant-based DNA delivery systems.

As a whole, the first objective of this thesis is to perform a preliminary evaluation of the physical stability characteristics of gemini surfactant-based DNA delivery systems. Prior to this evaluation, an investigation of the interactions between gemini surfactants and DNA, and between gemini surfactants and DOPE will be carried out.

1.3 Interactions of Gemini Surfactants with Pharmaceutical Surfactants

As already touched upon, gemini surfactants offer numerous advantages which make them intriguing candidates for use in pharmaceutical formulations. These advantages include their high efficiencies in reducing surface tension, low CMC values, superior wetting properties, and their ease and low cost of synthesis.[1-3] In order to further effectively assess the pharmaceutical applications of gemini surfactants towards drug delivery, an understanding of the interactions between gemini surfactants and other surfactants commonly found in pharmaceutical formulations is essential. This is of importance because pharmaceutical preparations normally contain mixtures of surface active compounds to provide the overall performance required for a particular application. In such instances, it is important to be aware that there can be substantial differences in the micellization tendencies of mixtures of surfactants, as compared to the single pure species.[24]

Mixtures of surface active agents form mixed micellar aggregates in aqueous solution, but the tendency towards aggregation can be different than that of the individual surfactants.[24] In ideal situations, there is no net-interaction between the amphiphile components, and the CMC of the mixed aggregate can be predicted using Clint's model, which relates the CMC of the mixed aggregate to the CMCs of the individual surfactants and the solution composition (note that Clint's equation will be discussed in more detail in Chapter 2.2).[25] Clint's ideal solution theory is an effective method for explaining the mixing behaviour of surfactants with chemically similar structures.[24] However, deviations in ideal mixing can occur in mixtures containing chemically distinct structures as a result of a net interaction between the amphiphiles.[24] This net interaction can be attractive or repulsive in nature, and can occur as a result of, i) electrostatic interactions between ionic groups, ii) ion-dipole interactions between ionic and nonionic groups, iii) steric interactions between bulky groups, iv) van der Waals interactions between hydrophobic groups, and v) hydrogen bonding between the constituent surfactant molecules.[26]

In systems exhibiting non-ideal mixing, the nature and strength of the net interaction between the amphiphiles can be assessed by determining the interaction parameter, β, which can be calculated using Rubingh's model for mixed micellar formation (discussed in more detail in Chapter 2.2).[24] Negative values of β indicate a synergistic/attractive mixing interaction between the amphiphiles, where the CMC of the mixed aggregate is lower than the ideal CMC value.[24] In contrast, positive values of β represent an antagonistic/repulsive interaction between the amphiphiles, where the CMC of the mixed aggregate is larger than the ideal CMC value.[24] Synergistic mixing interactions are typically moderately present in mixtures of ionic and nonionic surfactants,[27-29] and are relatively strong in mixtures of anionic and cationic

surfactants.[30] Antagonistic interactions have been observed between surfactants with similar headgroups but different chain structures.[31]

In pharmaceutical preparations, the use of surfactant mixtures which exhibit synergistic mixing interactions are beneficial due to their enhanced interfacial properties over the individual surfactants in the mixture. As a result of these enhanced interfacial properties (i.e. greater surface activities and lower CMCs), surfactant concentration requirements during formulation are reduced, which subsequently reduces cost and environment impact. In the literature, the interactions between gemini surfactants and conventional surfactants have been well documented and in most instances, mixtures of gemini surfactants and conventional cationic, anionic or nonionic surfactants have been found to exhibit synergistic mixing interactions.[32] To our knowledge, the interactions between gemini surfactants and pharmaceutical Tween surfactants has not been reported; an investigation of this interaction will form the basis of the last project in this thesis. The Tween surfactants are perhaps the most widely used surfactants in pharmaceutical formulations, in large part due to their nontoxic nature and approval as food grade surfactants.[33] In addition, Tween surfactants are commonly used in pharmaceutical dispersions because of their effectiveness at solubilisation, wetting, and reducing surface or interfacial tension.[34] As a result, they are widely used in the preparation of creams, emulsions and ointments for topical applications; as solubilising agents for a variety of substances, such as essential oils and oil-soluble vitamins; and as wetting agents in the preparation of oral and parental suspensions.[34]

As a whole, the second objective of this thesis is to investigate the mixing interactions between gemini surfactants and pharmaceutical Tween surfactants. This investigation will be

carried out using Clint's, Rubingh's, Motomura's and Maeda's theories of mixed micelle formation. These particular theories will be discussed in more detail in Chapter 3 of this thesis.

1.4 Thesis Objectives

In summary, this thesis involves two separate objectives, which can be broken down into four projects. The first main objective is to evaluate the physical stability characteristics of gemini surfactant-based DNA delivery systems. Prior to this analysis, the interactions between gemini surfactants and DNA, and between gemini surfactants and DOPE will be investigated.

The second objective of this thesis is to investigate the interaction properties between gemini surfactants and pharmaceutical Tween surfactants.

Chapter 2

Gemini Surfactants as Transfection Agents for Non-Viral Gene Delivery

2.1 Interaction between Gemini Surfactants and DNA

2.1.1 Introduction

Within this study, the interaction properties between gemini surfactants and DNA was investigated. As introduced in Chapter 1, the interaction between these two components is of great importance because complexation between the gemini/DOPE liposome and DNA is driven primarily by the electrostatic interactions between the cationic gemini headgroups and the negatively charged DNA molecules.

In the literature, properties such as the structure, morphology and thermodynamics of cationic surfactant/DNA complexes have been previously investigated using a variety of methods.[1-10] These studies have shown that variations in the length, degree of unsaturation, and chemical structure of the surfactant alkyl chain; along with the headgroup structure and nature of the counterion can each exhibit a significant effect on the interaction properties with DNA, and consequently the transfection efficiency.[1-10] Furthermore, the ratio of the cationic surfactant to DNA has been found to have a large effect on the lipoplex structural organization, and the transfection efficiency.[4-10] However, despite such studies, there still remain questions about the thermodynamical properties and the mechanism of association of cationic surfactants with DNA.

The thermodynamics and mechanism of association of cationic amphiphiles to DNA is known to be complex, and mediated by a variety of physical interactions. These include ionic interactions between the negatively charged DNA phosphate groups and the positively charged amphiphile headgroups; nonpolar interactions between the DNA and amphiphile molecules; repulsive interactions between DNA molecules and between amphiphile headgroups; and from hydration effects.[11,12] These forces, coupled with the individual structural properties of the plasmid DNA and cationic amphiphile, and the ratio of these two components, can lead to the

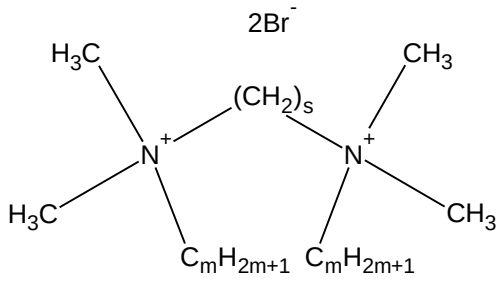
formation of a wide variety of supramolecular structures.[11,12] Furthermore, since the structures and solution properties of DNA and cationic amphiphiles are very different, one can expect that the properties of complexation between DNA and the amphiphile could differ depending upon the order in which the components are mixed.

The overall aim of this investigation was to evaluate the mechanism and thermodynamic properties of gemini surfactant/DNA complex formation. This was carried out in the forward (gemini surfactant into DNA) and in the reverse (DNA into gemini surfactant) titration manner. The energetics of complexation were assessed using isothermal titration calorimetry (ITC); while particle size and zeta potential measurements were carried out to give an insight into the structural properties of complex formation. Surface tension measurements were employed to evaluate complexation from a surface perspective. Overall, the combined results from this analysis were used to provide a better understanding of complex formation between gemini surfactants and DNA.

2.1.2 Experimental Procedures

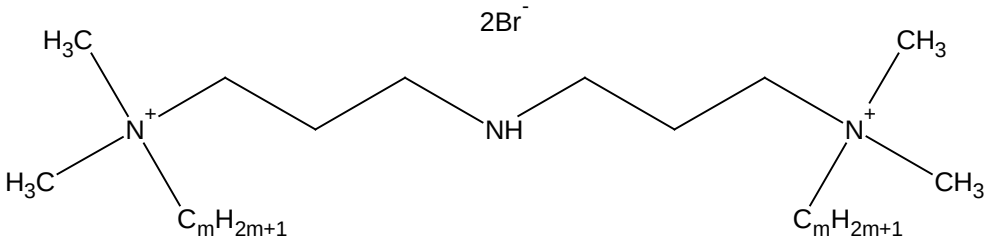
2.1.2.1 Materials

Two classes of gemini surfactants were employed in this study, referred to as nonsubstituted and amine-substituted. The nonsubstituted gemini surfactants used were of the *N*,*N*-bis(dimethylalkyl)-α,ω-alkanediammonium dibromide type, which can be represented by the notation *m*-*s*-*m*, where *m* refers to the length of the alkyl tail and *s* refers to the number of methylene units in the spacer. The general structure of these surfactants is shown below in Scheme 2.1.2.1.



Scheme 2.1.2.1 Representation of the general structure of the *m-s-m* type gemini surfactant.

The amine-substituted gemini surfactants used in this study were of the 1,9-bis(dialkyl)-1,1,9,9-tetramethyl-5-imino-1,9-nonanediammonium dibromide type. These surfactants can be represented by the notation *m*-7NH-*m*; a representation of the chemical structure is illustrated below in Scheme 2.1.2.2. As shown, these surfactants incorporate a secondary amine group in the spacer. Gemini surfactant-based DNA delivery systems formulated with 12-7NH-12 have demonstrated a 9-fold increase in transfection efficiency, in comparison to nonsubstituted gemini surfactants.[3] It is postulated that the presence of this pH active amine group has the potential to enhance the electrostatic interactions with the phosphate groups on the DNA backbone (particularly in a pH dependent fashion), as well as to facilitate the DNA escape from the endosome, which results in this increased transfection for the 12-7NH-12 based lipoplexes.[3]



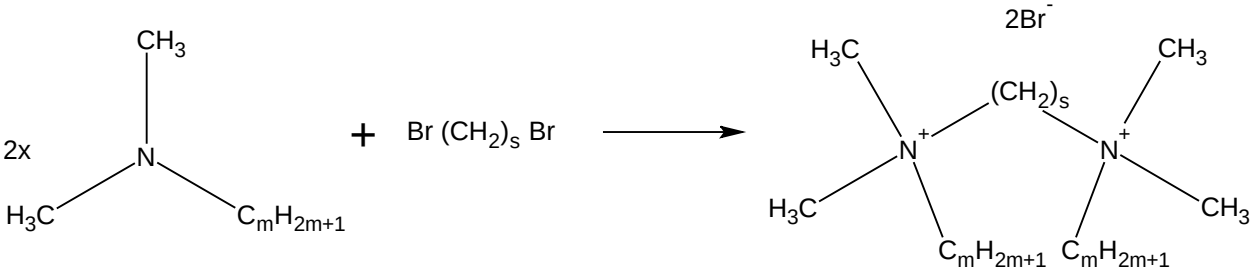
Scheme 2.1.2.2 Representation of the *m*-7NH-*m* type gemini surfactants.

For this investigation, the 12-3-12, 12-7-12, 12-7NH-12, 16-3-16, 16-7-6 and 16-7NH-16 gemini surfactants were used. The gemini surfactants were synthesized according to procedures

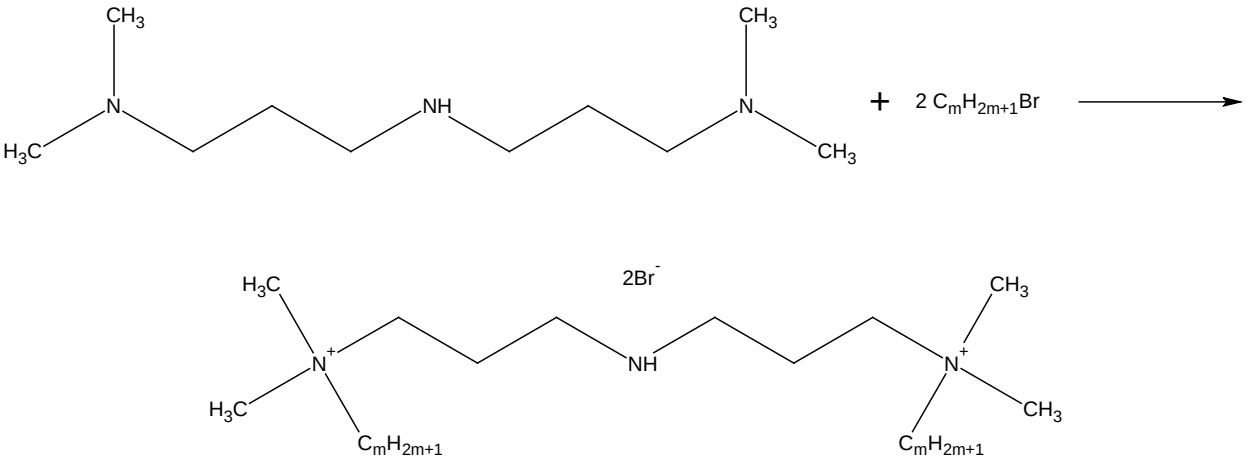
previously reported in the literature.[3,13] Briefly, the nonsubstituted gemini surfactants were synthesized by reflux of the appropriate α,ω-dibromoalkane with 2 molar equivalents (plus 10% excess) of the appropriate *N*,*N*-dimethylalkylamine in HPLC-grade acetone for 48 hours. The amine-substituted gemini surfactants were synthesized by reflux of 3,3'-iminobis(*N*,*N*-dimethylpropylamine) with 2 molar equivalents (plus 10% excess) of the appropriate 1-bromoalkane in HPLC-grate acetonitrile for 24 hours. The crude products were recovered by filtration and then purified by recrystallization; the structures were confirmed using ^{1}H NMR spectroscopy (Bruker 300 MHz). The surfactant purity was confirmed by the absence of a minimum in the post micelle region of the surface tension versus log concentration plot.

Scheme 2.1.2.3 Synthesis schemes for the A) nonsubstituted *m*-*s*-*m* type gemini surfactants, and B) amine-substituted *m*-7NH-*m* type gemini surfactants.

A)



B)



DNA solutions were prepared using double-stranded salmon sperm DNA obtained from Sigma, which was used as received. DNA stock solutions were prepared by sonication, and the concentration was calibrated spectrophotometrically using a Thermo Scientific NanoDrop 2000 spectrophotometer.

Water for all solutions was obtained from a Millipore Synergy purification system.

2.1.2.2 Methods

ITC measurements were performed using a MicroCal VP-ITC calorimeter. Aliquots (usually 5μL) of salmon DNA solution were injected from a Hamilton syringe into the sample cell containing the desired gemini surfactant; water was used as a reference. The experimental temperature was controlled at 25.00 ± 0.05°C. Experiments were repeated twice, and the experimental results were analyzed using Origin 7.0.

Particle size and zeta potential measurements were performed using a Malvern Zetasizer Nano ZS instrument. Both measurement types were performed in folded capillary cells (Malvern# DTS1060). The particle size distribution was determined using the non-negative least squares (NNLS) analysis method, where scattering was detected at 173°. Zeta potential measurements were carried out using laser Doppler microelectrophoresis at a frequency of 1000 Hz. All particle size and zeta potential measurements were repeated multiple times, and the average is reported.

Surface tension measurements were performed using a Lauda TE3 tensiometer, applying the du Noüy ring method. The temperature was kept constant at 25 ± 0.1°C using a circulating water bath. Surface tension values were corrected using the method of Harkins and Jordan.[14] Individual surface tension measurements were repeated multiple times.

2.1.3 Results and Discussion

The results and discussion section is broken down into two parts, which discuss the interaction properties between gemini surfactants and DNA in the forward and in the reverse titration manner separately.

2.1.3.1 Forward Titration: Gemini Surfactant into DNA

The properties of complex formation between gemini surfactants and DNA was first evaluated from a surface perspective, using surface tension measurements. Since gemini surfactants are surface active agents, complex formation should differ at the liquid/air interface than in the bulk, when DNA is initially in excess. Such a phenomenon has been observed before in the literature, where surface complexes (i.e. cationic amphiphile/DNA complexes) have been found to form before bulk complexes.[15,16]

Vongsetskul et al. have previously investigated the properties of complexation between gemini surfactants and DNA at the liquid/air interface using surface tension measurements.[16] However, in their study, only the 12-6-12 gemini surfactant was used to examine the interaction properties with DNA.[16] Therefore, the exact influence of the gemini surfactant alkyl tail length and the nature of the spacer group on the interaction properties with DNA at the liquid/air interface remains unknown.

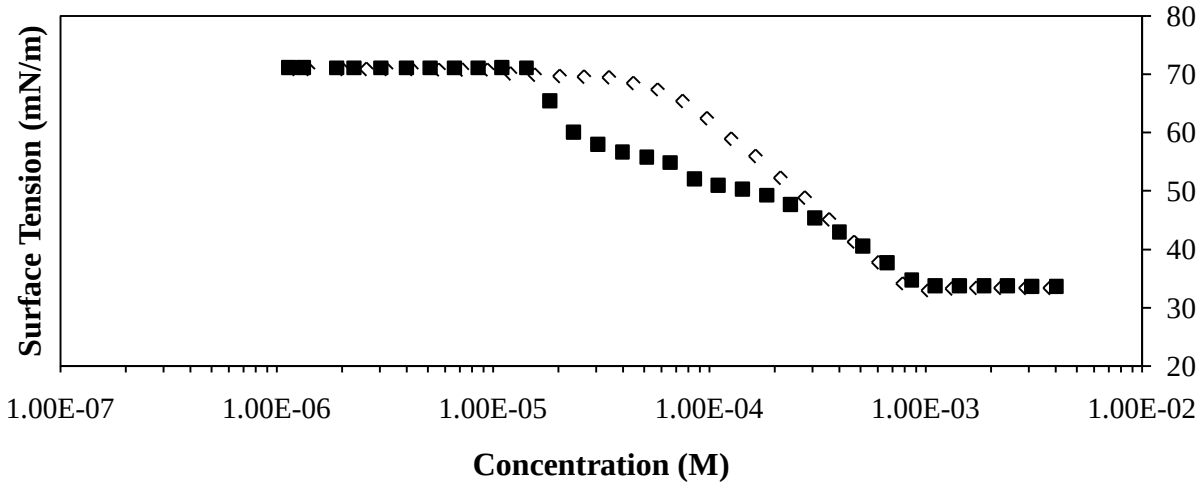
Within this study, complexation between gemini surfactants and DNA was examined as a function of, i) the gemini surfactant alkyl tail length (12-*s*-12 vs. 16-*s*-16) and, ii) the nature of the spacer group (*m*-3-*m* vs. *m*-7-*m* vs. *m*-7NH-*m*). Surface tension was measured as a function of the gemini surfactant concentration, as the gemini surfactant of interest was titrated into a 0.1 mM solution of salmon DNA. The combined results from this analysis are shown below in Figure 2.1.3.1. The surface tension plots for the titration of pure gemini surfactant into water are

shown for comparison purposes. Note, that in all cases, the surface tension of the pure DNA solution was nearly equal to that of pure water, which has been observed in the literature.[15,16]

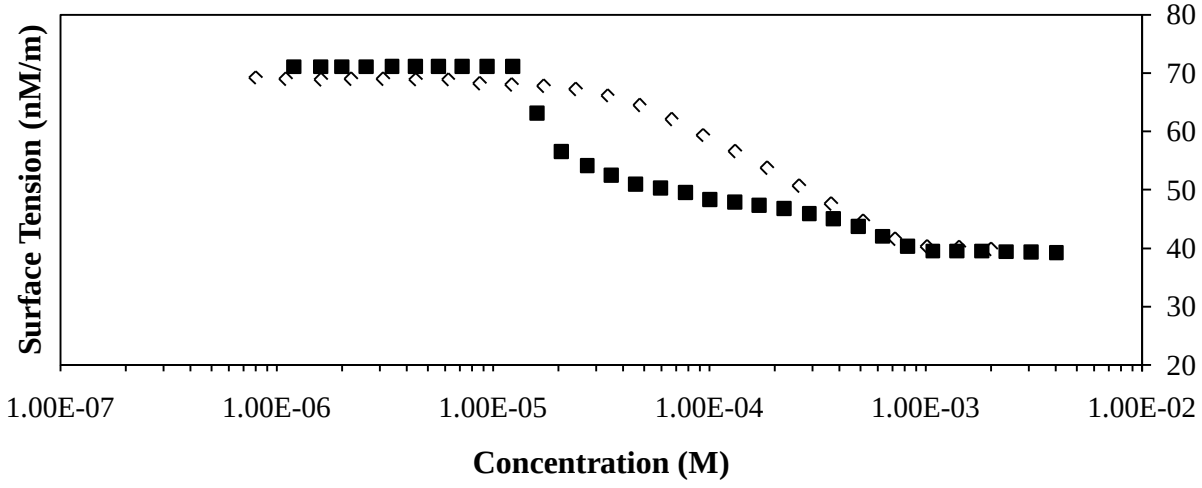
Figure 2.1.3.1 Surface tension plots for the titrations of gemini surfactant into water (◇), and into 0.1 mM salmon DNA solution (■) for A) 12-3-12, B) 12-7-12, C) 12-7NH-12, D) 16-3-16, E) 16-7-16, and F) 16-7NH-16.

12 Series

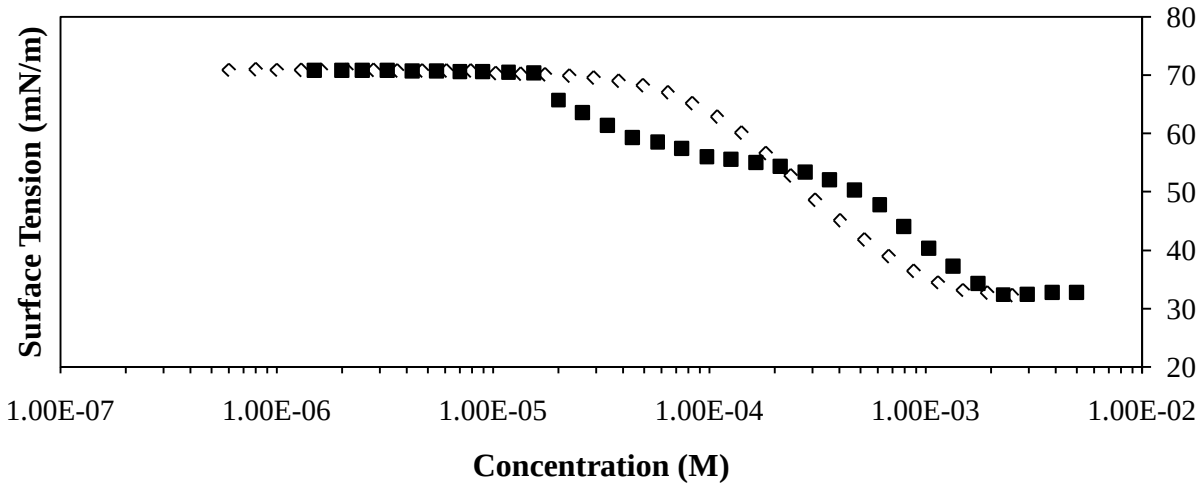
A) 12-3-12



B) 12-7-12

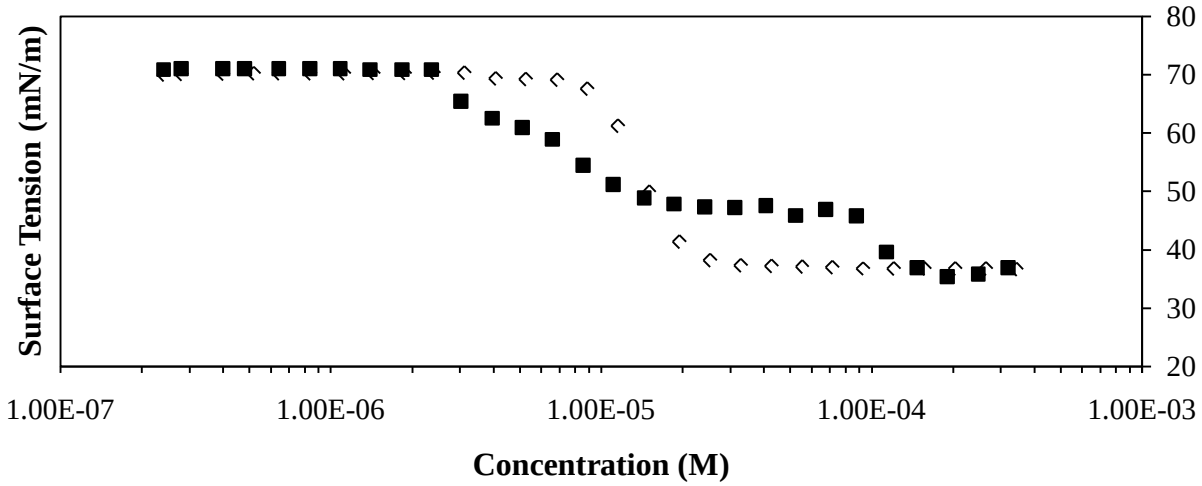


C) 12-7NH-12

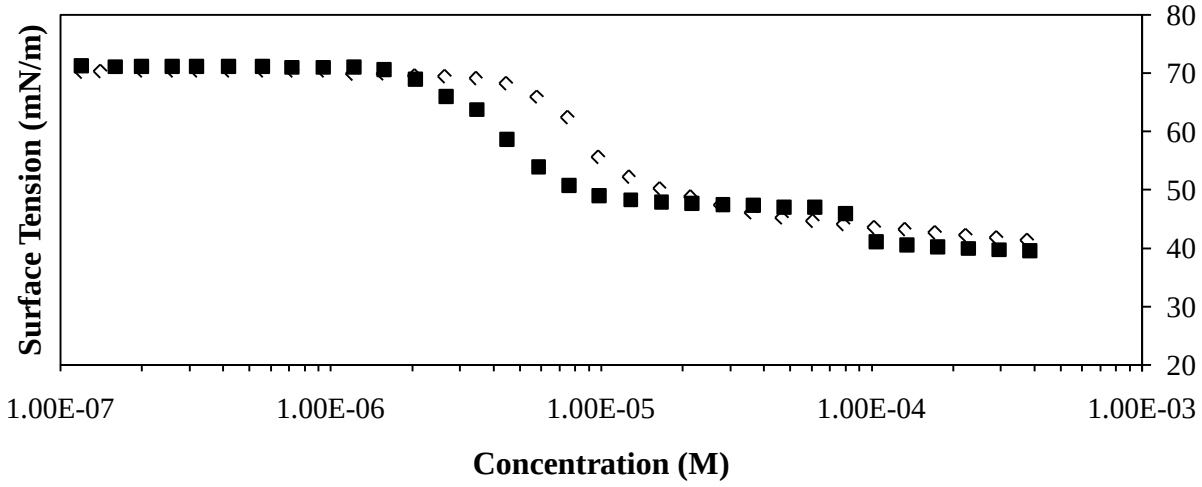


16 Series

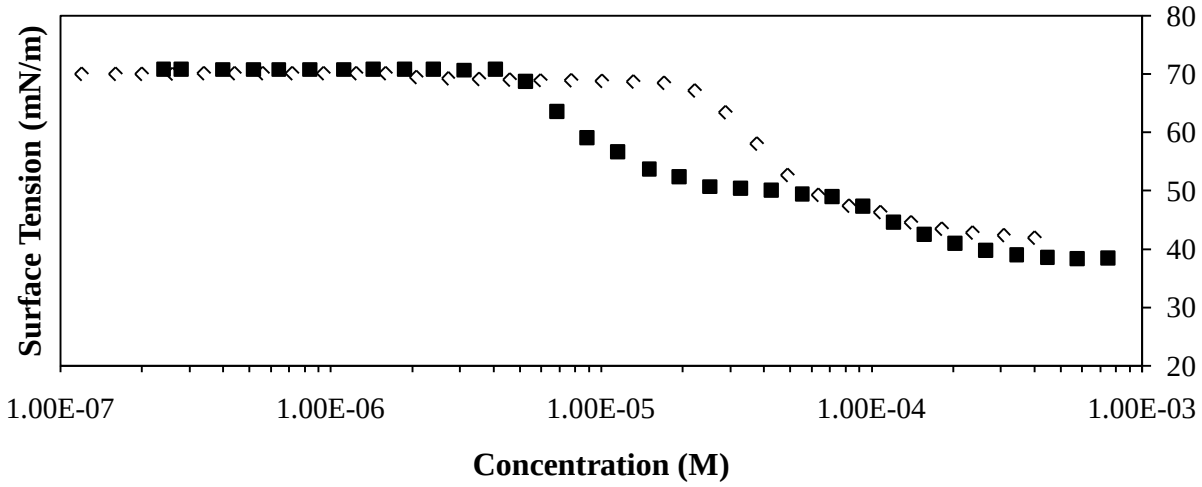
D) 16-3-16



E) 16-7-16



F) 16-7NH-16



As shown in all of the plots of Figure 2.1.3.1, there is a common pattern observed in the variation of surface tension as the gemini surfactant is titrated into the salmon DNA solution. The first immediate observation is that the surface tension starts to decrease at a significantly lower concentration when the gemini surfactant is titrated into the DNA solution, as opposed to when it is titrated into water. Such a pattern has been observed before in the literature, and is rationalized by the presence of DNA in solution inducing the adsorption of surfactant to the surface; more specifically, this behaviour indicates complex formation between DNA and the

gemini surfactant which attracts more surfactant to the surface.[15,16] As illustrated, the surface tension continues to decrease with increasing surfactant concentration but then eventually plateaus over a concentration range. This first breakpoint is referred to as the critical aggregation concentration (CAC), and is considered to correspond to the concentration of surfactant where cooperative binding first begins in the bulk.[15] The further addition of surfactant results in the continued binding of surfactant to DNA, and the surface tension remains fairly constant until the sites of DNA have been saturated with surfactant. Following this saturation point, further addition of surfactant leads to a lowering in the surface tension due to the adsorption of free gemini monomers to the surface. The surface tension continues to decrease until it reaches another plateau, which signifies the formation of regular gemini surfactant micelles (interface is saturated with gemini monomers, and thus gemini micelles begin to form in the bulk). This second breakpoint is represented as CMC*.

Overall, we see that the variation in surface tension from the titration of gemini surfactant into DNA solution is dependent upon the interplay of the assembly of surfactant molecules at the interface, and the complexation between gemini surfactants and DNA in the bulk and at the interface. The surface tension patterns observed in Figure 2.1.3.1 are consistent with those found in the literature, where it is proposed that surface aggregates form well before bulk aggregates.[15,16] The cumulative results from this analysis are tabulated below in Table 2.1.3.1.

Upon evaluation of the results in Table 2.1.3.1, it is apparent that the CAC is dependent upon the chemical structure of the gemini surfactant. The exact influences of the gemini alkyl tail length and the structure of the spacer group on the CAC are discussed separately below.

Table 2.1.3.1 Calculated critical concentrations for the titration of gemini surfactant into 0.1mM salmon DNA solution.

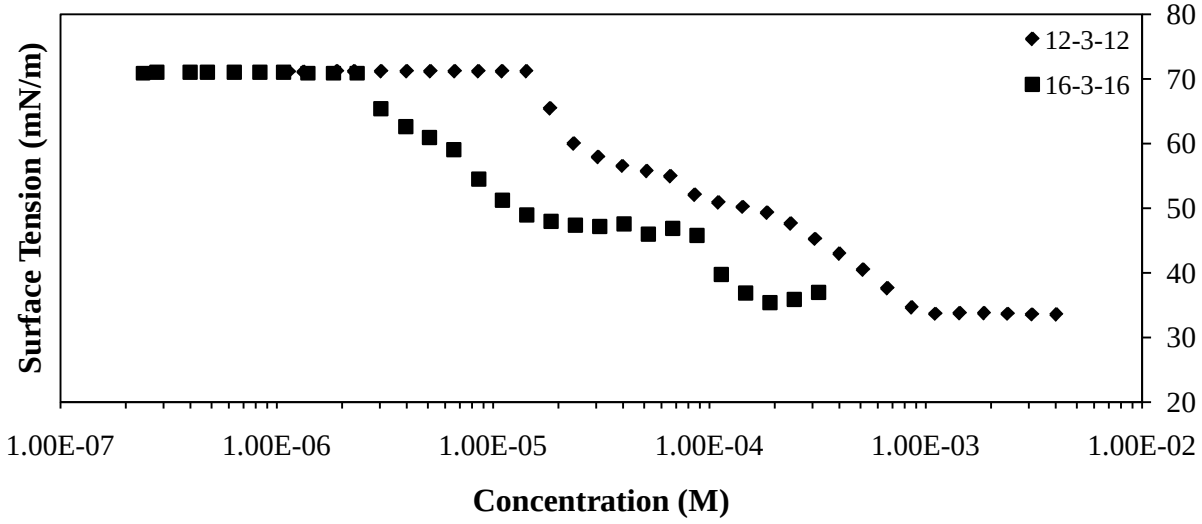
Surfactant	Titrand		
	Salmon DNA Solution		Water
	CAC (mM)	CMC* (mM)	CMC (mM)
12-3-12	0.028 ± 0.008	0.93 ± 0.02	0.89 ± 0.03
12-7-12	0.028 ± 0.008	0.91 ± 0.05	0.90 ± 0.04
12-7NH-12	0.033 ± 0.009	2.00 ± 0.09	1.17 ± 0.07
16-3-16	0.015 ± 0.002	0.15 ± 0.05	0.024 ± 0.003
16-7-16	0.011 ± 0.001	0.11 ± 0.01	0.026 ± 0.006
16-7NH-16	0.015 ± 0.003	0.27 ± 0.04	0.067 ± 0.006

Influence of the Gemini Alkyl Tail Length

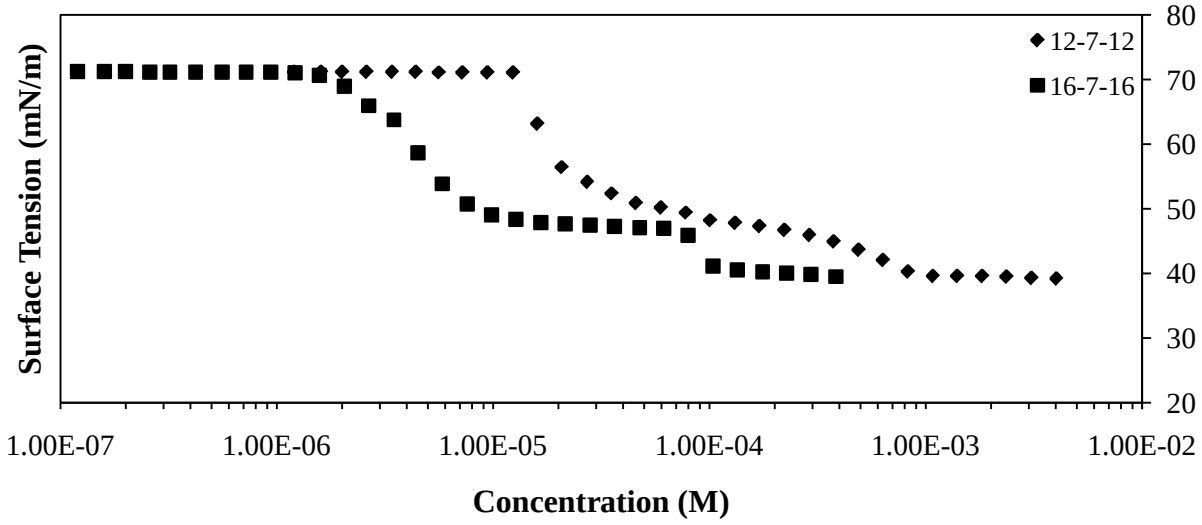
As observed in Table 2.1.3.1, it is apparent that in all cases when the spacer structure is the same, the 16-*s*-16 series of gemini surfactants experienced a significantly lower CAC in comparison to the 12-*s*-12 series. To help illustrate this, the surface tension plots of the titration of gemini surfactant into DNA solution as a function of the spacer group are shown below in Figure 2.1.3.2.

Figure 2.1.3.2 Surface tension plots for the titrations of gemini surfactant into 0.1mM salmon DNA solution for A) *m*-3-*m*, B) *m*-7-*m*, and C) *m*-7NH-*m*.

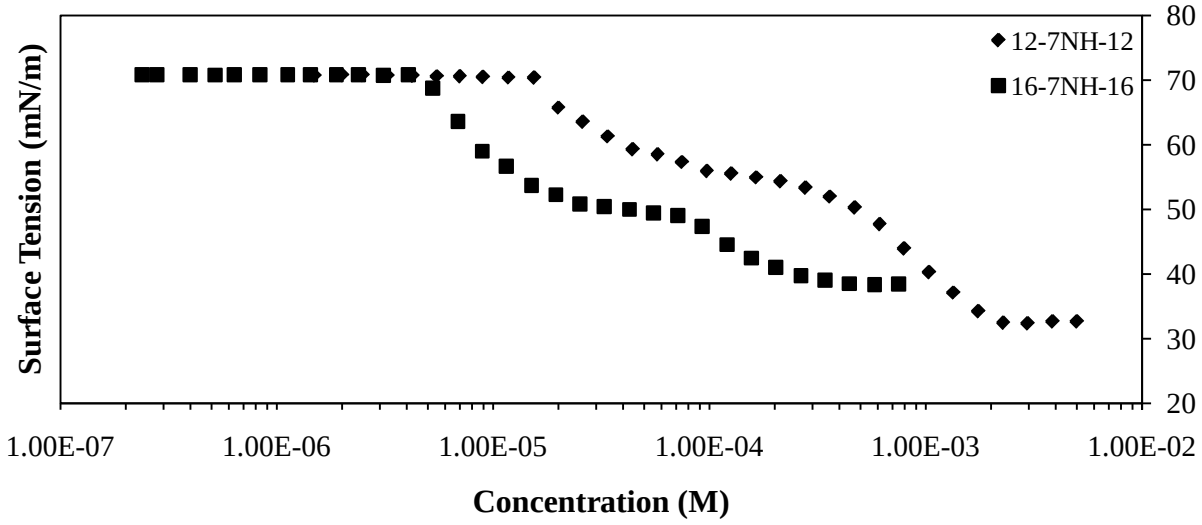
A) *m*-3-*m*



B) *m*-7-*m*



C) *m*-7NH-*m*



As clearly observed in all cases, the 16-*s*-16 series of surfactants exhibit a significantly lower CAC value in comparison to the 12-*s*-12 series. Based on this observation, it is evident that the length of the alkyl tail of the gemini surfactant plays a significant role in the complexation between gemini surfactants and DNA. More specifically, these results signify the importance of hydrophobic interactions between gemini surfactants and DNA, during complex formation.

The primary interaction between cationic amphiphiles and DNA is known to occur from an electrostatic interaction between the negatively charged phosphate sites of DNA and the cationic headgroups of the amphiphile.[17] There is an additional secondary contribution to cationic amphiphile/DNA complexation which occurs from hydrophobic interactions.[18,19] This hydrophobic contribution can occur from two perspectives. The first is from hydrophobic interactions between the alkyl tails of the amphiphile and the hydrophobic core of DNA.[18] The other influence stems from the hydrophobic interactions between the individual amphiphile molecules.[19] When the cationic amphiphile is initially added to the DNA solution, the first amphiphile molecule associated to a DNA molecule can promote more amphiphile association to the same DNA molecule through hydrophobic interactions between the amphiphile alkyl tails.[19]

Based on the just described contribution of hydrophobic interactions to cationic amphiphile/DNA complex formation, it is evident that increasing the alkyl tail length of the gemini surfactant should enhance these hydrophobic interactions, and therefore promote complexation with DNA. It is this enhanced interaction with DNA with likely accounts for the 16-*s*-16 series of gemini surfactants experiencing a lower CAC with DNA, in comparison to the 12-*s*-12 series. Such a pattern has been observed in the literature when the CAC was found to decrease as the alkyl tail length on a benzalkonium chloride surfactant was increased.[20]

Influence of the Spacer Group

Based on the results in Table 2.1.3.1, it is evident that there is no apparent trend in the CAC value as a function of the different spacer groups used in this study. This is interesting because one would expect the nature and/or length of the spacer group to influence the complexation between the gemini surfactant and DNA, and consequently have an influence on the CAC.

The *m*-7NH-*m* and *m*-3-*m* gemini surfactants are both expected to experience a more favourable electrostatic binding interaction with DNA, in comparison to the *m*-7-*m* gemini surfactant. The distances between the nitrogen centers of both the *m*-7NH-*m* and *m*-3-*m* gemini surfactants (5.08 and 5.25 Å, respectively)[21] correlate well with the distances between the phosphate groups of DNA (6.5-7.1 Å),[22] which can potentially make the electrostatic interactions between these components more favourable. In contrast, the distance between the nitrogen centers of the *m*-7-m gemini surfactants is expected to be much larger and closer to the literature value for the 12-8-12 surfactant, which is 11.52 Å.[21] Accordingly, one could expect greater complexation between DNA and the *m*-7NH-*m* / *m*-3-*m* gemini surfactants, and potentially a lower CAC value. Interestingly, as observed in Table 2.1.3.1, this was not the case in this experiment.

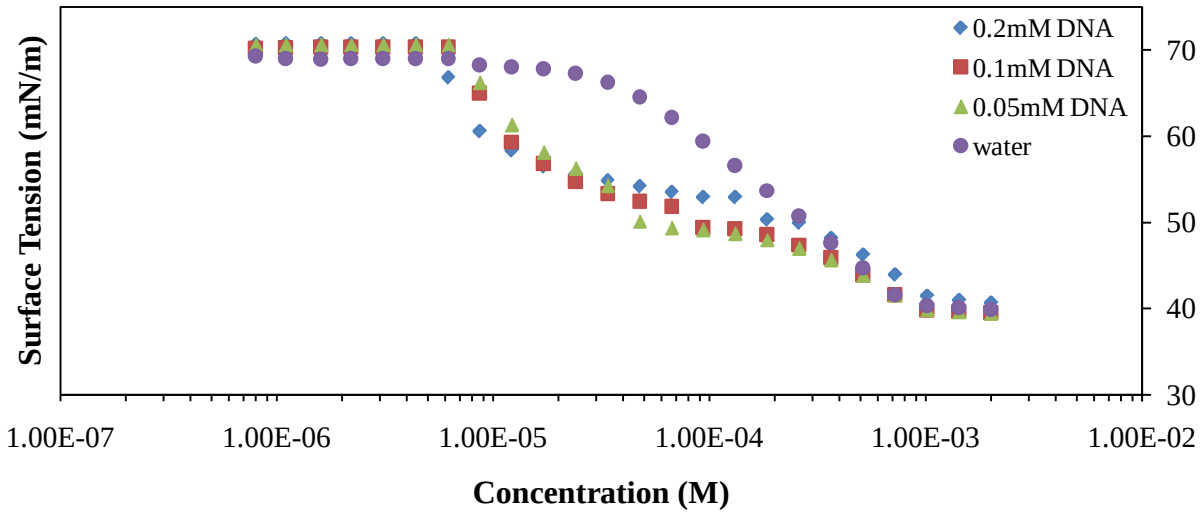
Influence of the DNA Concentration

The final investigation made in this surface tension study was an examination of the influence of the DNA concentration on complex formation between gemini surfactants and DNA. Figure 2.1.3.3 illustrates the variation of surface tension as the gemini surfactant is titrated into salmon DNA solution, at different DNA concentrations. The critical concentrations calculated from this analysis are tabulated below in Table 2.1.3.2.

Table 2.1.3.2 Tabulated results for the influence of DNA concentration on the surface tension plots for the titration of 12-7-12 into DNA solution.

DNA Concentration (mM)	CAC (mM)	CMC/CMC* (mM)
0	-	0.90 ± 0.04
0.05	0.035 ± 0.006	0.92 ± 0.01
0.1	0.026 ± 0.007	0.92 ± 0.01
0.2	0.019 ± 0.005	1.04 ± 0.05

Figure 2.1.3.3 Influence of DNA concentration on the surface tension plots for the titration of the 12-7-12 gemini surfactant into salmon DNA solution.



As observed in Figure 2.1.3.3, and reported in Table 2.1.3.2, the CAC was found to decrease as the DNA concentration increased. The behaviour can be rationalized by the fact that increases in the DNA concentration should enhance the occurrence of gemini/DNA complexation, which therefore results in this decrease of the CAC. This seems reasonable because as observed in Figure 2.1.3.3, when the gemini surfactant was titrated into the more concentrated 0.2 mM DNA solution, there is a greater initial decrease in the surface tension, which is likely the result of increased gemini/DNA complexation and adsorption of gemini surfactant to the liquid/air interface. Interestingly, previous studies in the literature have shown that the DNA concentration does not have an effect on the CAC; it has been reported that a 5-fold increase in DNA concentration did not have an influence on the CAC for a benzalkonium chloride/DNA and a 12-6-12/DNA system.[16,20]

As also observed in Table 2.1.3.2, when the gemini surfactant was titrated into the more concentrated 0.2 mM salmon DNA solution, the CMC* value (which is the concentration at which regular micelles form) was shifted to 1.04 mM in comparison to the actual CMC value of

0.90 mM in water. Such a pattern has been observed before in the literature for a benzalkonium chloride/DNA system, which was rationalized by the fact that an increased DNA concentration will require more surfactant monomers to saturate the sites on DNA, therefore prolonging the concentration at which regular micelles can form (i.e. the CMC* value).[20] Interestingly for a 12-6-12/DNA system, a 5-fold increase in the DNA concentration was not shown to influence the CMC* value.[16]

As a whole, this investigation examined the properties of complex formation between gemini surfactants and DNA at the liquid/air interface using surface tension measurements. The patterns observed in the surface tension plots from the titration of gemini surfactant into salmon DNA solution were consistent with those found in the literature, where it is proposed that surface aggregates form well below bulk aggregates.[15,16] Our results showed that the CAC value is dependent on the gemini surfactant alkyl tail length, as the CAC was significantly reduced when the tail length was increased from 12 carbons to 16 carbons; this behaviour was rationalized in terms of the enhanced hydrophobic interactions as the tail length is increased. The nature of the spacer group (*m*-3-*m* vs. *m*-7-*m* vs. *m*-7NH-*m*) was however not shown to have an effect on the CAC value.

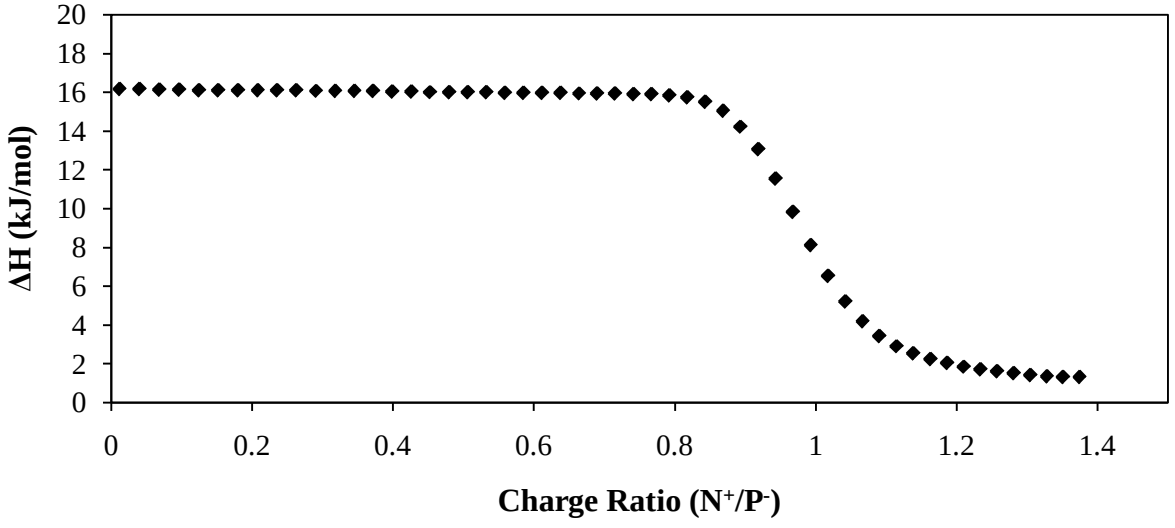
Bulk Complexation

The properties of complexation between gemini surfactants and DNA was next evaluated using ITC. ITC measurements were carried out to investigate the enthalpy profile of complexation between gemini surfactant and DNA. Figure 2.1.3.4 illustrates a typical enthalpogram obtained for the titration of gemini surfactant into DNA solution. As shown, the

enthalpy of binding between gemini surfactants and DNA is endothermic, and remains fairly constant until the reaction endpoint, where it drops abruptly to approximately 0 kJ/mol.

The pattern observed in Figure 2.1.3.4 is in good agreement with those previously reported by Wang et al.[9] In their study, the authors suggested that the lack of any peak regions in the enthalpograms implies that complex formation in an excess of DNA does not lead to any major morphological changes prior to precipitation.[9] Concurrent particle size measurements were carried out where it was found that the particle diameter decreased as the gemini surfactant was added to the DNA solution, due to the condensation of DNA by the added surfactant.[9] However, as the surfactant/DNA charge ratio approached approximately 1.0, the particle size increased abruptly as a result of precipitation.[9] Their results were further supported by atomic force microscopy (AFM) images.[9] Within their study, the properties of complexation was evaluated as a function of the gemini surfactant structure.[9]

Figure 2.1.3.4 Observed enthalpies for the titration of the 12-3-12 gemini surfactant into salmon DNA solution at 25°C. N^+/P^- refers to the nitrogen/phosphate charge ratio.



2.1.3.2 Reverse Titration: DNA into Gemini Surfactant

Following the evaluation of complex formation between gemini surfactants and DNA in the forward titration manner, the properties of complexation was examined in the reverse order. This was carried out using ITC, and as shown in Figure 2.1.3.5, the enthalpy profile obtained from the reverse titration of DNA into gemini surfactants is dramatically different than it is in the forward manner, as there are two peaks present on the enthalpogram in Figure 2.1.3.5.

Figure 2.1.3.5 Observed enthalpies for the titration of salmon DNA into 12-7-12 gemini surfactant solution at 25°C. P^-/N^+ refers to the phosphate/nitrogen charge ratio.

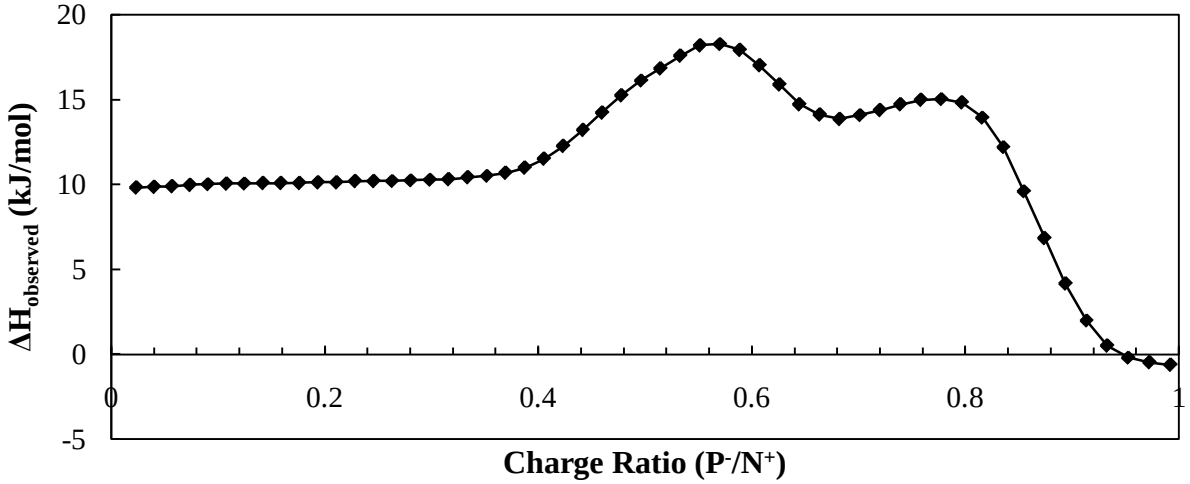


Table 2.1.3.3 Measured properties of complex formation from the titration of DNA into 12-7-12 gemini surfactant solution. X_1 and X_2 correspond to the P^-/N^+ charge ratios for peaks 1 and 2 respectively. The acronym "bp" refers to base pairs.

$\Delta H_{initial}$ (KJ/mol bp)	ΔH_1 (KJ/mol bp)	X_1	ΔH_2 (KJ/mol bp)	X_2
10.1	18.3	0.57	15.0	0.78

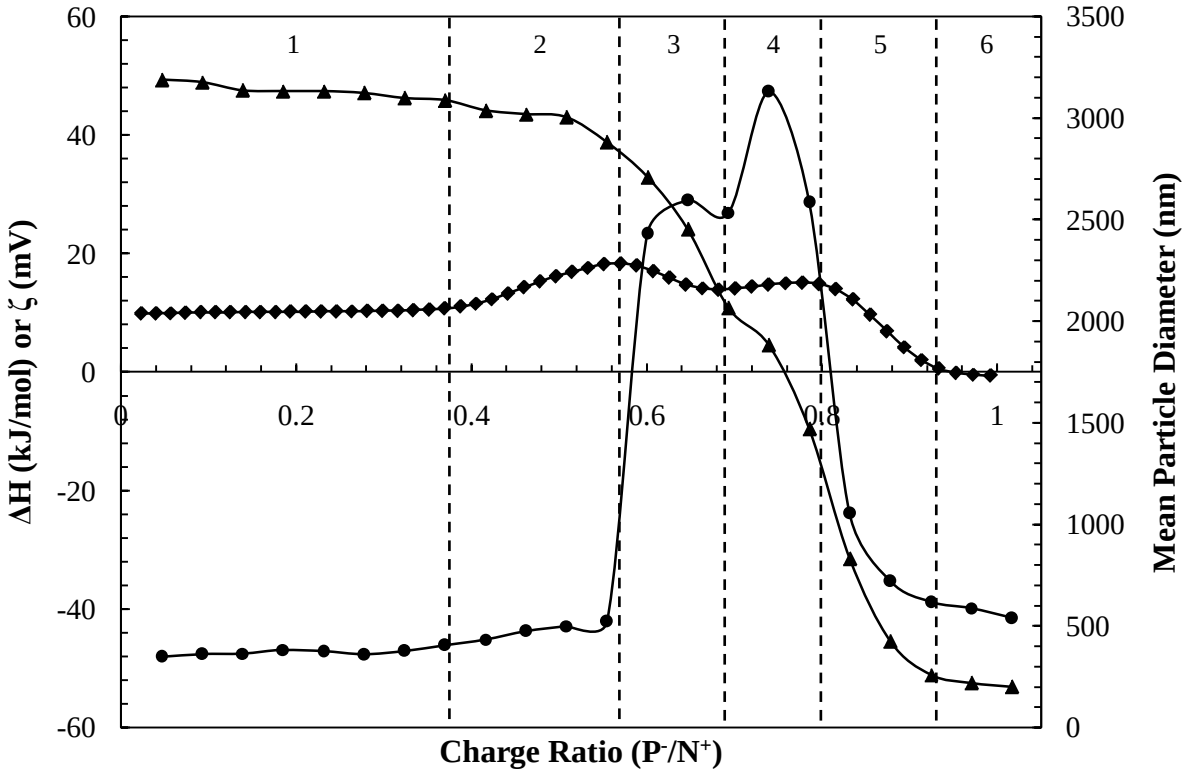
Complexation between *m-s-m* type gemini surfactants and DNA in the reverse titration manner was previously investigated by Wang et al., who used a combination of ITC, AFM, particle size and zeta potential measurements to characterize the energetics and mechanism of complex formation.[9] The enthalpograms obtained in their study showed a single broad peak.[9]

They proposed that complex formation in the reverse titration manner proceeded in three stages, i) at low DNA concentrations, the "beads on a string" structure was proposed to occur, which was then proceeded by ii) the reorganization of gemini/DNA complexes into more discrete, compact particles that were hypothesized to flocculate over a narrow region of charge ratios, followed by iii) the precipitation of the neutral complexes at higher DNA concentrations.[9]

Based on our experimental results, it appears that the mechanism of complex formation previously reported by Wang et al. is an oversimplification of the actual process. As shown in Figure 2.1.3.5, the enthalpogram obtained in our study showed two peaks as oppose to a single broad peak (which was obtained by Wang et al.), which suggests a more complex mechanism of association between gemini surfactants and DNA. A possible explanation for the differences in the enthalpograms is the greater sensitivity of the instrument used in our study, which was able to resolve the broad peak as previously observed by Wang et al. into two separate peaks.

To assist in the explanation of the enthalpy profile observed in Figure 2.1.3.5, particle size and zeta potential measurements were concurrently taken under identical experimental conditions. The combined evolution of the mean particle diameter (z-average), zeta potential, and observed enthalpy as DNA is titrated into gemini surfactant solution is illustrated in Figure 2.1.3.6. Note that Figure 2.1.3.6 is broken down into 6 regions, to assist in the explanation of the enthalpy profile. As well, scheme 2.1.3.1 is provided as an illustration of the proposed mechanism.

Figure 2.1.3.6 Observed enthalpies (♦, left axis), zeta potentials (▲, left axis) and mean particle diameters (●, right axis) for the titration of DNA into 12-7-12 gemini surfactant solution.



As observed in Figure 2.1.3.6, when DNA is initially titrated into the gemini surfactant solution, a near constant endothermic enthalpy of 10.1 kJ/mol is observed in the system (designated as Region 1). Such a pattern was observed by Wang et al., where it was proposed that this initial region corresponds to the interaction of DNA molecules with gemini surfactant micelles, forming the "beads on a string structure" (see Part B in scheme 2.1.3.1 for an illustration of this structure).[9]

As DNA is further added to the system, an endothermic transition is observed in the enthalpy profile; designated as Region 2. One can postulate that within this region, the number of available gemini micelles that can adopt the beads on a string conformation decreases, which consequently shifts the gemini monomer-micelle equilibrium towards demicellization. The

process of transforming micelles into monomers is endothermic, i.e. the opposite sign of the exothermic heat of micellization.[9] The enthalpy for this transition is 8.2 kJ/mol, which is in good agreement with the estimated enthalpy of demicellization for 12-7-12, which is predicted to be between 8.5 and 9.0 kJ/mole (i.e. an intermediate value of the enthalpy of demicellization for 12-6-12 and 12-8-12).[23] The Wettig group has shown that the enthalpy for this transition from the titration of DNA into the 12-3-12 surfactant is 20.0 kJ/mol, which is similar to the 19.3 kJ/mol enthalpy of demicellization for 12-3-12.[24] It is worth noting that within Region 2, there are no significant changes in particle size and zeta potential, which further supports that this endothermic transition is a result of gemini demicellization.

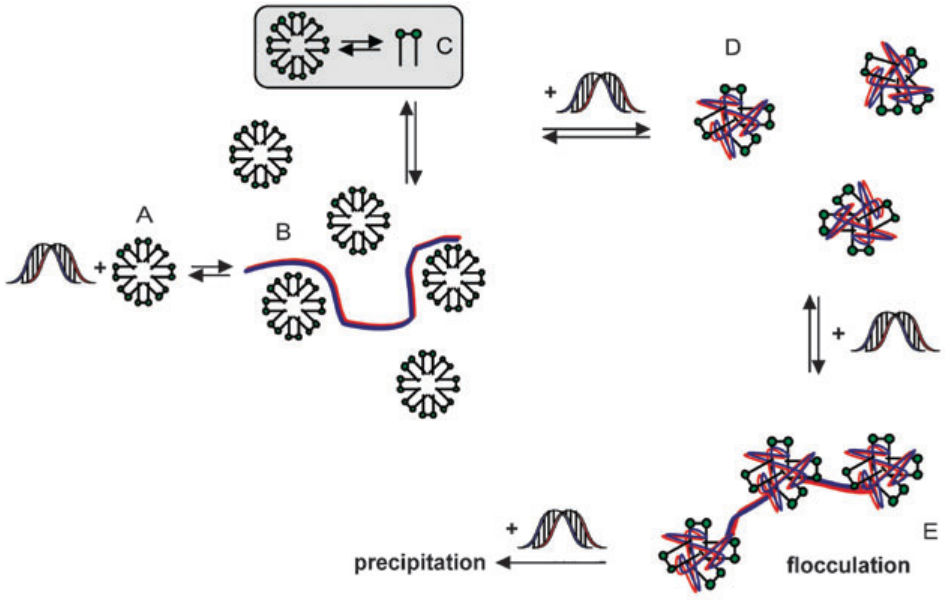
As shown in Region 3, when DNA is further added to the system, there are significant changes in all 3 measured properties. There is an exothermic transition in the enthalpy profile, decrease in the zeta potential, and an increase in the particle diameter. It is postulated that within this region, as DNA is added, the system reaches a point where there are no additional gemini micelles present to interact with DNA. As such, there is a significant reorganization in the system, to most effectively reduce the electrostatic repulsions between the DNA molecules (see section D in scheme 2.1.3.1). This results in a near complete charge neutralization of the system (shown by the decrease in zeta potential), and the formation of large complexes as shown by the increases in particle diameter. The exothermic contribution observed in this region can potentially be attributed to the release of a substantial amount of structured water along with an exothermic binding between DNA molecules and gemini monomers.

Within Region 4, there is a second endothermic transition within the system, a near plateau in the zeta potential, and a further increase in the particle size. A reasonable assignment

for this endothermic transition is the flocculation of the gemini/DNA system. This is supported by the near 0 mV value of the zeta potential, and the increase in the mean particle diameter.

Region 5 can essentially be correlated to the precipitation of the gemini/DNA system. As shown, the observed enthalpy essentially decreases to 0 kJ/mol, and simply corresponds to the enthalpy of dilution for DNA, since no additional interactions can take place within the system.

Scheme 2.1.3.1 Representation of the mechanism of complex formation from the titration of DNA into the 12-7-12 gemini surfactant. From reference [25]



As a whole, it is evident that the previously reported interaction mechanism between gemini surfactants and DNA (by Wang et al.) in the forward titration manner appears to be an oversimplification of the actual process. From our work, the actual mechanism clearly involves a complex series of equilibria as the system progresses from a “beads on a string” type interaction to precipitation.

2.1.4 Conclusions

Overall, the broad aim of this investigation was to examine the thermodynamics and mechanism of complexation between gemini surfactants and DNA. This was carried out in a series of ITC, particle size, zeta potential, and surface tension experiments, which examined the properties of this complexation in the forward and reverse titration manner.

In the forward titration manner, complexation between gemini surfactants and DNA was initially examined from a surface perspective using surface tension measurements. Our experimental results were found to be in good agreement with those previously reported, which suggest that surface aggregates form before bulk aggregates.[15,16] Furthermore, our experimental results demonstrated that the CAC is dependent upon the alkyl tail length of the gemini surfactant (12-*s*-12 vs. 16-*s*-16), but not on the structure of the spacer group (*m*-3-*m* vs. *m*-7-*m* vs. *m*-7NH-*m*). Complexation in the forward sequence was further evaluated using ITC, and the results obtained in our study were found to be in good agreement with those previously reported by Wang et al.; that is, the enthalpy of binding between gemini surfactants and DNA is endothermic, and remains fairly constant until the reaction endpoint, where it drops abruptly to approximately 0 kJ/mol.[9]

In the reverse titration manner, our experimental results demonstrated that the previously reported mechanism of association between gemini surfactants and DNA in the literature is an oversimplification of the actual process. Based on a series of ITC, particle size and zeta potential measurements, our results demonstrated that the actual process is likely a complex series of linked equilibria where the system progresses from a “beads on a string” type interaction to a complete reorganization and the formation of neutral complexes, followed by flocculation and precipitation. It is worth mentioning that the Wettig group is currently evaluating the influence of

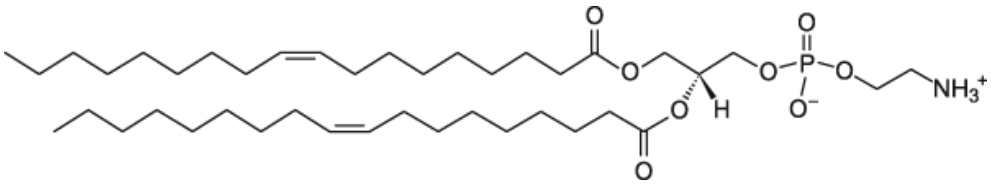
the gemini surfactant structure on the enthalpy profile of the titration of gemini surfactant into DNA.

2.2 Interaction between Gemini Surfactants and DOPE

2.2.1 Introduction

Within this study, the mixing interaction between gemini surfactants and DOPE was investigated. Despite being just as important as the interaction with DNA, the study of the interactions between gemini surfactants and neutral lipids, such as DOPE, has received little attention. In fact, upon a literature search, the mixing interaction properties between gemini surfactants and DOPE has not been previously characterized. As introduced in chapter 1, the interaction between these two amphiphiles is of great significance because the liposome structure used in the preparation of gemini surfactant-based lipoplexes is a mixed aggregate of these two components.

In chapter 1 it was introduced that mixed amphiphile systems can exhibit an ideal or nonideal mixing behaviour in solution. Whether or not an ideal or non-ideal mixing behaviour is observed is dependent upon the individual structures of the amphiphiles.[1] Scheme 2.2.1.1 illustrates the chemical structure of DOPE, and it can be seen that it has a significantly different chemical structure in comparison to the *m-s-m* type gemini surfactants. Typically, mixtures of cationic and nonionic amphiphiles exhibit a nonideal mixing behaviour in solution due to a net interaction between the headgroups.[2-6]



Scheme 2.2.1.1 Chemical structure of DOPE.

Within this study, the nature and strength of the interaction between gemini surfactants and DOPE was evaluated using Rubingh's theory for mixed micellar systems. The interaction with DOPE was assessed as a function of the gemini surfactant spacer group and alkyl tail length. In regards to assessing the effect of the spacer group, the 16-3-16, 16-7-16 and 16-7NH-16 gemini surfactants were used. These particular surfactants were chosen to allow us to see the effects of i) the spacer length (16-3-16 vs. 16-7-16), and ii) the influence of the secondary amine group (16-7NH-16 vs. 16-7-16) on the interaction properties with DOPE. In regards to the effect of the alkyl tail length, the 12-7-12, 14-7-14 and 16-7-16 surfactants were used.

2.2.2 Experimental Procedures

2.2.2.1 Materials

The 16-3-16, 16-7-16, 16-7NH-16, 14-7-14 and 12-7-12 gemini surfactants used in this study were synthesized according to procedures previously reported in the literature.[7,8] The surfactants were purified by recrystallization, and the structures were confirmed using ^{1}H NMR spectroscopy (Bruker 300 MHz). The purity was confirmed by the absence of a minimum in the post micelle region of the surface tension versus logarithmic concentration plot.

1,2-dioleyl-*sn*-glycerophosphatidylethanolamine (DOPE) was obtained from Avanti Polar Lipids (Alabaster, Alabama, USA), and was used without any further purification.

Water for all solutions was obtained from a Millipore Synergy purification system.

2.2.2.2 Methods

To prepare the binary gemini/DOPE systems, the particular gemini surfactant studied was mixed with DOPE in ethanol (sonicated for 10 minutes) and then deposited as a thin film on a round bottom flask using a Heidolph rotary evaporator. The amphiphile mixture was then suspended in water using a water bath sonicator.

The mixed gemini and DOPE systems were prepared in the gemini:DOPE molar ratios of 0, 0.2, 0.4, 0.6, 0.8 and 1, to a final concentration of 2 mM for the 16-3-16, 16-7-16 and 16-7NH-16 systems, and to a final concentration of 5 mM for the 14-7-14 and 12-7-12 systems.

Surface tension measurements were performed using a Lauda TE3 tensiometer, applying the du Noüy ring method. The temperature was kept constant at 25 ± 0.1°C using a circulating water bath. Surface tension was measured as a function of concentration of the single or binary amphiphile system. The surface tension values were corrected using the method of Harkins and Jordan,[9] and individual surface tension measurements were repeated multiple times.

2.2.3 Results and Discussion

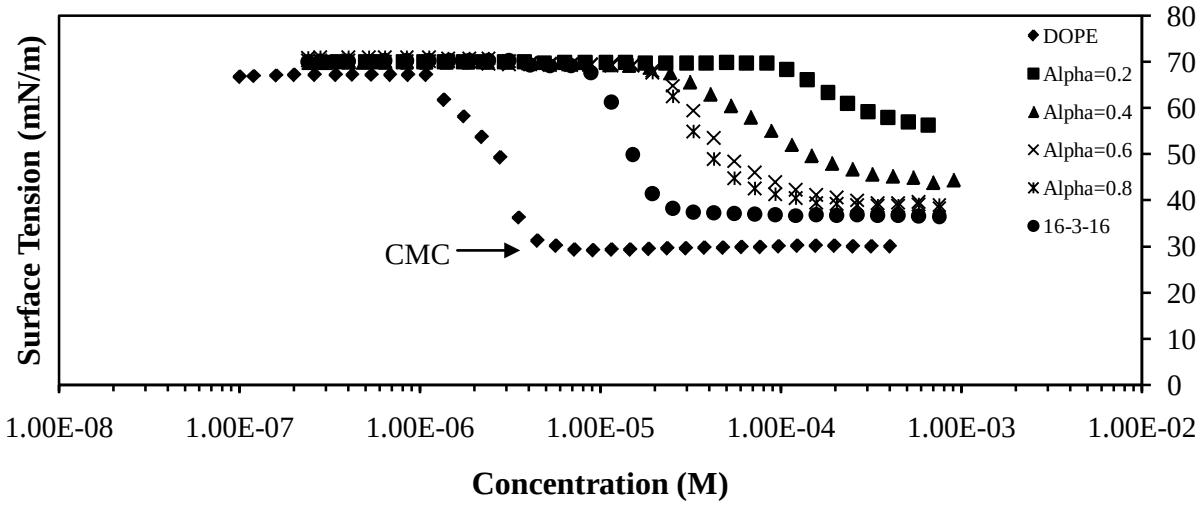
The classical way of evaluating the interactions between amphiphiles is by determining the CMC of the mixed system at different molar ratios between 0 and 1.[1] As discussed in chapter 1, amphiphile mixtures do form mixed aggregates in aqueous solution, but the tendency towards aggregation can be different from that of the pure components.[1] Within this study, the CMCs of the mixed gemini/DOPE systems were evaluated at the gemini:DOPE molar ratios of 0, 0.2, 0.4, 0.6, 0.8 and 1.

The CMC values of the pure and mixed gemini/DOPE systems were determined using surface tension measurements. On a plot of surface tension versus the logarithmic amphiphile concentration, the CMC corresponds to the breakpoint in the curve (indicated by the arrow in Figure 2.2.3.1), which can be determined through linear fitting of the pre-micellar and post-micellar regions. The surface tension plots for the mixed gemini/DOPE systems are shown below in Figure 2.2.3.1. It should be noted that CMC experiments for the mixed systems composed of DOPE with 12-7-12 and 14-7-14 were attempted but the CMCs could not be reached, despite using 5 mM stock solutions of these mixtures. Even though more concentrated solutions could

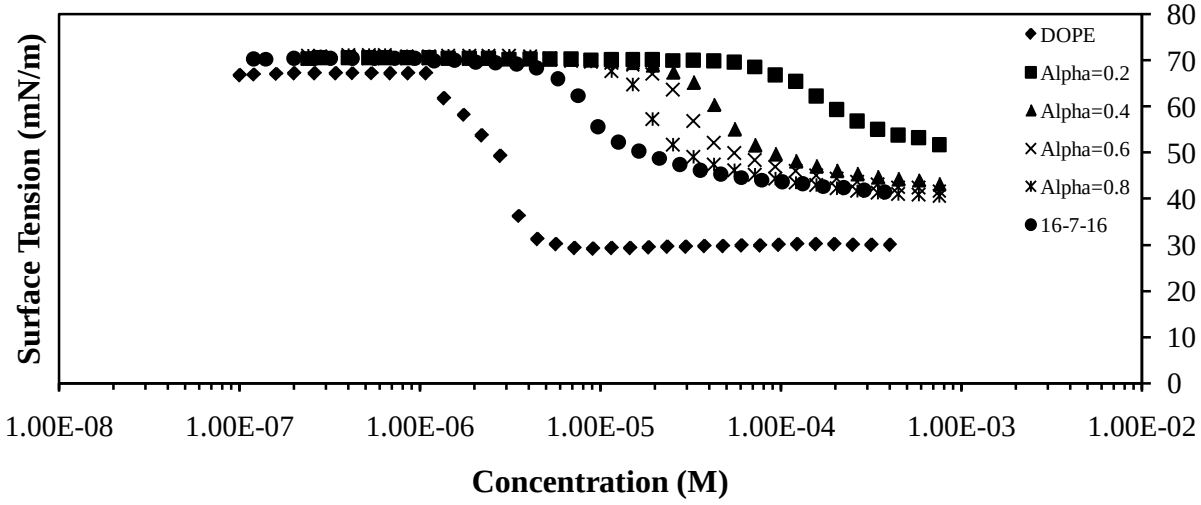
have been prepared, the high cost of DOPE made this irrational. As such, only the interactions between 16-3-16, 16-7-16 and 16-7NH-16 with DOPE are presented in this study, and the effect of the variation in surfactant alkyl tail length was abandoned.

Figure 2.2.3.1 Surface tension plots for the mixed amphiphile systems of A) 16-3-16 + DOPE, B) 16-7-16 + DOPE, and C) 16-7NH-16 + DOPE, at 25°C. Note, alpha represents the mole fraction of the gemini surfactant in solution.

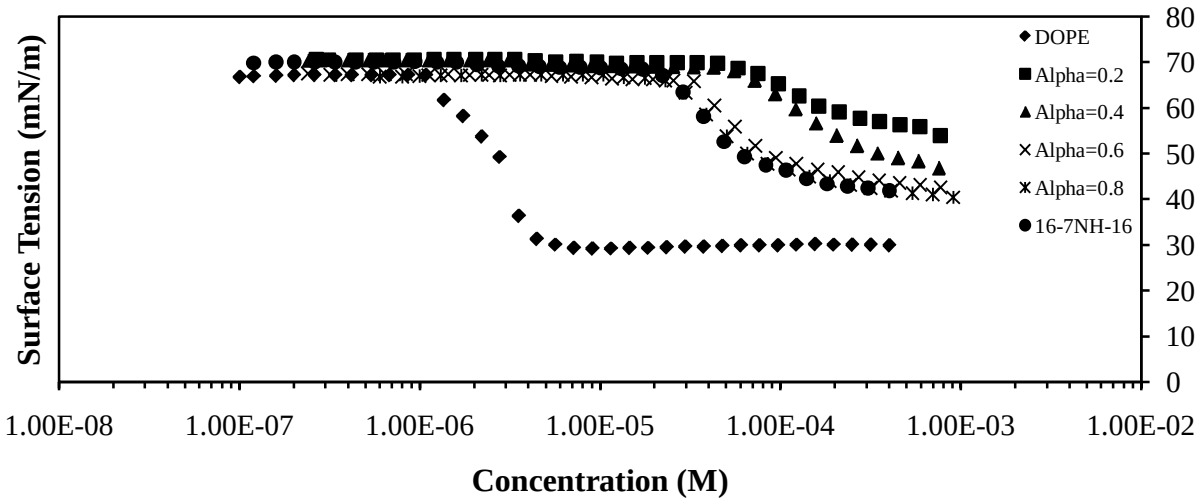
A)



B)



C)



The first step in the evaluation of the interaction properties of amphiphile mixtures is to compare the experimental CMC values with the ideal values, at the different molar ratios studied. The ideal CMCs are calculated using Clint's model for mixed micellar systems, which assumes that there is no net interaction between the amphiphiles (i.e. ideal mixing).[10] Clint's equation is represented by

$$\frac{1}{\mathrm{CMC_{mix}}} = \sum_{i=1}^{2} \frac{\alpha_i}{\mathrm{CMC}_i} \qquad \text{Eq. 2.2.1.}$$

where the ideal CMC of the mixture, $\mathrm{CMC_{mix}}$, can be determined by the mole fraction of component i in solution, α_i, and the CMC of pure component i, CMC_i.[10]

Figure 2.2.3.2 illustrates the comparison of the experimental CMC values with the ideal values for the mixed gemini/DOPE systems, as a function of α_{gemini}; the results are tabulated in Table 2.2.3.1. At first glance, it is seen that in all cases, the experimental CMC values are larger than the ideal values. These positive deviations are indicative of a net interaction between the amphiphiles, which is repulsive in nature; that is, an antagonistic mixing interaction is evident

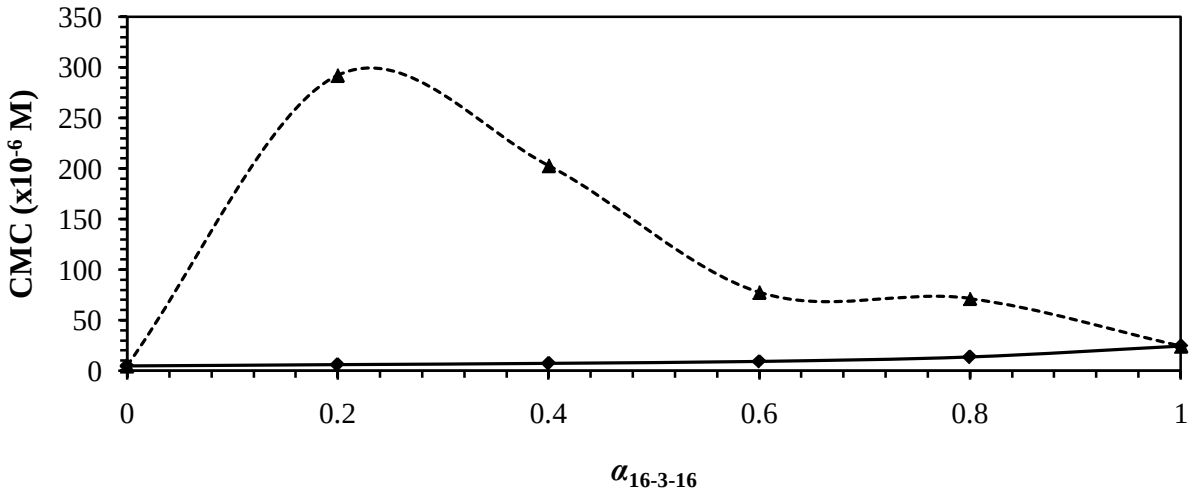
between gemini surfactants and DOPE which results in the experimental CMCs being larger than the ideal CMCs.[1] Two important points to note in Figure 2.2.3.2 is that the degree of deviation in the experimental CMC values from the ideal values appears to be dependent upon i) the structure of the gemini surfactant, and ii) the molecular composition in solution (i.e. α_{gemini}). These interaction differences were further investigated and quantified using Rubingh's approach.

Table 2.2.3.1 Comparison of the measured and calculated CMC values for the single and binary gemini/DOPE systems at 25°C.

16-3-16 + DOPE			**16-7-16 + DOPE**			**16-7NH-16 + DOPE**		
α_{gemini}	$CMC_{Exp.}$ ($x10^{-6}M$)	CMC_{Clint} ($x10^{-6}M$)	α_{gemini}	$CMC_{Exp.}$ ($x10^{-6}M$)	CMC_{Clint} ($x10^{-6}M$)	α_{gemini}	$CMC_{Exp.}$ ($x10^{-6}M$)	CMC_{Clint} ($x10^{-6}M$)
0	5.03	-	0	5.03	-	0	5.03	-
0.2	292	5.98	0.2	294	6.00	0.2	291	6.17
0.4	203	7.36	0.4	97.2	7.43	0.4	282	7.98
0.6	77.6	9.59	0.6	56.87	9.76	0.6	120	11.3
0.8	71.6	13.7	0.8	40.83	14.2	0.8	91.6	19.3
1	24.2	-	1	26.2	-	1	67.0	-

Figure 2.2.3.2 Plots of CMC versus α_{gemini} for the A) 16-3-16 + DOPE, B) 16-7-16 + DOPE, and C) 16-7NH-16 + DOPE binary systems at 25°C. Measured CMC values are represented as (▲); calculated ideal CMC values from Clint's model are represented as (♦).

A)



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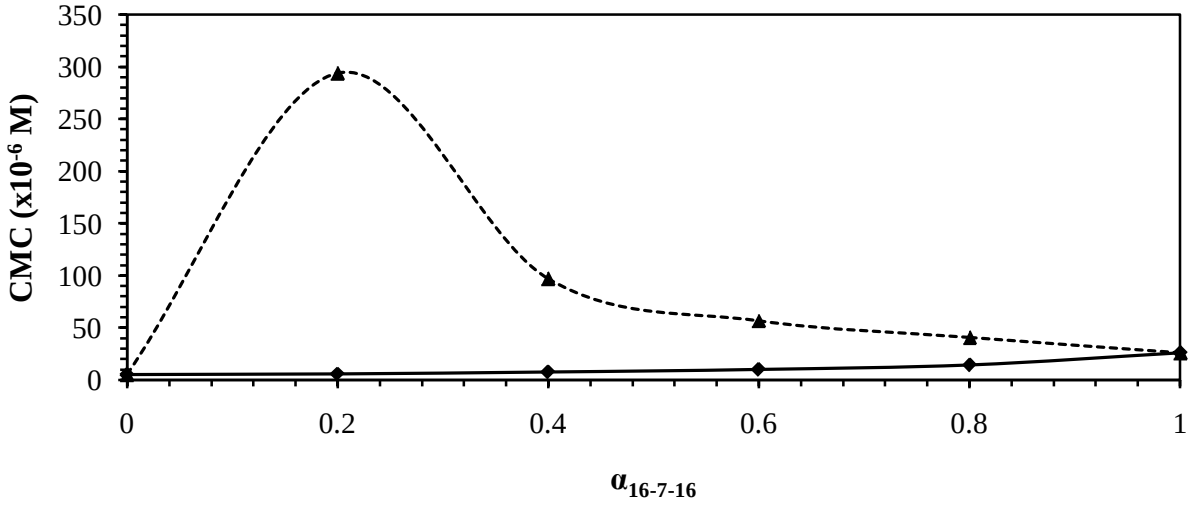

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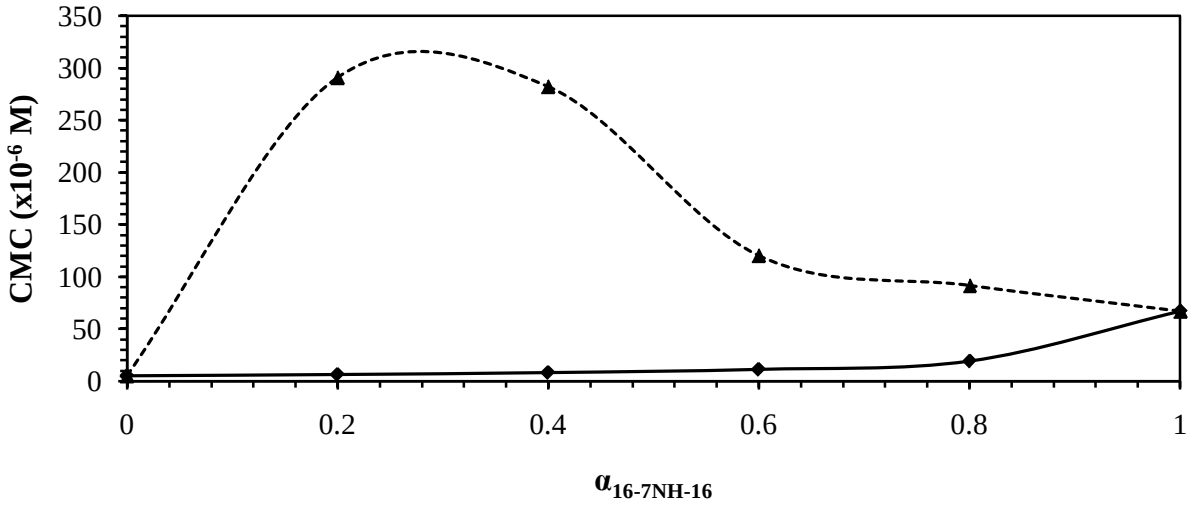


Rubingh's model is an effective approach classically found in the literature to evaluate the nature and strength of the interactions in mixed surfactant systems. Rubingh's model can be represented by the following equation

$$\frac{X_1^2 \ln(\mathrm{CMC}_{\mathrm{mix}}\,\alpha_1/\mathrm{CMC}_1 X_1)}{(1-X_1)^2 \ln(\mathrm{CMC}_{\mathrm{mix}}\,(1-\alpha_1)/\mathrm{CMC}_2(1-X_1))} = 1 \qquad \text{Eq. 2.2.2.}$$

which can be used to calculate the composition of the mixed micellar phase, where X_1 is the mole fraction of surfactant 1 (in our case, the gemini surfactant) in the mixed micelle.[1] X_1 is calculated through an iterative process, which subsequently allows for the determination of the interaction parameter, β, which can be calculated using the equation

$$\beta = \frac{\ln(\mathrm{CMC}_{\mathrm{mix}}\,\alpha_i/\mathrm{CMC}_i X_i)}{(1-X_i)^2} \qquad \text{Eq. 2.2.3.}$$

where the variables are the same to those previously described.[1] As briefly introduced in chapter 1, the value of β is indicative of the nature and strength of the interaction between the amphiphiles in the mixed system. Negative values of β represent a synergistic interaction, while positive values of β represent an antagonistic interaction.[1] Furthermore, the magnitude of β is indicative of the strength of the interaction; that is, the larger the magnitude of β, the stronger the interaction between the amphiphiles.[1]

The micellar mole fractions of the gemini surfactant in the mixed gemini/DOPE aggregates were first calculated using the previously described Eq. 2.2.2, and then compared to the ideal values calculated using Motomura's equation. Motomura's equation allows for the determination of the micelle mole fractions in the ideal state, X_{ideal}, which can be calculated using the relationship given in equation 2.2.4.[11]

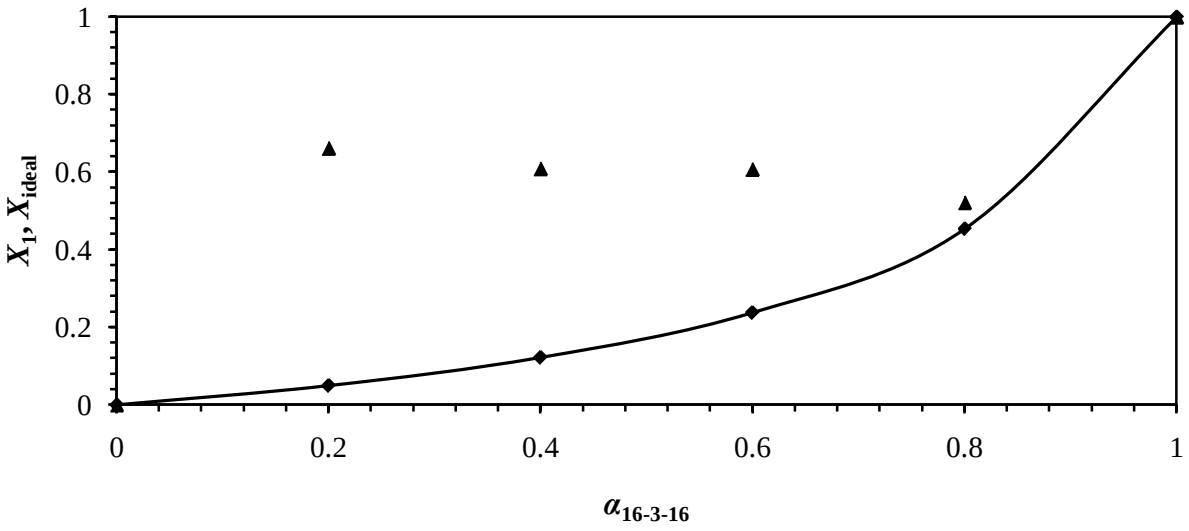
$$X_{\mathrm{ideal}} = [(\alpha_1 \mathrm{CMC}_2)/(\alpha_1 \mathrm{CMC}_2 + (1-\alpha_1)\mathrm{CMC}_1] \qquad \text{Eq. 2.2.4.}$$

Figure 2.2.3.3 illustrates the comparison of X_1 and X_{ideal} as a function of α_{gemini} for the mixed gemini/DOPE systems. As shown in all 3 binary systems, X_1 is always larger than X_{ideal} over the entire range of α_{gemini}. These positive deviations of X_1 from X_{ideal} demonstrate that the mixed micellar aggregates are enriched with the gemini surfactant component, and poorer in the

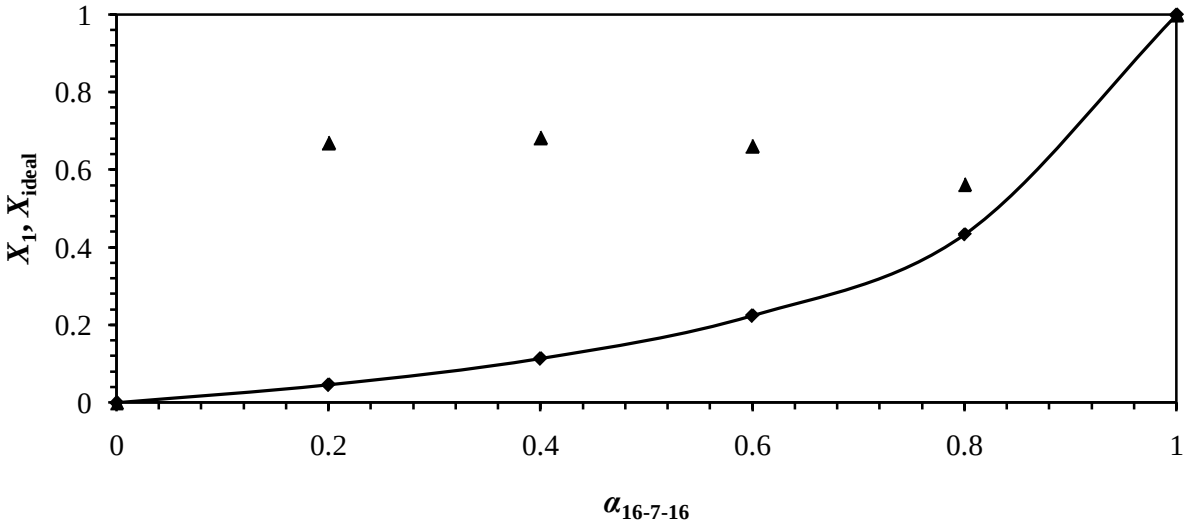
DOPE component than the intended ideal state. Interestingly, the gemini molar contribution to the mixed micellar aggregates (i.e. magnitude of X_1) appears to be somewhat consistent over the entire range of α_{gemini}, for all 3 binary systems. In other words, the solution composition (i.e. α_{gemini}) appears to have only a mild effect on the composition of the mixed micellar aggregates. Note that X_1 could not be solved for the mixed 16-7NH-16/DOPE system at a α_{gemini} molar ratio of 0.2 because no convergence was found for Equation 2.2.2; such a problem has been observed in the literature.[12-14]

Figure 2.2.3.3 Micellar mole fractions, X_1 (▲) and X_{ideal} (♦), as a function of α_{gemini} for the A) 16-3-16 + DOPE, B) 16-7-16 + DOPE, and C) 16-7NH-16 + DOPE binary systems at 25°C.

A)



B)



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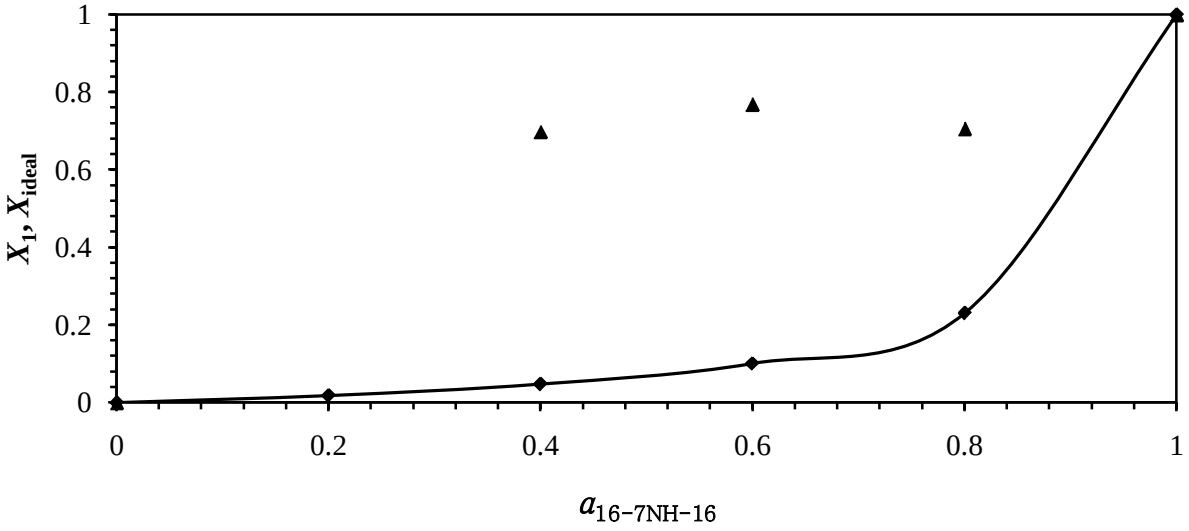


The values of β were subsequently calculated using Eq. 2.2.3 and the results are illustrated graphically in Figure 2.2.3.4. The computed β values were then used to determine the activity coefficients of the individual amphiphiles with the equations

$$\ln\gamma_1 = \beta(1 - X_1)^2 \qquad \text{Eq. 2.2.5.}$$

$$\ln\gamma_2 = \beta(X_1)^2 \qquad \text{Eq. 2.2.6.}$$

where γ_1 and γ_2 represent the activity coefficients of amphiphile 1 (i.e. the gemini surfactant) and amphiphile 2 (DOPE), respectively.[1] The activity coefficients are indicative of the effect and contribution of the individual components in the mixed micelles.[1] The determined values of γ_1 and γ_2 were subsequently used to calculate the excess free energy of mixing, ΔG_{ex}^{0}, which can be determined by the equation

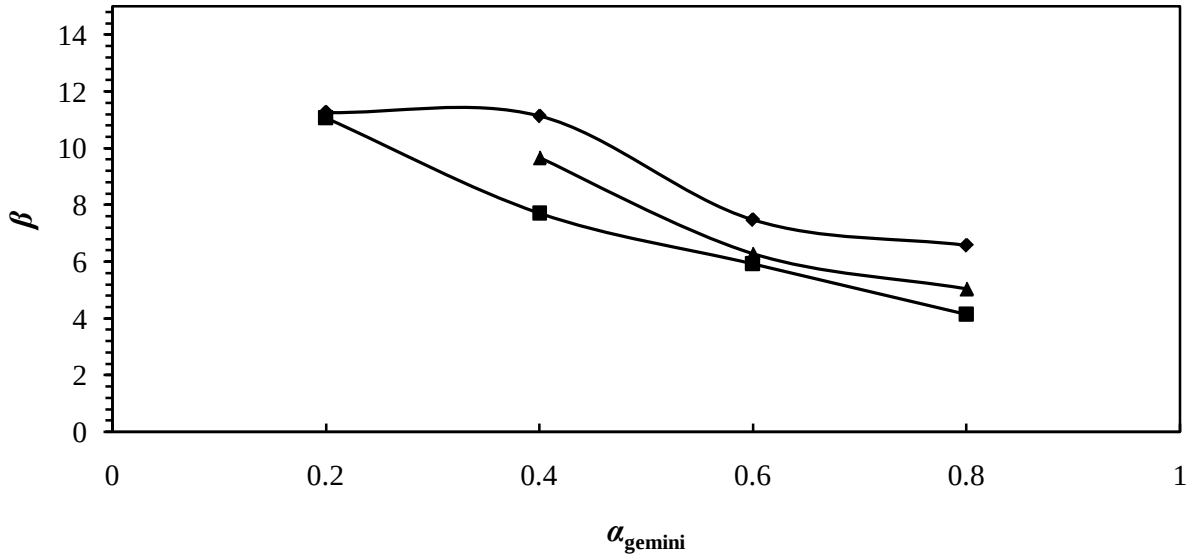
$$\Delta G_{ex}^{0} = RT \sum_{i=1}^{2} X_i \ln \gamma_i \qquad \text{Eq. 2.2.7.}$$

where R and T represent the gas constant and absolute temperature respectively.[1]

Table 2.2.3.2 Cumulative results obtained from Rubingh's analysis for the mixed gemini/DOPE systems.

System	α_{gemini}	X_1	$CMC_{Exp.}$ ($x10^{-6}M$)	β	γ_1	γ_2	ΔG_{ex}^{0} (KJ/mole)
16-3-16	0	0	5.03	-	-	-	-
+	0.2	0.66	292	11.3	3.65	137	6.26
DOPE	0.4	0.61	203	11.1	5.51	62.1	6.58
	0.6	0.61	77.6	7.47	3.17	15.7	4.42
	0.8	0.52	71.6	6.58	4.55	5.95	4.07
	1	1	24.2	-	-	-	-
16-7-16	0	0	5.03	-	-	-	-
+	0.2	0.67	294	11.1	3.36	142	6.07
DOPE	0.4	0.68	97.2	7.71	2.17	36.4	4.14
	0.6	0.66	56.9	5.92	1.97	13.4	3.28
	0.8	0.56	40.8	4.15	2.22	3.70	2.53
	1	1	26.2	-	-	-	-
16-7NH-16	0	0	5.03	-	-	-	-
+	0.2	-	291	-	-	-	-
DOPE	0.4	0.70	282	9.67	2.41	111	5.05
	0.6	0.77	120	6.30	1.40	41.4	2.77
	0.8	0.71	91.6	5.06	1.55	12.4	2.60
	1	1	67.0	-	-	-	-

Figure 2.2.3.4 Interaction parameter as a function of α_{gemini}. β values are represented as (♦) for the 16-3-16/DOPE; (■) for the 16-7-16/DOPE; and (▲) for the 16-7NH-16/DOPE binary systems at 25°C.



The cumulative results from Rubingh's analysis are tabulated in Table 2.2.3.2. As observed in this table, and as illustrated in Figure 2.2.3.4, it is confirmed that an antagonistic mixing interaction is present between gemini surfactants and DOPE, as implied by the positive values of β.[1] As also observed in Table 2.2.3.2., the activity coefficients are always greater than unity, thus confirming a nonideal mixing behaviour, which is repulsive in nature.[1] The positive values of the excess free energy of mixing (i.e. ΔG_{ex}^{0}) imply that the mixed micelles are less stable than the individual micelles of the gemini and DOPE components.[1] Interestingly, the mixed micelles generally become more stable as the gemini surfactant molar fraction in solution (i.e. α_{gemini}) is increased.

The antagonistic mixing interaction experienced between the gemini surfactants and DOPE can potentially be attributed to the differences in the preferred curvature of the molecules in the mixed aggregate. As developed by Israelachvili in 1992, the type of aggregate structure

formed by an amphiphile is based upon its critical packing parameter, P, which is a relative ratio of, i) the surface area occupied by a surfactant, and ii) the volume in the micelle core required for the surfactant.[15] More specifically, the packing parameter can be calculated by the equation

$$\mathrm{P} = \frac{v}{a_0 l_\mathrm{c}} \qquad \text{Eq. 2.2.8.}$$

where v is the volume of the hydrophobic portion of the molecule, a_0 is the area occupied by the headgroup, and l_c is the length of the hydrocarbon tail.[15] The magnitude of P can be used to predict the type of aggregate structure using Table 2.2.3.3.

Table 2.2.3.3 Expected aggregate characteristics in relation to the amphiphile critical packing parameter, P.[15]

Critical Packing Parameter	**General Surfactant Type**	**Expected Aggregate Structure**
< 0.33	Simple surfactants with single chains and relatively large headgroups	Spherical or ellipsoidal micelles
0.33 – 0.5	Simple surfactants with relatively small headgroups, or ionic surfactants in the presence of large amounts of electrolytes	Relatively large cylindrical or rod-shape micelles
0.5 – 1.0	Double-chain surfactants with large headgroups and flexible chains	Vesicles and flexible bilayer structures
1.0	Double-chain surfactants with small headgroups or rigid, immobile chains	Planar extended bilayer structures
> 1.0	Double-chain surfactants with small headgroups, very large, bulky hydrophobic groups	Reverse or inverted micelles

Since DOPE is composed of two bulky alkyl tails (as a result of the unsaturation on each tail), a packing parameter greater than 0.5 is expected, which suggests that DOPE forms bilayer structures, as observed experimentally.[16] In contrast, the gemini surfactants used in this study are expected and known to form micellar structures.[17-19] As a whole, it is apparent that gemini

surfactants and DOPE have different preferences in the type of aggregate structure formed once their respective CMCs are reached. These different preferences can potentially account for the antagonistic mixing interaction experienced between them.

As observed, there are two trends present in Figure 2.2.3.4. The first is that the strength of the antagonistic interaction (i.e. the magnitude of β) with DOPE is dependent upon the structure of the gemini surfactant spacer group. The second trend observed is that the magnitude of β is dependent upon the solution composition (i.e. α_{gemini}). An explanation for these two patterns is proposed below.

Influence of the Gemini surfactant structure on β

As shown in Figure 2.2.3.4, for a given solution composition (i.e. a particular value of α_{gemini}), 16-3-16 experiences the most antagonistic interaction with DOPE, followed by 16-7NH-16, and then 16-7-16. Therefore it is evident that both the gemini spacer length (16-3-16 vs. 16-7-16), and the presence of the secondary amine group (16-7NH-16 vs. 16-7-16) influences the interaction properties with DOPE.

The influence of the spacer length on the interaction properties with DOPE can likely be attributed to the proximity of the cationic headgroups in the gemini surfactant. Intuitively, the distances between the cationic headgroups are shorter in the 16-3-16 gemini surfactant than they are in the 16-7-16 surfactant. As a result of the headgroups being closer in proximity in the 16-3-16 surfactant, the mixed aggregate formed with DOPE will most likely have an increased charge density. This increased charge density will likely make the formation of the mixed micellar aggregate less favourable (because of the greater repulsive interactions), and should accordingly result in a stronger antagonistic interaction between the gemini surfactant and DOPE. In fact, it

has been reported in the literature that for gemini surfactants of the *m-s-m* type, when the spacer is less than 6 ± 1, the unfavourable and strong coulombic repulsions between the two quaternary ammonium headgroups results in the spacer chain being fully stretched to reduce these interactions.[7,18,20] In contrast to the 16-3-16 surfactant, the increased spacer length for the 16-7-16 surfactant allows for the headgroups to be at an optimal distance to balance both the repulsive interactions between the headgroups and the unfavourable contact of the hydrophobic spacer with water.[7,18,20]

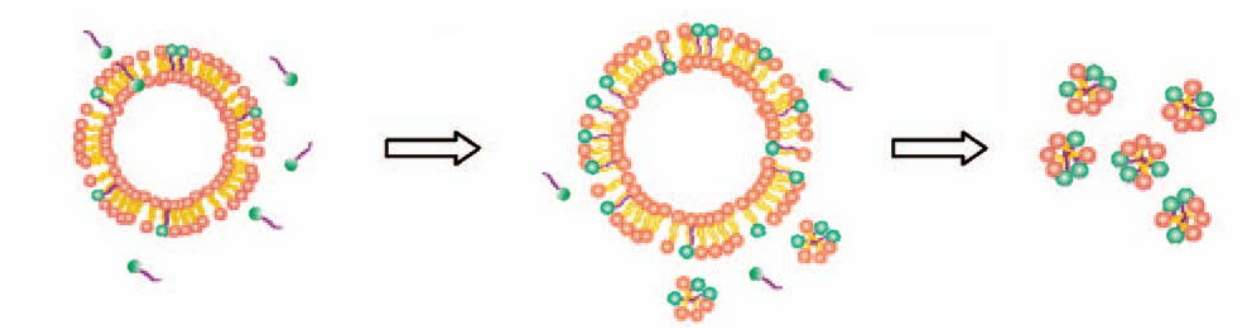
The second trend observed in Figure 2.2.3.4 is that 16-7NH-16 experiences a stronger antagonistic interaction with DOPE, in comparison to 16-7-16. This observation can likely be explained in terms of the increased polar nature of the spacer group in the 16-7NH-16 gemini surfactant. The 16-7NH-16 surfactant contains an ionisable secondary amine group, which likely increases the repulsive interactions between the headgroups in the mixed gemini/DOPE aggregate. These enhanced repulsive interactions will likely make the formation of the gemini/DOPE aggregate less favourable, thus accounting for the greater antagonistic interaction experienced in the 16-7NH-16/DOPE system, in comparison to the 16-7-16/DOPE system. This interpretation is supported with results reported by Shiloach et al. who concluded that mixed micelle formation in binary ionic/nonionic systems is governed primarily by the electrostatic interactions between the amphiphile headgroups.[21]

Influence of the solution composition on β

As observed in Figure 2.2.3.4, the magnitude of the interaction parameter decreases as the gemini surfactant molar fraction in solution (i.e. α_{gemini}) increases. This is quite interesting because according to Rubingh's theory, β should remain constant over the entire range of

composition.[1] However, numerous studies, in particular studies of nonionic and ionic amphiphiles, have shown β to vary with composition.[22-25]

To offer a possible explanation for the variation of β with α_{gemini}, one must understand how surfactants and lipids interact as a function of the surfactant molar fraction. In a mixed surfactant/lipid system, as the surfactant concentration increases, lipid vesicle solubilisation occurs which can be described by a three stage hypothesis; this includes a vesicular region, a vesicle-micelle coexistence region, and a mixed micellar region.[26] Within the vesicular region, the surfactant concentration is low, and the surfactant monomers are partitioned between the aqueous solution and the lipid bilayer (note that the lipid vesicle is not yet solubilised).[26] As the surfactant concentration is increased, surfactant monomers increasingly enter the bilayer until it becomes saturated.[26] Further addition of surfactant subsequently results in a coexistence stage of surfactant monomers, mixed micelles and vesicles.[26] Upon the further addition of surfactant, the vesicles become completely solubilised into mixed micellar aggregates.[26] An illustration of this three stage hypothesis is shown below. Note that a reverse pattern is observed as the lipid concentration is increased.



Scheme 2.2.3.1 Illustration of the three stage hypothesis for the solubilisation of vesicles by surfactants. From reference [27]

This three stage hypothesis can be used as an aid to offer a possible explanation for the β variation with α_{gemini}. At low α_{gemini} values, the mixed gemini/DOPE system is predicted to exist predominantly in the vesicular stage as a result of the high DOPE concentration and the low gemini concentration. Within this stage, the partitioning of the gemini surfactant into the vesicle structure is likely not favourable because of the dicationic nature of the gemini surfactant. More specifically, it is not favourable for the gemini surfactant monomers to partition into the bilayer because the cationic gemini headgroups will increase the charge density of the aggregate structure, and therefore destabilize the vesicle through the increased repulsive interactions. As such, at lower α_{gemini} values, one can potentially expect a larger antagonistic interaction between the gemini surfactant and DOPE.

In contrast, at high α_{gemini} values, the mixed gemini/DOPE system will likely exist in the micellar stage, as a result of the high gemini concentration and the low DOPE concentration. Although the mixing behaviour is still antagonistic, there is a favourable contribution from the DOPE molecules partitioning within the micellar aggregates; the DOPE monomers are able to reduce the destabilizing repulsive interactions in the aggregate structure by organizing themselves between the cationic gemini monomers. As such, one can potentially expect the antagonistic interaction to be weaker at higher α_{gemini} values.

2.2.4 Conclusions

Within this investigation, the interaction properties between gemini surfactant and DOPE were examined. As discussed, the interaction between gemini surfactants and DOPE is of great importance because the liposome structure used in the formation of gemini surfactant-based DNA delivery systems is a mixed aggregate of these two components.

In this study, the interaction properties between gemini surfactants and DOPE were evaluated using Rubingh's theory for mixed micellar formation. It was found that in all cases, the interaction between the 16-3-16, 16-7-16, and 16-7NH-16 gemini surfactants and DOPE is antagonistic in nature. This behaviour is rationalized in terms of the differences in the preferred aggregate structures of these two components. The strength of the antagonistic interaction was found to be dependent upon the spacer group, where 16-3-16 experienced the strongest antagonistic interaction with DOPE, followed by 16-7NH-16 and then 16-7-16. Furthermore, the strength of the antagonistic interaction was found to decrease as the gemini surfactant molar fraction in solution was increased.

The combined results from this analysis are of great significance in that we were able to determine that both the nature of the spacer group, and the relative ratio of the gemini surfactant to DOPE influences the mixing interaction and stability of the mixed gemini/DOPE aggregate. As will be discussed in the next section of this thesis, the stability of the mixed gemini/DOPE aggregate carries significant implications with respect to the efficacy characteristics of gemini surfactant-based DNA delivery system.

Future studies should involve examining the effect of the gemini surfactant alkyl tail length on the interaction properties with DOPE. This can perhaps be conducted using experimental methods that do not require such large sample requirements for CMC determination, possibly using conductivity measurements or titration calorimetry. As well, transmission electron microscopy images would be useful in examining how the gemini/DOPE aggregate structure evolves as the relative ratio of these components are altered.

2.3 Physical Stability Analysis of Gemini Surfactant-based DNA Delivery Systems

2.2.1 Introduction

The concluding project carried out for this study was an evaluation of the physical stability of gemini surfactant-based DNA delivery systems. As introduced in Chapter 1, the physical stability of these systems has direct implications with respect to their safety and efficacy characteristics within the human body, which in turn allows for an assessment of the practicality of these lipoplexes for gene delivery applications. Ideally, the results from this analysis should enable us to establish storage conditions and a shelf-life for these systems; and should additionally allow us to assess whether or not additional measures will have to be taken to modify the preparation procedure or formulation of these lipoplexes to enhance their stability characteristics.

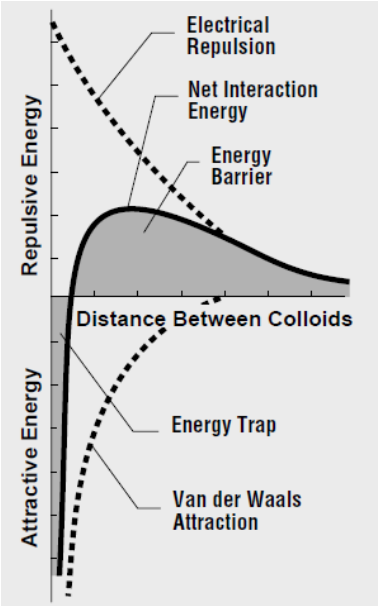
The physical stability analysis of the gemini surfactant-based DNA delivery systems was performed in accordance to the FDA's definition of the physical stability of liposome drug products, which is "a function of the integrity and size distribution of the lipid vesicles."[1] Based on this definition it is evident that there are two main evaluations that must be made in order to assess the physical stability of liposome drug products, i) the susceptibility of the liposome particles to undergo fusion and/or aggregation, and ii) the ability of the liposome to effectively encapsulate and protect the active therapeutic agent during delivery.[1] The significance of these two evaluations will be addressed separately below.

Liposome Fusion and Aggregation:

The susceptibility of liposomes to undergo fusion and/or aggregation can be rationalized by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory.[2-4] The DLVO theory is represented by the equation, $V_T = V_A + V_R$, where V_T represents the total potential energy of particle

interaction, which is a function of the attractive forces between particles, V_A, and the repulsive forces between particles, V_R.[2-4] The attractive interactions arise from van der Waals forces, while the repulsive interactions arise from the electrostatic repulsion between the electric double layers of particles.[2-4] Based on the DLVO theory, the stability of a colloidal system can be rationalized in terms of the balance between the attractive and the repulsive forces of interaction among particles, as they approach each other due to Brownian motion.[2-4]

A representation of the DLVO theory is illustrated below in scheme 2.3.1.1. As shown, the free energy of particle interaction is dependent upon the distance of particle separation.[2-4] The attractive forces between particles predominate at smaller distances of separation, while the repulsive forces predominate at intermediate distances.[2-4] Implicit in this representation is that there is an energy barrier (i.e. the primary maximum) from the repulsive forces that prevent particles from adhering to each other.[2-4] However, if particles are able to collide with sufficient energy to overcome that barrier, they will adhere strongly and irreversibly together.[2-4]



Scheme 2.3.1.1 Variation of the free energy of interaction with respect to particle separation according to the DLVO theory. From reference [5]

Based on the DLVO theory, it is evident the physical stability of gemini surfactant-based lipoplexes is dependent upon the ability of the particles to electrostatically repel each other.[2-4] If the lipoplex particles do not effectively repel each other, fusion or aggregation may occur, leading to the formation of larger colloidal structures. In general, particles with zeta potentials more positive than +30 mV or more negative than -30 mV are considered to be stable.[4]

The particle size distribution is of great significance as it carries several implications with respect to the safety and efficacy characteristics of the lipoplex system within the body.[6-10] In regards to safety, it is known that largely sized particles are capable of causing embolisms within the circulatory system.[6] Embolism occurs when an object causes a blockage of a blood vessel as a result of being too large to pass through it.[6] Consequently, the cells that obtain their blood supply from this passage are starved of oxygen and can possibly die.[6] Depending on where these embolisms occur, the end results can be devastating and possibly fatal.[6] As such, there is a common consensus that intravenously administered agents should not have particle sizes greater than 5 μm.[7] In regards to the lipoplex efficacy, the particle size distribution is of significance for two main reasons, i) larger sized liposomes are eliminated from the circulation much more rapidly than smaller liposomes,[8] and ii) the particle size can influence the lipoplex transfection efficiency, since lipoplex-cellular association and uptake are size dependent processes.[9,10]

Liposome Membrane Integrity:

The other evaluation constituting a physical stability analysis for liposome drug products is an assessment of the membrane integrity. Liposomes are vesicular structures consisting of a bilayer membrane, where the integrity of this membrane is dependent upon the ordering and packing of the lipid molecules.[11] Generally, this packing is determined by the curvature of the liposome structure,[12] and by the interactions between the alkyl chains and the head groups.[11]

These interactions depend on the length and saturation of the alkyl tails, and on the structure of the headgroups.[11]

An insufficient packing of the lipid membrane of the lipoplex delivery system can result in the degradation and elimination of the incorporated plasmid DNA.[13] Exposed DNA can be degraded enzymatically by nucleases present within the serum.[13] These nucleases exert their effect by cleaving the phosphodiester bonds between the nucleotide subunits.[13] Furthermore, exposed DNA has been shown to be rapidly removed from the circulation by liver uptake, which occurs predominantly by the liver non-parenchymal cells.[14,15] The combination of these two factors make it evident that the membrane of lipoplex systems must be of a sufficient integrity to protect the incorporated DNA from the site of administration to the site of gene expression.

For small molecule liposome-based delivery systems, the liposome membrane integrity is typically examined using a dye leakage study. In our work, we are concerned with the ability of the lipoplex structure to protect the incorporated DNA from nucleases present in the bulk environment surrounding the lipoplex. As such, within this study, the membrane integrity characteristics of the gemini surfactant-based lipoplexes were evaluated using DNase sensitivity assays. These assays involved exposing the lipoplex systems to DNase I to evaluate how well the incorporated DNA is protected from DNase degradation. Liposomal membranes of sufficient integrity should offer adequate protection to the incorporated DNA from digestion

Project Aim:

Collectively, the objective of this study was to assess the physical stability of gemini surfactant-based lipoplexes by assessing the particle size distribution and membrane integrity characteristics of these systems. The particle size distribution characteristics were evaluated using dynamic light scattering (DLS) and by performing zeta potential measurements; the

membrane integrity characteristics were evaluated by performing DNase sensitivity assays. The physical stability of these delivery systems was monitored as a function of time, temperature and pH.

The physical stability analysis was carried out on two different carrier systems. The first system was formulated with the 12-7NH-12 gemini surfactant, as this particular complex has demonstrated the highest transfection efficiency out of the complexes studied by our group.[16] For comparison purposes, the second system evaluated was formulated with the 12-7-12 gemini surfactant. The 12-7NH-12 gemini surfactant differs from the 12-7-12 surfactant by the presence of a pH active secondary amine group in the spacer, which can potentially result in differences in the respective stability properties of the two systems.

2.3.2 Experimental Procedures

2.3.2.1 Materials

The gemini surfactants used in this study were synthesized according to procedures previously reported in the literature.[17,18] The surfactants were purified by recrystallization, and the structures were confirmed using ^{1}H NMR spectroscopy (Bruker 300 MHz). The purity was confirmed by the absence of a minimum in the post micelle region of the surface tension versus log concentration plot.

The pGL2 plasmid was obtained from Promega (Madison, Wisconsin, USA), and amplified in Escherichia Coli to obtain the yields required for the stability analysis. The DNA used was purified with a Promega PureYield Plasmid Midiprep System (Madison, Wisconsin).

1,2-dioleyl-*sn*-glycerophosphatidylethanolamine (DOPE) was obtained from Avanti Polar Lipids (Alabaster, Alabama, USA), and was used without any further purification.

Water for all solutions was obtained from a Millipore Synergy purification system.

2.3.2.2 Methods

Preparation of gemini surfactant-based DNA delivery systems

The gemini surfactant-based DNA delivery systems were formulated with the respective gemini surfactant, DOPE, and DNA. The pGL2 plasmid was added to the gemini surfactant solution to obtain a gemini:DNA charge ratio of 10:1 (this ratio was previously determined to be the most effective)[19], and the mixture was incubated for 15 minutes. DOPE vesicles were subsequently added to the mixture in a gemini:DOPE molar ratio of 2:5, and the final complex was incubated for 30 minutes.

The DOPE vesicles were prepared using the sonication method. DOPE and α-tocopherol (used as a preservative; DOPE:α-tocopherol weight ratio of 5:1) were dissolved in ethanol via sonication, and the mixture was then deposited as a thin film on a round bottom flask using a Heidolph rotary evaporator. The mixture was next suspended in an isotonic sucrose solution (at pH 9) using a water bath sonicator (60°C for 3 hours), and the DOPE vesicles were then filtered through 0.45 μm filters.

Particle Size and Zeta Potential Measurements

Particle size and zeta potential measurements were both carried out using a Malvern Zetasizer Nano ZS instrument. The particle size distribution was determined using the NNLS analysis method, where scattering was detected at 173°. Size measurements were performed in disposable polystyrene cuvettes. Zeta potential measurements were carried out using laser Doppler microelectrophoresis at a frequency at 1000 Hz. Measurements were performed in folded capillary cells (Malvern# DTS1060). All particle size and zeta potential measurements were repeated multiple times, and the average is reported. Significant differences between particle size and zeta potential results were assessed using a t-test at a 95% confidence level.

DNase Sensitivity

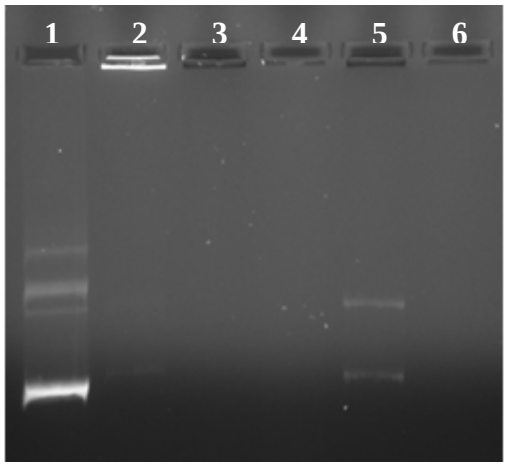
The DNase sensitivity assays were carried out by incubating the desired lipoplex system with DNase I (1 unit DNase I per 1 μg DNA) and the DNase reaction buffer (composed of Tris-HCl, $MgSO_4$, and $CaCl_2$), for 30 minutes at 37°C. Following the 30 minute exposure period, the DNase I enzymes were inactivated by the addition of the DNase stop solution (composed of ethylene glycol tetraacetic acid), and then denatured by incubating the complexes at 60°C for 10 minutes. The liposomal membranes were then disrupted through the addition of a phenol/chloroform mixture (1:1, v/v), and the aqueous DNA phase was separated from the liposome-forming components through centrifugation.

The extent of DNA degradation by DNase I was assessed using gel electrophoresis. An aliquot of the extracted DNA phase was loaded onto a 0.8% (w/v) agarose containing 0.5 μL/mL ethidium bromide. Electrophoresis experiments were carried out for 1 hour at 100V, in TBE buffer solution. The resulting gels were analyzed using an Alpha Innotech Gel Imaging System.

Since DNase sensitivity assays on gemini surfactant-based lipoplexes were never carried out in the past, the experimental procedure used was first validated. There were two aims in the validation process: i) to ensure that only intact DNA fragments can be visualized on the imaged gel, and ii) to ensure that the phenol/chloroform extraction method can effectively separate DNA from the liposome forming components

As shown in Figure 2.3.2.1, a series of controls was ran to ensure that only intact DNA fragments can be visualized on the imaged gel. The main conclusions drawn from this gel are, i) only DNA separated from the liposome or gemini surfactant can effectively be visualized, and ii) pure DNA plasmid exposed to DNase resulted in no apparent bands.

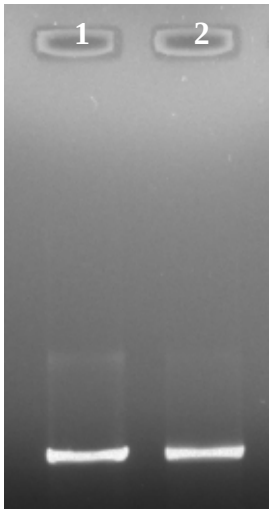
Figure 2.3.2.1 Imaged gel of the experimental controls for the DNase sensitivity assays.



Lane	Component
1	pGL2 DNA plasmid
2	gemini surfactant-based lipoplex
3	gemini surfactant
4	DOPE
5	gemini surfactant + DNA (gemini:DNA charge ratio = 10:1)
6	DNA exposed to DNase I

The aim of the second validation test was to ensure that the chloroform/phenol extraction method can be used to effectively separate DNA from the gemini surfactant and DOPE components. To conduct this, DNA separated from a gemini surfactant-based lipoplex using the chloroform/phenol extraction method was compared to an aqueous solution containing an equal concentration of DNA. The results are illustrated below in Figure 2.3.2.2, and as shown, the chloroform/phenol extraction method allowed for the effective separation of DNA from the gemini/DOPE liposome. This was confirmed by a 5.2% variation in the band intensities shown in Figure 2.3.2.2.

Figure 2.3.2.2 Validation of the chloroform/phenol extraction method.



Lane	Component
1	Aqueous DNA solution
2	DNA extracted from gemini lipoplex using chloroform/phenol extraction method

2.3.3 Results and Discussion

Physical Stability as a Function of Time and Temperature:

12-7NH-12 Complex

The physical stability of the gemini surfactant-based DNA delivery systems was first assessed as a function of time and temperature. Figures 2.3.3.1 and 2.3.3.2 illustrate the evolution of the mean particle diameter and zeta potential of the 12-7NH-12 lipoplex system as a function of time, during storage at 4, 22 and 60°C.

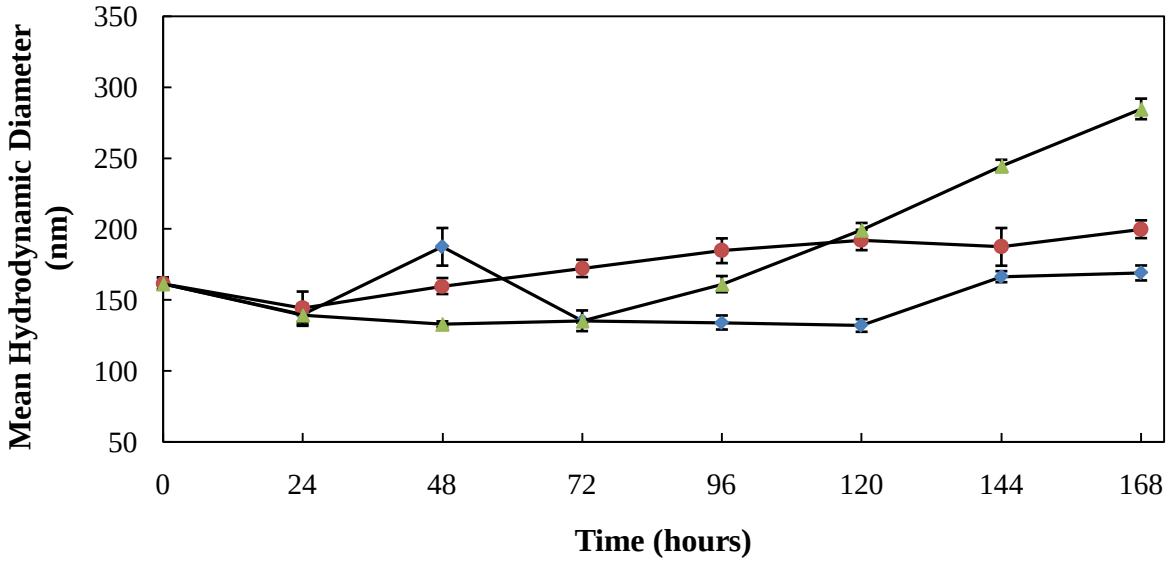
As shown in Figure 2.3.3.1, there is initially no apparent trend in the evolution of the mean particle diameter over time as a function of the storage temperature. However, after 96 hours of storage, the lipoplex system stored at 4°C was found to have a consistently and significantly ($p < 0.05$) smaller mean particle diameter in comparison to the lipoplex systems stored at 22 and 60°C. As well, after 144 hours, the mean particle diameter of the system stored at 22°C was found to be significantly ($p < 0.05$) smaller than that of the system stored at 60°C (note that particle size measurements of the system stored at 60°C was halted after 2 weeks due to discoloration of the system and the formation of visible precipitates). Over the 5 week interval, the lipoplex stored at 4°C showed no significant changes in the mean particle diameter, which always remained below 190 nm. The mean particle diameter of the system stored at 22°C was found to increase from 161.4 to 234.6 nm during this 5 week interval.

Figure 2.3.3.2 illustrates the evolution of the zeta potential as a function of time, for the 3 storage temperatures. As shown, there is initially no apparent trend in the variation of the zeta potential before 48 hours of storage. However, after 72 hours, the zeta potential of the lipoplex system stored at 60°C was found to be consistently and significantly ($p < 0.05$) smaller than the zeta potentials of the systems stored at 4 and 22°C. Furthermore, the zeta potential of the system

stored at 22°C was found to be consistently and significantly ($p < 0.05$) smaller than that of the system stored at 4°C after 2 weeks of storage. However, over the 5 week storage interval, the zeta potentials of the systems stored at 4 and 22°C were always found to be larger in magnitude than +30 mV, characteristic of a stable colloidal system.

Figure 2.3.3.1 Variation of the mean hydrodynamic diameter as a function of time for the 12-7NH-12 lipoplex system stored at 4°C (♦), 22°C (●), and 60°C (▲) over a time period of A) 1 week, and B) 5 weeks.

A)



B)

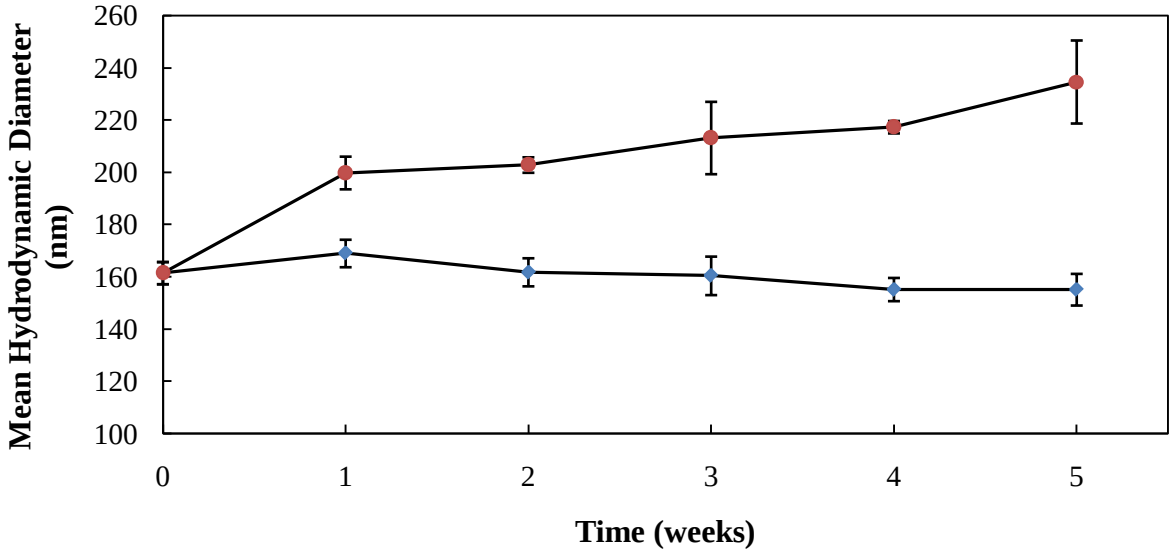
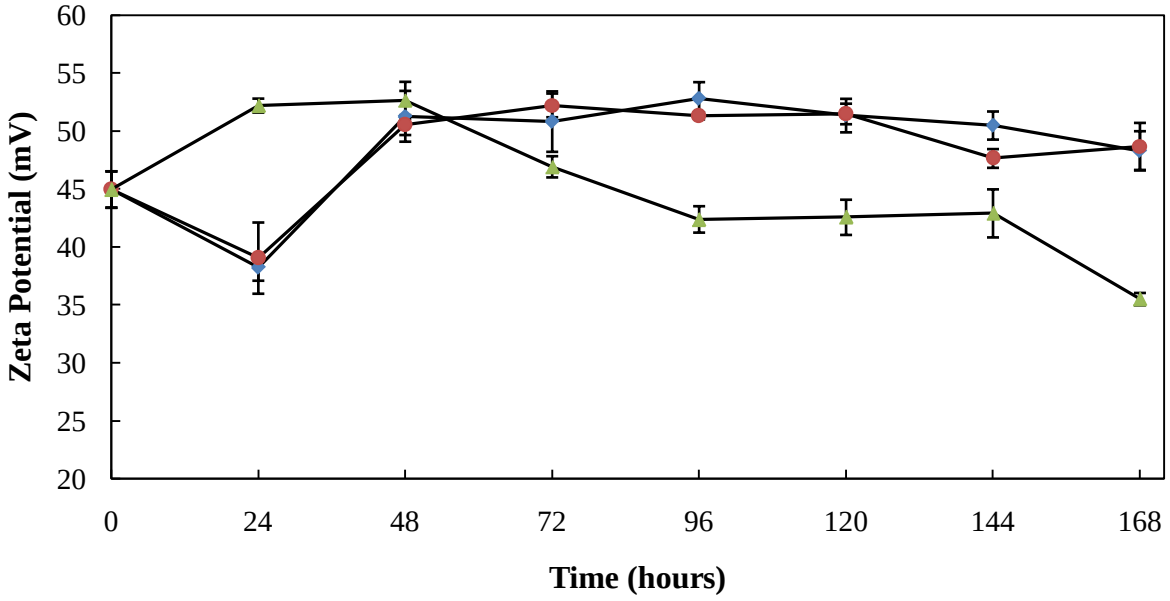
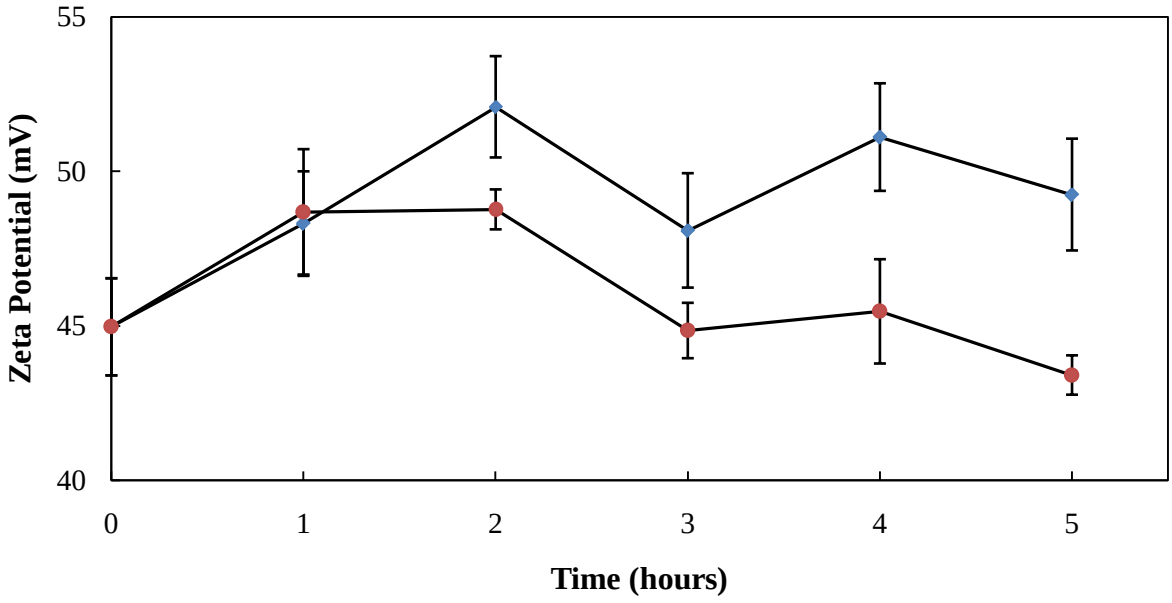


Figure 2.3.3.2 Variation of the zeta potential as a function of time for the 12-7NH-12 lipoplex system stored at 4°C (♦), 22°C (●), and 60°C (▲) over a time period of A) 1 week, and B) 5 weeks.

A)



B)



12-7-12 Complex

For the 12-7-12 lipoplex, the evolution of the mean particle diameter as a function of time for the 3 storage temperatures is illustrated below in Figure 2.3.3.3. As clearly observed, the

mean particle diameter of the system stored at 60°C was found to increase over time, and became significantly ($p < 0.05$) larger than the particle diameters of the systems stored at 4 and 22°C after 72 hours of storage. The mean particle diameter of the system stored at 22°C was found to be consistently and significantly ($p < 0.05$) larger than that of the system stored at 4°C after 120 hours of storage. Over the 5 week storage interval, the system stored at 4°C was always found to have a mean particle diameter less than 150 nm. In regards to the system stored at 22°C, the mean diameter was found to increase to 207.1 nm over the 5 weeks interval.

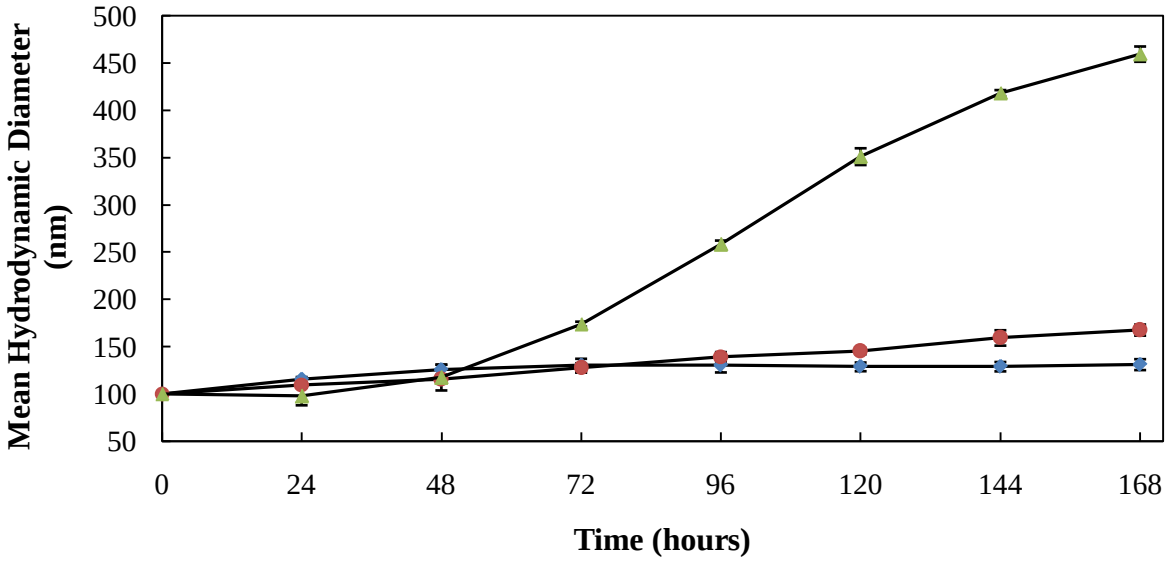
Overall, a general pattern is apparent in both of the 12-7-12 and 12-7NH-12 lipoplexes; aggregation is more prevalent as the storage temperature of the lipoplex system is increased. This particular behaviour is expected because increases in the temperature of the system will result in an increase of the thermal energy of lipoplex particles in solution. As a result, more particles are able to collide with sufficient energy to overcome the primary maximum energy barrier (as described by the DLVO theory), thus resulting in increased aggregation.[2-4]

The evolution of the zeta potential for the 12-7-12 lipoplex as a function of time for the 3 storage temperatures is illustrated in Figure 2.3.3.4. As observed, the zeta potential of the lipoplex system stored at 60°C was found to decrease with time, and was additionally found to be consistently and significantly ($p < 0.05$) smaller than those of the systems stored at 4 and 22°C after 72 hours of storage. The reason for this decrease in zeta potential is unknown, but can most likely be attributed to changes in the structural organization of the lipoplex particles over time. A similar pattern has been observed in the literature for a cationic solid lipid nanoparticle/DNA vector stored at 40°C, where the zeta potential of the system was found to decrease significantly after a 6 month storage period.[20] For the lipoplex systems stored at 4 and 22°C, there is no apparent trend in the evolution in the zeta potential over time; in all cases the

zeta potential was larger in magnitude than +30 mV. Such a pattern has been observed in a DOTAP/DOPE/DNA lipoplex system, where no apparent trends in the zeta potentials were observed over a 104 week interval when the lipoplex systems were stored at various temperatures between -20 and 60°C.[21]

Figure 2.3.3.3 Variation of the mean hydrodynamic diameter as a function of time for the 12-7-12 lipoplex system stored at 4°C (♦), 22°C (●), and 60°C (▲) over a time period of A) 1 week, and B) 5 weeks.

A)



B)

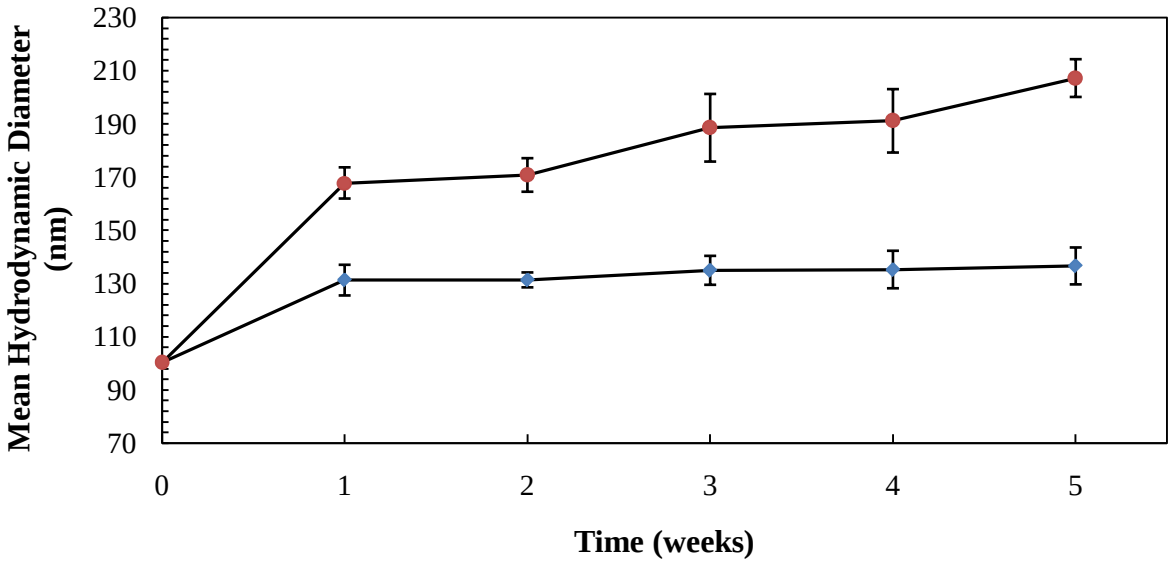
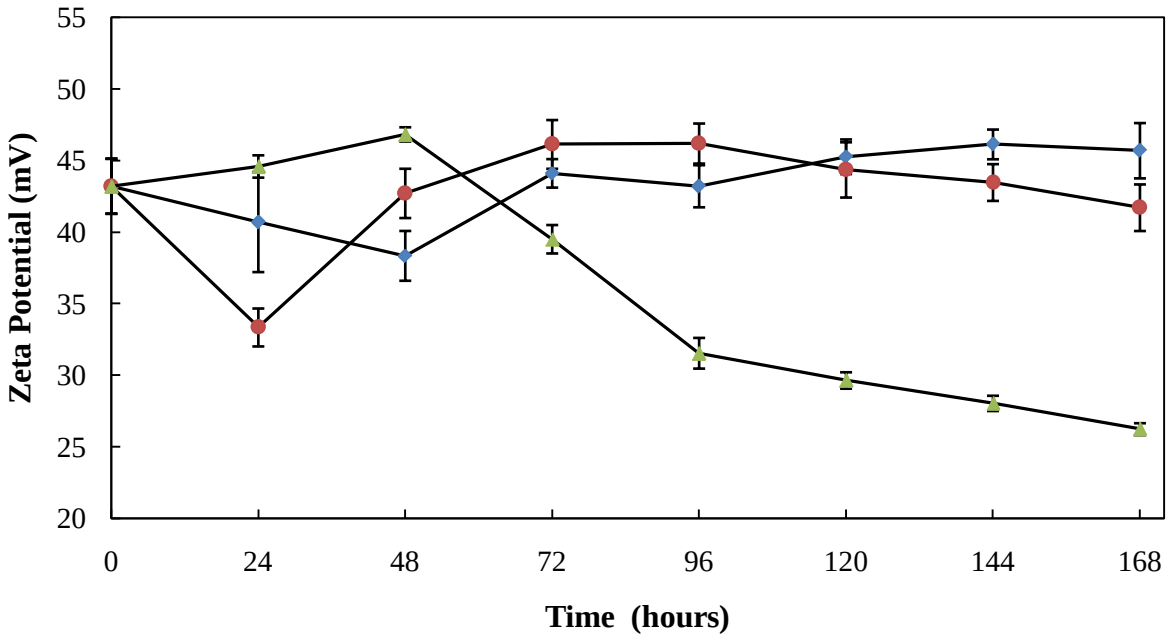
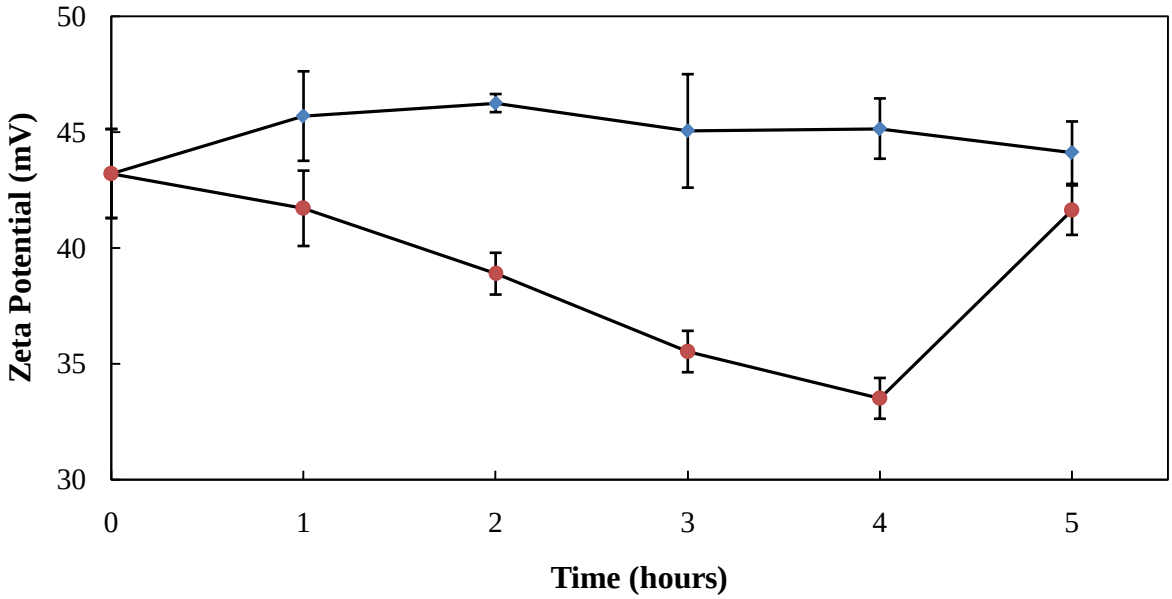


Figure 2.3.3.4 Variation of the zeta potential as a function of time for the 12-7-12 lipoplex system stored at 4°C (♦), 22°C (●), and 60°C (▲) over a time period of A) 1 week, and B) 5 weeks.

A)



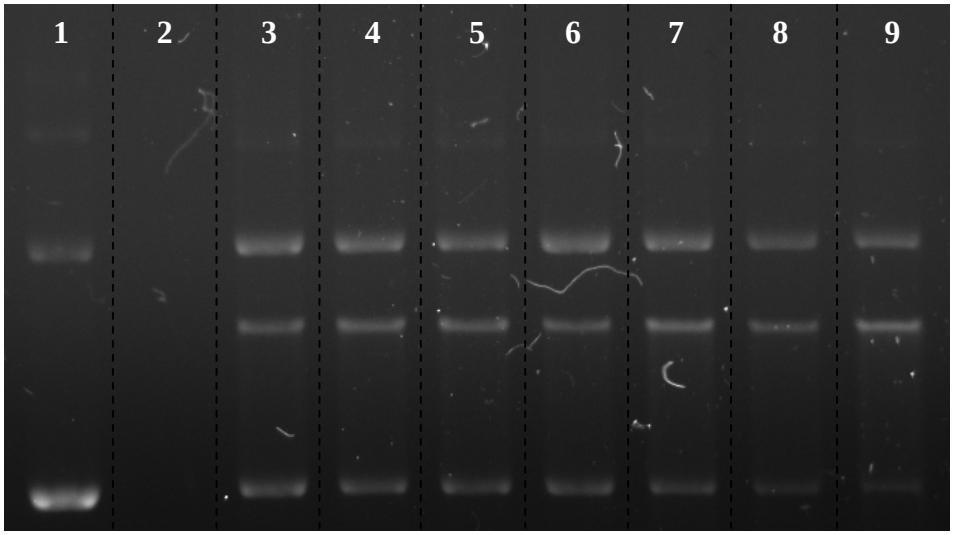
B)



During the evaluation of the particle size distribution characteristics of the gemini surfactant-based lipoplex systems, the membrane integrity properties were simultaneously evaluated using DNase sensitivity assays. Figures 2.3.3.5 and 2.3.3.6 illustrate the DNase sensitivity of the gemini lipoplex systems as a function of time during storage at 4°C.

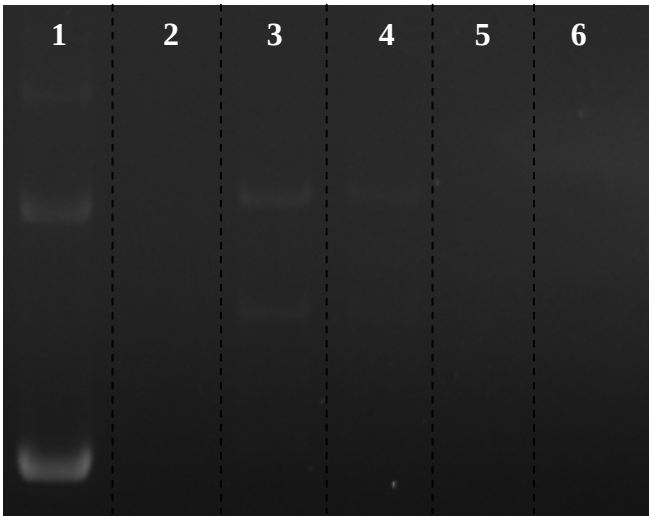
Figure 2.3.3.5 DNase sensitivity as a function of storage time for the 12-7NH-12 lipoplex system over a time period of A) 1 week, and B) 5 weeks. Note, lane 1 represents the standard amount of DNA in each lipoplex.

A)



Lane #	Lane Content
1	DNA
2	-
3	24 hours
4	48 hours
5	72 hours
6	96 hours
7	120 hours
8	144 hours
9	168 hours

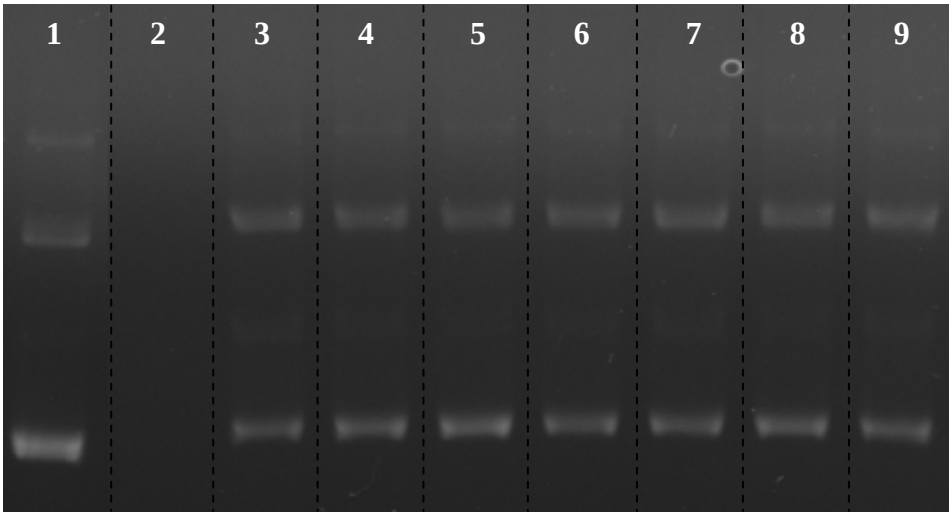
B)



Lane #	Lane Content
1	DNA
2	-
3	2 weeks
4	3 weeks
5	4 weeks
6	5 weeks

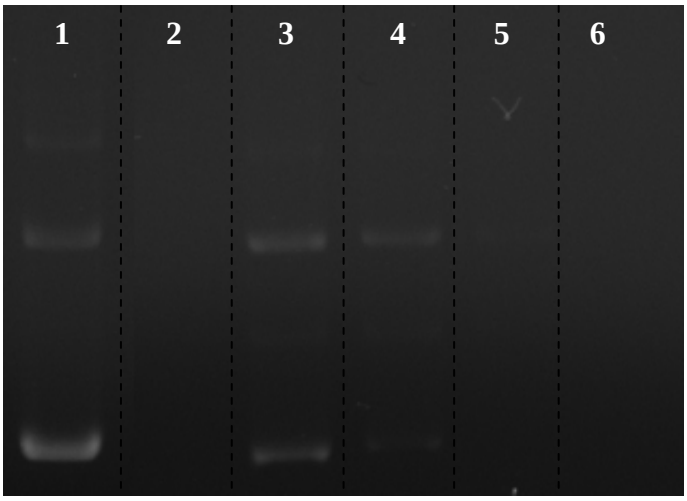
Figure 2.3.3.6 DNase sensitivity as a function of storage time for the 12-7-12 lipoplex over a time period of A) 1 week, and B) 5 weeks. Note, lane 1 represents the standard amount of DNA in each lipoplex.

A)



Lane #	Lane Content
1	DNA
2	-
3	24 hours
4	48 hours
5	72 hours
6	96 hours
7	120 hours
8	144 hours
9	168 hours

B)



Lane #	Lane Content
1	DNA
2	-
3	2 weeks
4	3 weeks
5	4 weeks
6	5 weeks

As shown in both Figures 2.3.3.5 and 2.3.3.6, the gemini lipoplexes became more sensitive to DNase degradation over time. For both of the 12-7-12 and 12-7NH-12 lipoplexes, no evident bands were detected after 4 weeks of storage, implying a near complete degradation of the incorporated DNA.

The increased sensitivity of the gemini lipoplexes to DNase degradation over time can potentially be attributed to the dissociation of the lipoplex structure. A previous study conducted by Lai et al. has suggested that lipoplex formulations dissociate over time, as supported by the formation of less dense particles with increasing time of storage.[22] Despite increases in the geometric sizes of the DOTIM/cholesterol/DNA lipoplexes used in their study (note that in some cases, the geometric sizes remained fairly constant), the molar masses of the particles were found to decrease over time.[22]

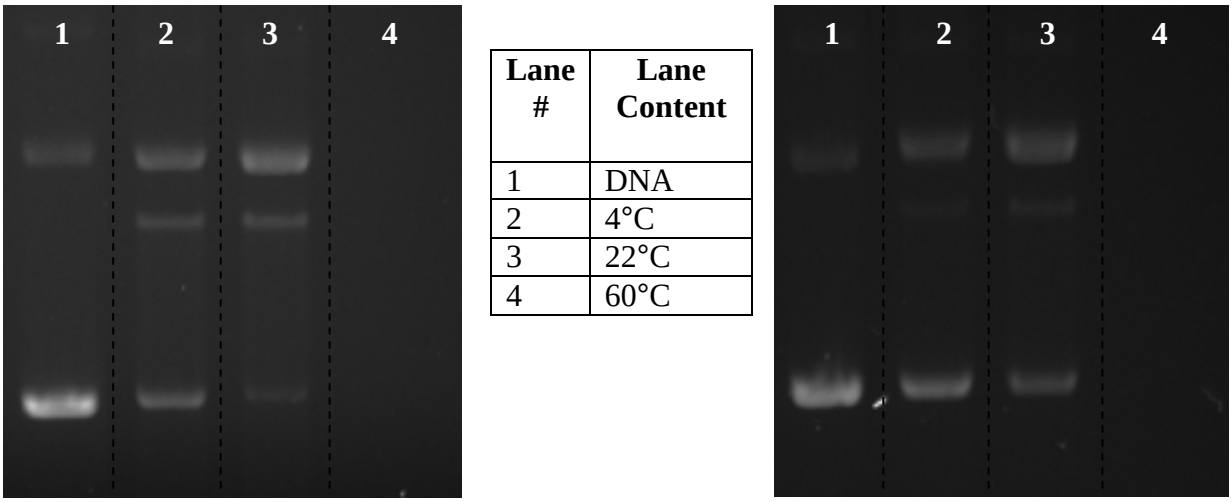
Although it is unknown whether or not dissociation occurs in the gemini lipoplexes, the antagonistic mixing interaction between gemini surfactants and DOPE can possibly offer a driving force for this phenomenon. As revealed in Chapter 2.2 of this thesis, the mixing interaction between gemini surfactants and DOPE is repulsive in nature, which was rationalized in terms of the differences in the preferred curvature of the aggregate structures of these two amphiphiles in solution. Is it therefore reasonable that over time, the mixed gemini/DOPE aggregates may potentially dissociate into gemini-rich aggregates and DOPE-rich aggregates. In the literature, mixed amphiphile systems exhibiting antagonistic mixing interactions have been shown to demix into separate single component-rich aggregate structures.[23-27]

Lai et al. additionally showed that the rate of lipoplex dissociation increased as the storage temperature was increased.[22] To examine the temperature dependence of the lipoplex membrane integrity, DNase sensitivity was examined as a function of storage temperature (4, 22 and 60°C) over a time period of 24 hours; an illustration is shown below in Figure 2.3.3.7. As observed, the lipoplex delivery system stored at 60°C demonstrated an inferior membrane integrity, in comparison to the other two systems stored at 22 and 4°C. This was shown by the

increased sensitivity of the lipoplex system stored at 60°C to DNase degradation, as there were no evident bands in lanes of the gels corresponding to these complexes.

Figure 2.3.3.7 DNase sensitivity as a function of storage temperature over a time period of 24 hours for the A) 12-7NH-12 lipoplex, and B) 12-7-12 lipoplex. Note that lane 1 represents the standard amount of DNA in each lipoplex.

A) B)



Lane #	Lane Content
1	DNA
2	4°C
3	22°C
4	60°C

As a whole, it is evident that the physical stability of gemini surfactant-based DNA delivery systems is dependent upon both the time and temperature of storage. As shown by the particle size distribution results, lipoplex aggregation occurs over time, and becomes more prevalent as the storage temperature is increased. Upon examination of the DNase sensitivity assays, it is evident that the gemini lipoplex systems becomes more sensitive to DNase degradation over time, and as the storage temperature is increased.

The combined results from this analysis suggest that the overall physical stability of the gemini surfactant-based lipoplex systems is limited by the membrane integrity of the liposome structure. As shown in both Figures 2.3.3.5 and 2.3.3.6, after 4 weeks of storage at 4°C, the

exposure of the lipoplexes to DNase resulted in no apparent DNA bands in the gel lanes. However, as shown in Figures 2.3.3.1 and 2.3.3.3., the mean particle diameter was found to remain fairly consistent over this 4 week interval.

Physical Stability as a Function of pH

The physical stability of the gemini surfactant-based lipoplex systems was further investigated as a function of pH. Figures 2.3.3.8 and 2.3.3.9 illustrate the evolution of the mean particle diameter and zeta potential as a function of pH, for the 12-7NH-12 and 12-7-12 lipoplex systems respectively.

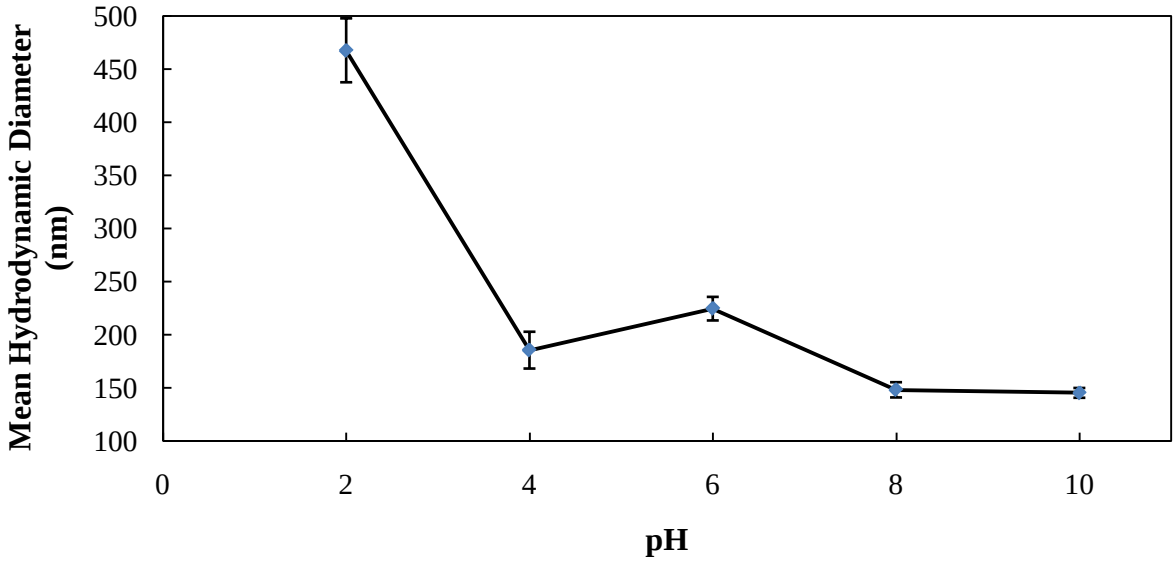
For the 12-7NH-12 lipoplex system, the mean particle diameter was found to increase as the pH decreased. In contrast, for the 12-7-12 lipoplex, there is no apparent trend in the evolution of the mean particle diameter as a function of pH. Based on these two observations, it is evident that the secondary amine group on the spacer of the 12-7NH-12 surfactant is responsible for the pH-dependent size distribution of the 12-7NH-12 based lipoplex system. It has been previously reported that the pKa for this secondary amine group is 5.0 ± 0.4.[16] Therefore, upon acidification of the system to pH values less than approximately 5, the protonation of this secondary amine group should result in increased headgroup repulsive interactions in the aggregate structure, which likely accounts for the observed increases in particle size. As shown in Figure 2.3.3.8A, the mean diameter of the 12-7NH-12 lipoplex was found to increase at pH 6, which is close to the pKa value of the secondary amine group in the spacer.

Figures 2.3.3.8B and 2.3.3.9B illustrate the dependence of the lipoplex zeta potential as a function of pH. As shown in both of the 12-7NH-12 and 12-7-12 lipoplexes, the zeta potential decreased as the pH of the system was increased. Such a pattern has been observed before in the

literature, and is rationalized by the increased counterion binding of hydroxide ions to the lipoplex particle as the pH becomes more basic.[16] As a result, charge screening of the lipoplex particles occurs, which results in the observed decrease in zeta potential.

Figure 2.3.3.8 Variation of the A) mean hydrodynamic diameter, and B) Zeta potential as a function of pH for the 12-7NH-12 lipoplex system.

A)



B)

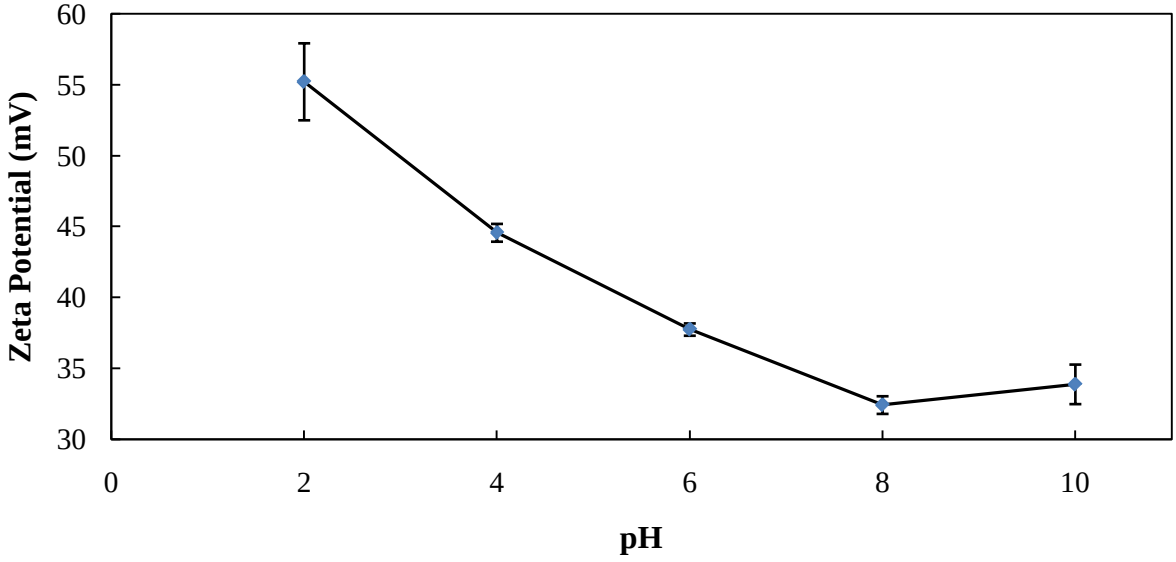
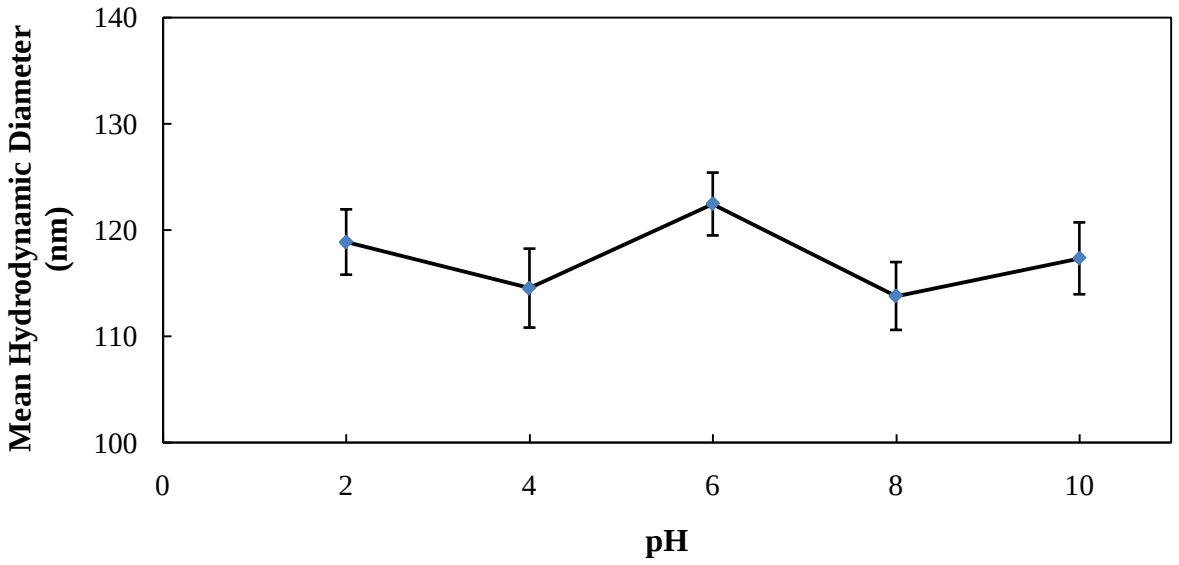
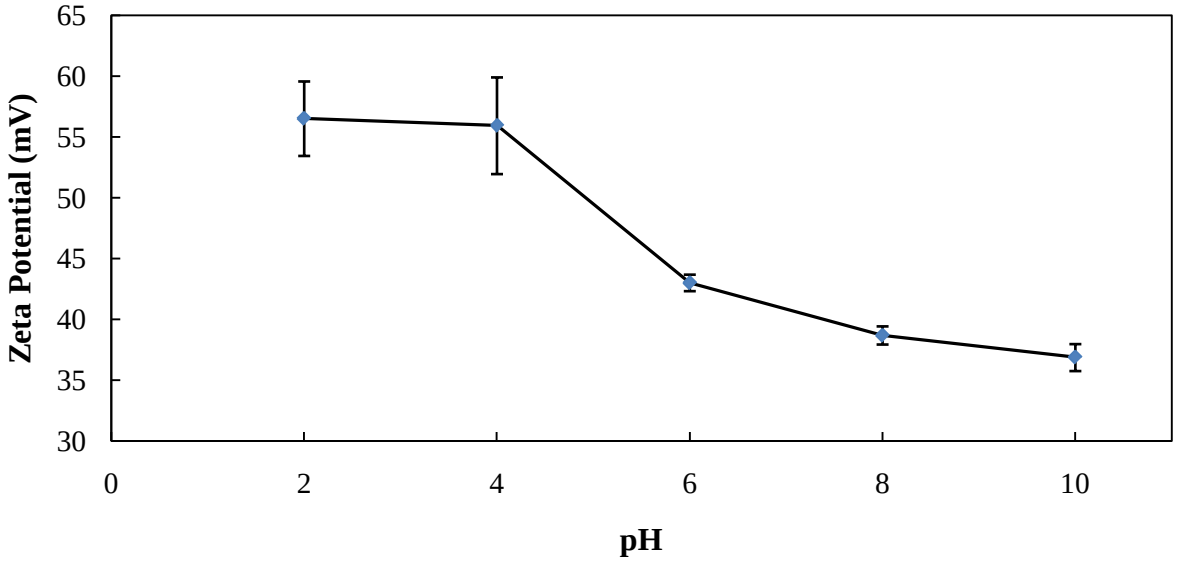


Figure 2.3.3.9 Variation of the A) mean hydrodynamic diameter, and B) Zeta potential as a function of pH for the 12-7-12 lipoplex system.

A)



B)

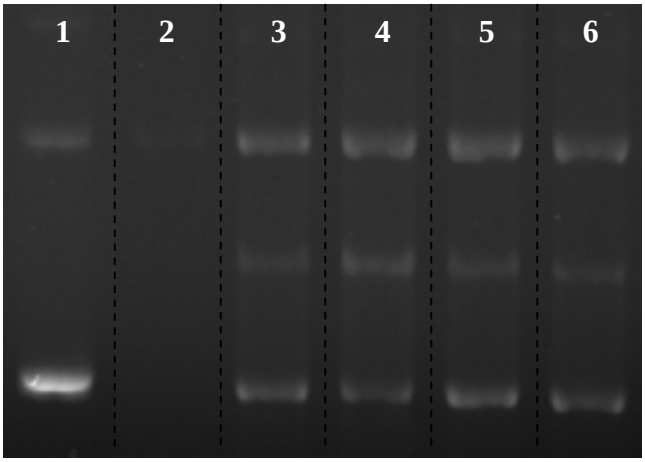


The membrane integrity characteristics of the lipoplex systems as a function of pH are illustrated below in Figure 2.3.3.10. Interestingly, it is observed that for both of the 12-7NH-12 and 12-7-12 lipoplex systems, the membrane integrity of the lipoplex structure is reduced

significantly as the pH is decreased to a value of approximately 2, as there are no visible bands on the gel for this treatment. The exact reason for this observation is unknown, but can potentially be attributed to a pH-dependent change in the packing arrangements of the lipoplex structure, which makes the incorporated DNA more susceptible to degradation by DNase. DNA depurination, which is the removal of purine bases (adenine or guanine) from the deoxyribose moiety by hydrolysis of the glycosidic bond, may also provide another explanation for the observed behaviour at pH 2.[28] As found in the literature, the rate of depurination is pH dependent, and is significantly increased under acidic conditions (i.e. the hydrolysis of the purine-deoxyribose glycosyl bond is acid-catalyzed).[28]

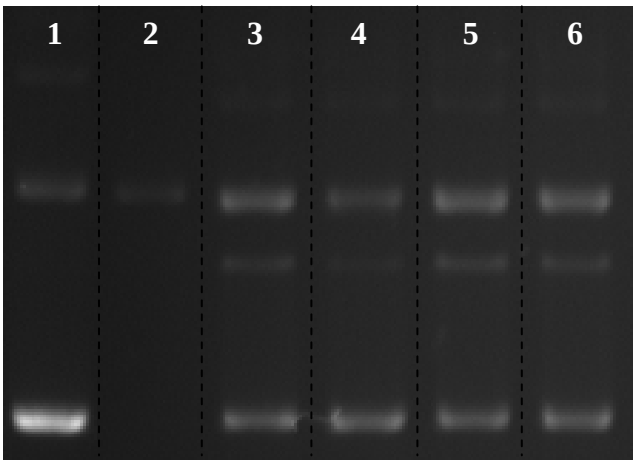
Figure 2.3.3.10 DNase sensitivity as a function of pH for the A) 12-7NH-12 lipoplex, and B) 12-7-12 Lipoplex. Note that lane 1 represents the standard amount of DNA in each lipoplex.

A)



Lane #	Lane Content
1	DNA
2	pH 2
3	pH 4
4	pH 6
5	pH 8
6	pH 10

B)



Lane #	Lane Content
1	DNA
2	pH 2
3	pH 4
4	pH 6
5	pH 8
6	pH 10

2.3.4 Conclusions

Within this investigation, a preliminary physical stability analysis on gemini surfactant-base lipoplex systems was performed. The physical stability was assessed by examining the changes in the particle size distribution and membrane integrity of the gemini lipoplex system as a function of time, temperature and pH.

With respect to the particle size distribution, the gemini lipoplexes were found to aggregate over time, where the rate of aggregation was dependent upon the storage temperature. Lipoplex systems stored at higher temperatures were found to aggregate at a faster rate. In terms of the membrane integrity, the lipoplex systems were found to become more sensitive to DNase degradation over time, and as the storage temperature was increased.

Based on the combined experimental results from this analysis, it is evident that the physical stability of gemini lipoplex systems is limited by the membrane integrity. As shown in Figures 2.3.3.5 and 2.3.3.6, both of the 12-7NH-12 and 12-7-12 lipoplexes showed no evidence

of DNase protection after 4 weeks of storage. In contrast, there were no dramatic changes in the mean particle diameter of these lipoplexes over this 4 week period.

Chapter 3

Interactions between Gemini surfactants and pharmaceutical Tween surfactants

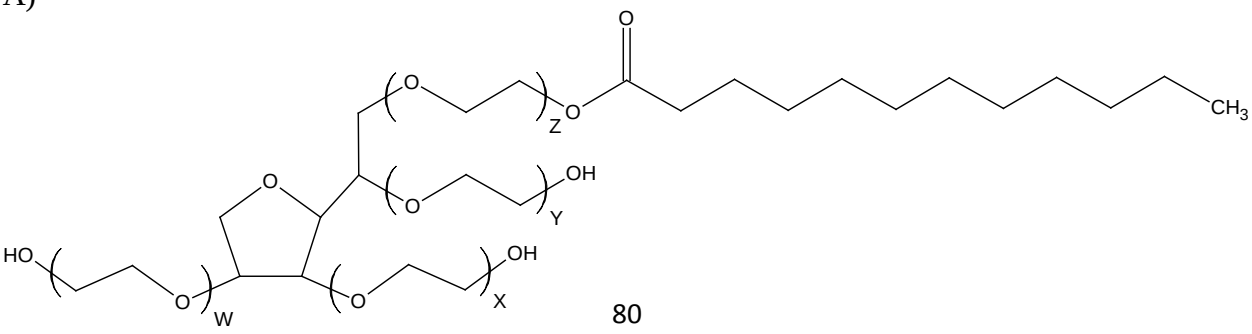
3.1 Introduction

As discussed extensively in Chapter 1, the unique solution properties of gemini surfactants make them intriguing candidates for use in pharmaceutical formulations. In order to further effectively assess the pharmaceutical applications of gemini surfactants, an understanding of their interactions with other surfactants typically found in pharmaceutical formulations is essential. Within this investigation, the interactions between gemini surfactants and the nonionic polyoxyethylene (20) sorbitan ester family of surfactants (known as the Tween surfactants) was examined. The Tween surfactants are perhaps the most commonly used surfactants in pharmaceutical formulations, in large due to their nontoxicity characteristics and approval as food grade surfactants.[1]

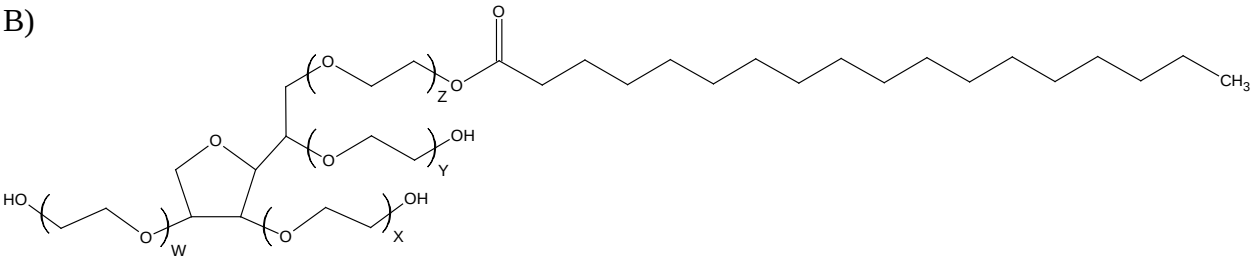
In this study, the interaction properties between the 16-3-16 gemini surfactant and a homologous series of polyoxyethylene (20) sorbitan ester surfactants having laurate (Tween 20), stearate (Tween 60) or oleate (Tween 80) alkyl tails was evaluated. The chemical structures of Tween 20, 60 and 80 are shown below in Scheme 3.1.1. As observed, all three Tweens share the same headgroup, but differ in the length and saturation of their alkyl tails. There are 12 carbons in the alkyl tail of Tween 20, which is in contrast to the 18 carbons in the alkyl tails of Tween 60 and Tween 80. Tween 80 differs from Tween 60 by the presence of an unsaturation at carbon 9 on the alkyl tail.

Scheme 3.1.1 Chemical structures of A) Tween 20, B) Tween 60, and C) Tween 80. Note in all cases, W+X+Y+Z = 20.

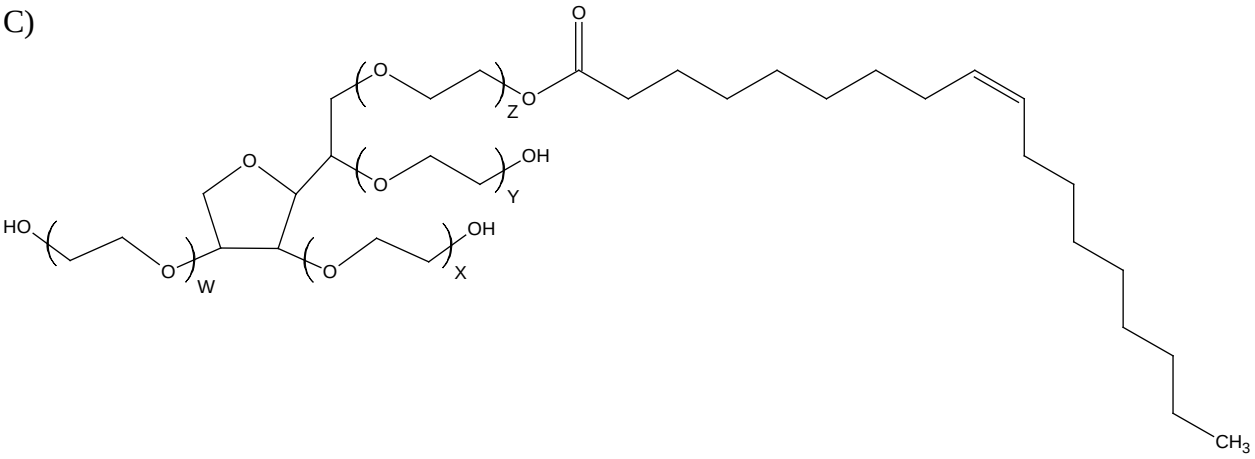
A)



B)



C)



In this investigation, the interaction properties between the gemini and Tween surfactants were examined and analyzed using Clint's, Rubingh's and Motomura's theories of mixed micellar systems. These theories were previously discussed in Chapter 2.2 of this thesis.

3.2 Experimental Procedures

3.2.1 Materials

The polyoxyethylene (20) sorbitan monolaurate (Tween 20), polyoxyethylene (20) sorbitan monostearate (Tween 60), and polyoxyethylene (20) sorbitan monooleate (Tween 80) surfactants were obtained from PCCA (Houston, Texas) and were used without any further purification.

The 16-3-16 gemini surfactant used was synthesized according to procedures previously reported in the literature.[2,3] The crude product was purified by recrystallization, and the structure

was confirmed using ^{1}H NMR spectroscopy (Bruker 300 MHz). The purity was confirmed by the absence of a minimum in the post micelle region of the surface tension versus log concentration plot.

Water for all solutions was obtained from a Millipore Synergy purification system.

3.2.2 Methods

Surface tension measurements were performed using a Lauda TE3 tensiometer, applying the du Noüy ring method. The temperature was kept constant at 25 ± 0.1°C using a circulating water bath. Surface tension values were corrected using the method Harkins and Jordan.[4] Individual surface tension measurements were repeated multiple times. The CMC was determined by the break in the plot of surface tension versus concentration.

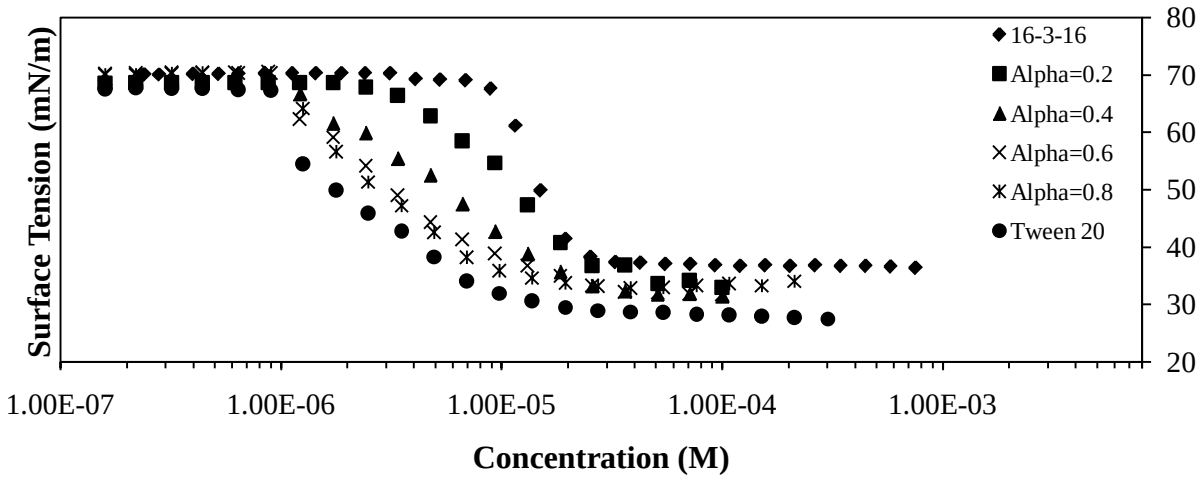
Mixed 16-3-16 and Tween solutions were prepared in the molar ratios of 0, 0.2, 0.4, 0.6, 0.8 and 1 (to a final concentration of 1 mM), and separately titrated into water. Surface tension was measured as a function of concentration of the single or binary amphiphile system.

3.3 Results and Discussion

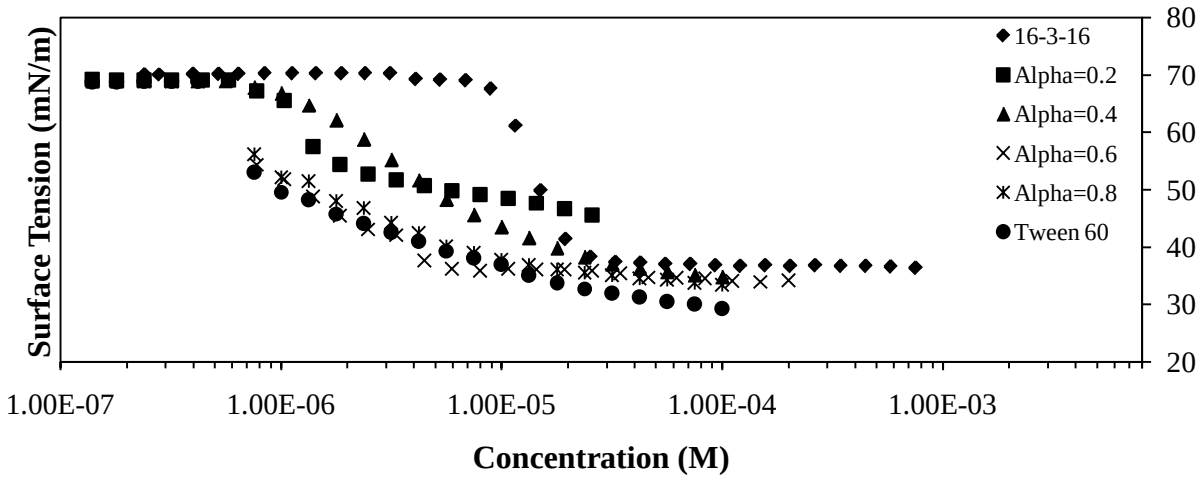
The results from this investigation were analyzed identically to those described in Chapter 2.2 of this thesis. Briefly, the CMCs of the mixed gemini/Tween systems were evaluated at different molar ratios between 0 and 1, using surface tension measurements (see Figure 3.3.1). The determined CMC values were then compared to the ideal values calculated using Clint's equation (Eq. 2.2.1). Rubingh's approach was subsequently used to calculate the mole fractions in the mixed micelle, and the results were compared to those in the ideal state as determined by Motomura's equation. Rubingh's approach was further used to calculate the interaction parameters, the activity coefficients, and the excess free energies of mixing for the binary gemini/Tween systems.

Figure 3.3.1 Surface tension plots for the mixed amphiphile systems of A) Tween 20 + 16-3-16, B) Tween 60 + 16-3-16, and C) Tween 80 + 16-3-16, at 25°C.[5] Note, alpha represents the mole fraction of the Tween surfactant in solution.

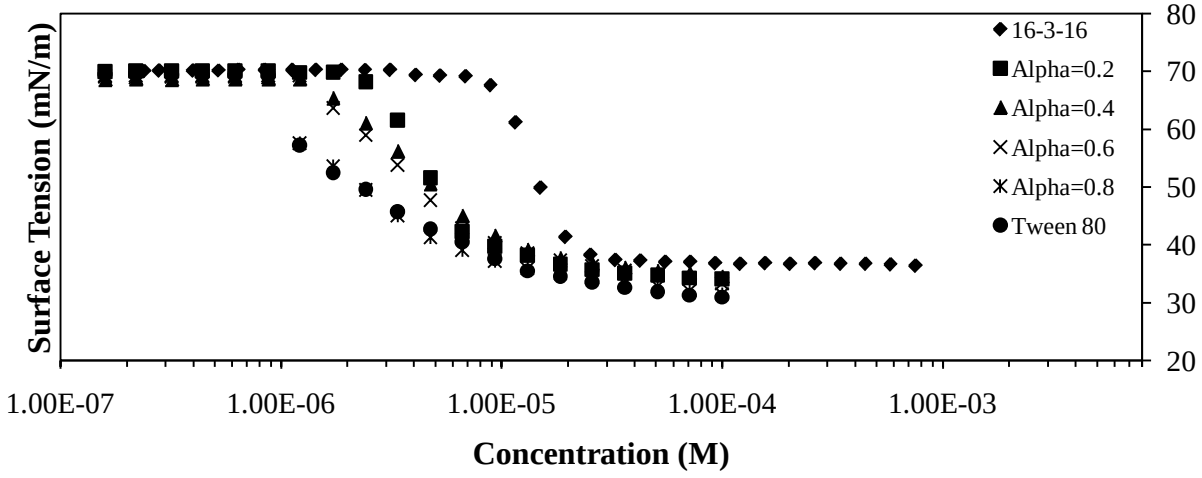
A)



B)



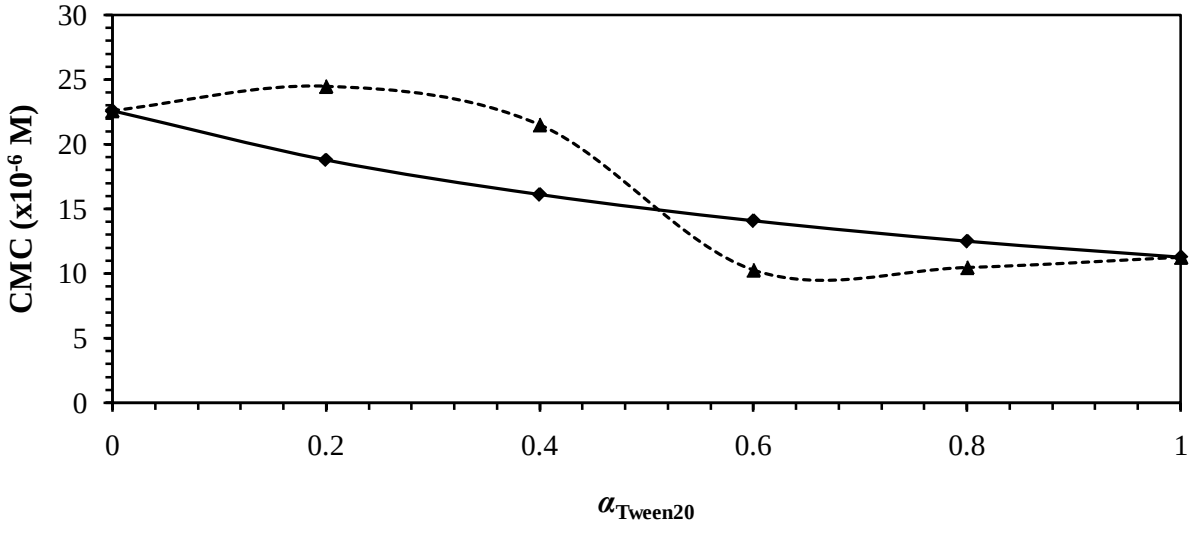
C)



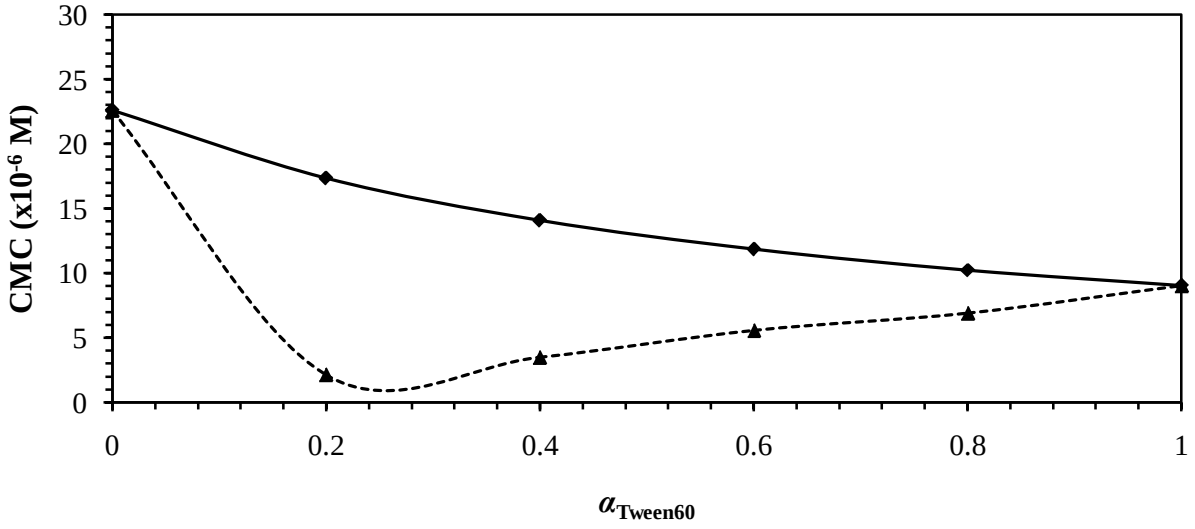
In Figure 3.3.2, the relationship between the CMC values of the mixed gemini/Tween systems and the molecular composition in solution is shown. The solid lines represent the ideal CMC values calculated using Clint's equation, while the dotted lines represent the determined experimental CMC values. As illustrated, the variations of the CMC values with composition appear to be different depending on the Tween surfactant in the mixture. In particular, the experimental CMC values for the Tween 20/16-3-16 system were initially larger than the ideal values; however, the experimental CMC values became smaller than the ideal values at larger mole fractions of Tween 20. For both the Tween 60 and Tween 80/16-3-16 systems, the experimental CMC values are always lower than those calculated ideally.

Figure 3.3.2 Plots of CMC versus α_{Tween} for the A) Tween 20 +16-3-16, B) Tween 60 + 16-3-16, and C) Tween 80 + 16-3-16 binary systems at 25°C.[5] Measured CMC values are represented as (▲); calculated ideal CMC values from Clint's model are represented as (♦).

A)



B)



C)

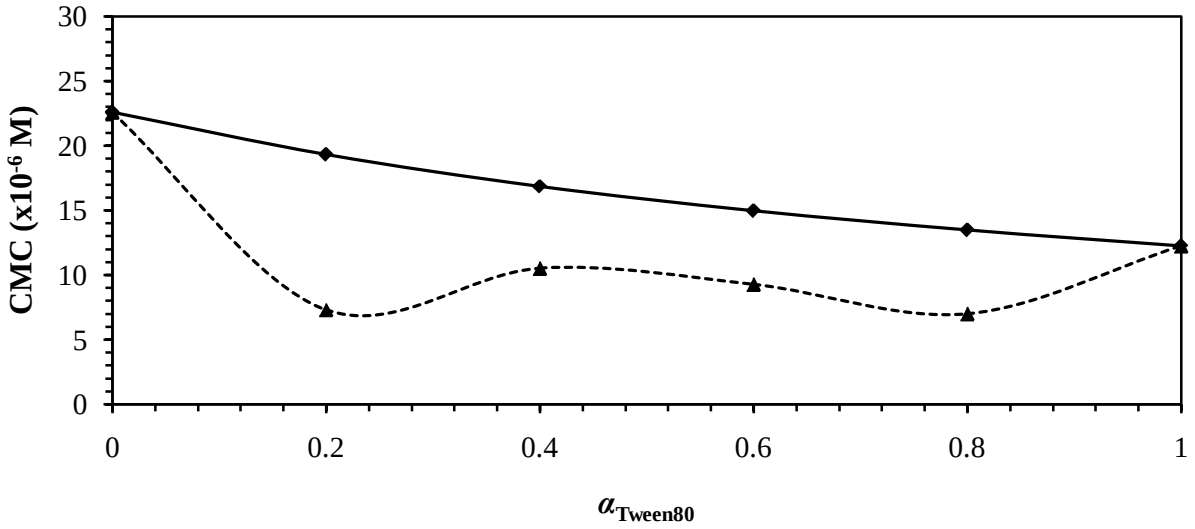


Table 3.3.1 Comparison of the measured and calculated CMC values for the single and binary 16-3-16 and Tween systems at 25°C.[5] Literature values are in brackets.

Tween 20 + 16-3-16			**Tween 60 + 16-3-16**			**Tween 80 + 16-3-16**		
$\alpha_{Tween20}$	**$CMC_{Exp.}$** ($x10^{-6}$M)	**CMC_{Clint}** ($x10^{-6}$M)	$\alpha_{Tween60}$	**$CMC_{Exp.}$** ($x10^{-6}$M)	**CMC_{Clint}** ($x10^{-6}$M)	$\alpha_{Tween80}$	**$CMC_{Exp.}$** ($x10^{-6}$M)	**CMC_{Clint}** ($x10^{-6}$M)
0	22.6	-	0	22.6	-	0	22.6	-
0.2	24.5	18.8	0.2	2.15	17.4	0.2	7.34	19.3
0.4	21.5	16.1	0.4	3.50	14.1	0.4	10.5	16.9
0.6	10.3	14.1	0.6	5.59	11.9	0.6	9.29	15.0
0.8	10.5	12.5	0.8	6.91	10.3	0.8	7.01	13.5
1	11.3 (11)[6]	-	1	9.02 (5.5)[6]	-	1	12.3 (18)[6]	-

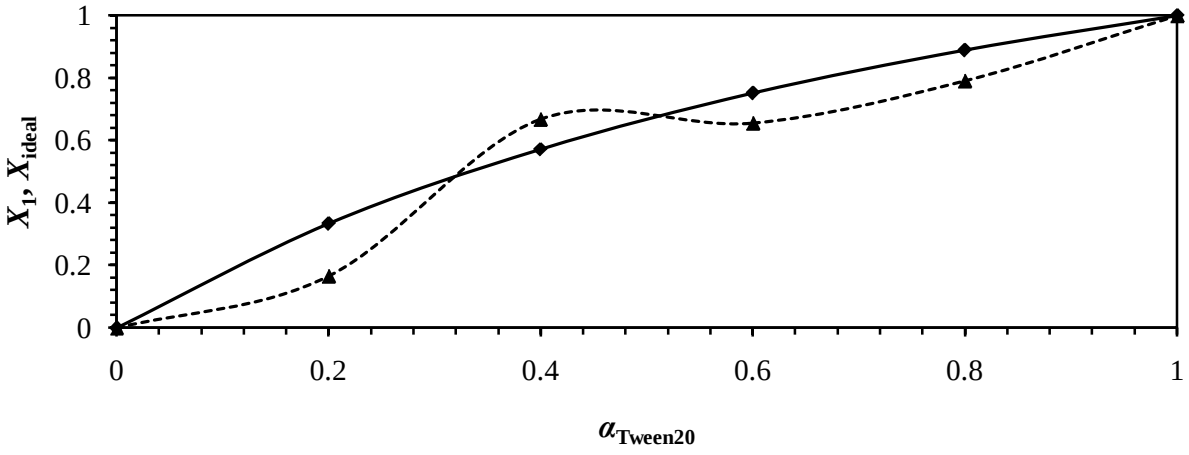
The evident deviations in the experimental CMC values from the ideal CMC values observed in Figure 3.3.2 suggest a nonideal mixing behaviour between the gemini and Tween surfactants.[7] As discussed in Chapter 1, a nonideal mixing behaviour is indicative of a net interaction between the amphiphiles, which can be attractive or repulsive.[7] The nature and strength of these interactions in the mixed gemini/Tween systems were evaluated and characterized using Rubingh's model for mixed micellar formation.

The mole fractions of the Tween surfactant in the mixed micelles were first determined using the previously described equation 2.2.2. These values were then compared to the ideal ones as calculated using Motomura's equation (Eq. 2.2.4). Figure 3.3.3 illustrates the comparison of X_1 and X_{ideal} as a function of α_{Tween} for the mixed Tween/16-3-16 systems. It can be seen that in the case of the Tween 60/16-3-16 and Tween 80/16-3-16 systems, X_1 was found to deviate positively from X_{ideal} until α_{Tween} approached approximately 0.35. These initial positive deviations from X_{ideal} demonstrate that within these low α_{Tween} regions, the mixed micelles are richer in the Tween surfactants and poorer in 16-3-16 than its intended ideal state. For α_{Tween} values greater than approximately 0.35, the mixed micelles became enriched with the 16-3-16 surfactant. In the case of the Tween 20/16-3-16 system, there is no apparent trend in the deviation of X_1 from X_{ideal}. It can be seen that the mixed micelle is initially enriched with 16-3-16 at low α_{Tween} values; and becomes enriched again with 16-3-16 at higher α_{Tween} values.

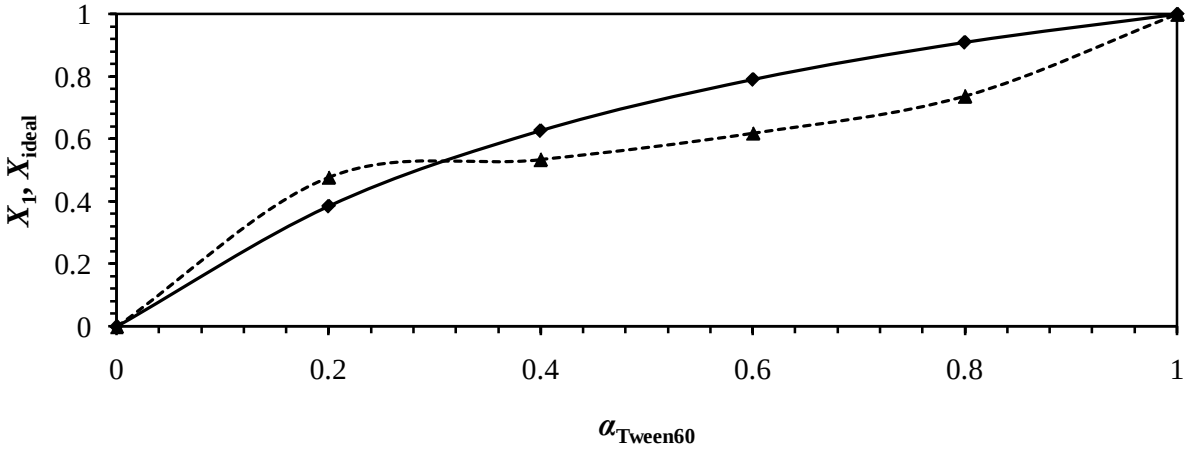
The interaction parameters for the mixed Tween/16-3-16 systems were subsequently calculated using equation 2.2.3, and the results are illustrated graphically in Figure 3.3.4. The computed β values were then used to calculate γ_1, γ_2, and ΔG_{ex}^{0} using equations 2.2.5, 2.2.6, and 2.2.7 respectively.

Figure 3.3.3 Micellar mole fractions, X_1 (▲) and X_{ideal} (♦), as a function of α_{Tween} for the A) Tween 20 + 16-3-16, B) Tween 60 + 16-3-16, and C) Tween 80 + 16-3-16 binary systems at 25°C.[5]

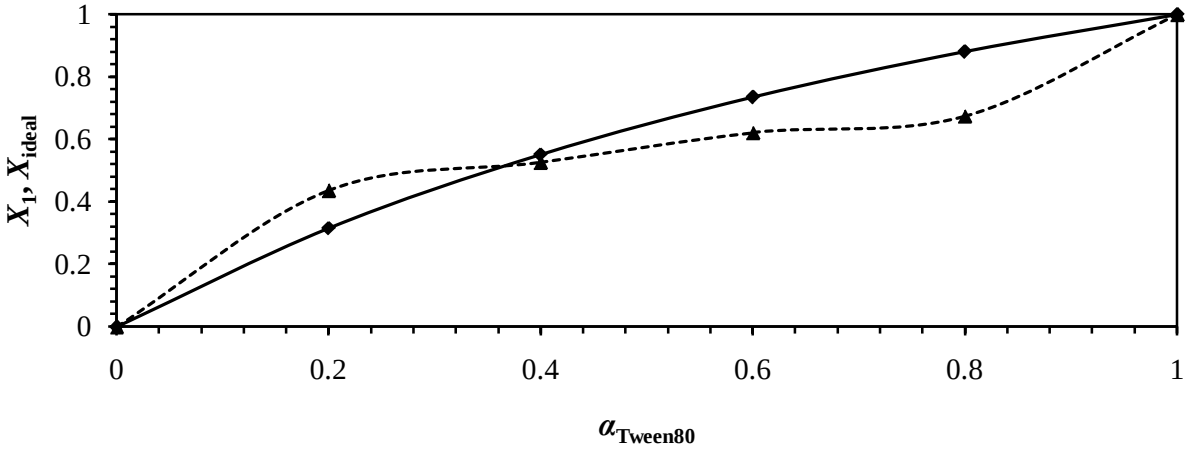
A)



B)



C)



The cumulative results of Rubingh's analysis for the mixed Tween/16-3-16 systems are tabulated in Table 3.3.2. Upon initial inspection, it is evident that there is a synergistic mixing interaction present in the Tween 60/16-3-16 and Tween 80/16-3-16 systems, due to the negative values of β over all solution compositions. Furthermore, the activity coefficients (γ_1, γ_2) for these two systems were always found to be less than unity, thus confirming a nonideal mixing behaviour which is attractive in nature.[7] As well, the negative values for the excess free energies of mixing (ΔG_{ex}^{0}) suggest that the mixed micelles in these systems are more stable than the micelles of the individual components.[7] For the mixed Tween 20/16-3-16 system, it is evident that there is an initial slight antagonistic interaction at low α_{Tween} values, due to the positive values of β. However, the interaction becomes synergistic at α_{Tween} values of 0.6 or higher.

Figure 3.3.4 Interaction parameter as a function of α_{Tween}. β values are represented as (♦) for the Tween 20; (■) for the Tween 60; and (▲) for the Tween 80 binary systems with 16-3-16 at 25°C.[5]

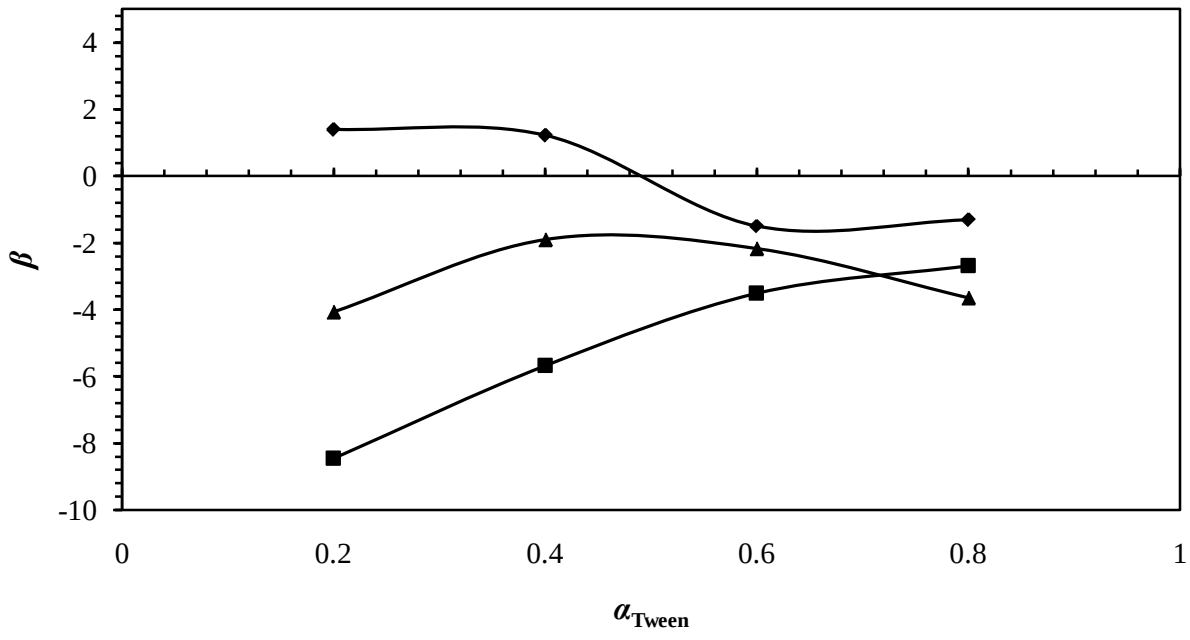


Table 3.3.2 Results obtained from Rubingh's analysis for the mixed Tween and 16-3-16 systems.[5]

System	α_{Tween}	X_1	$CMC_{Exp.}$ ($x10^{-6}M$)	β	γ_1	γ_2	ΔG_{ex}^0 (KJ/mole)
Tween 20	0	0	22.6	-	-	-	-
+	0.2	0.17	24.5	1.4	2.6	1.0	0.47
16-3-16	0.4	0.67	21.5	1.2	1.1	1.7	0.67
	0.6	0.66	10.3	-1.5	0.84	0.53	-0.83
	0.8	0.79	10.5	-1.3	0.94	0.44	-0.54
	1	1	11.3	-	-	-	-
Tween 60	0	0	22.6	-	-	-	-
+	0.2	0.48	2.15	-8.5	0.10	0.15	-5.2
16-3-16	0.4	0.53	3.50	-5.7	0.29	0.20	-3.5
	0.6	0.62	5.59	-3.5	0.60	0.26	-2.1
	0.8	0.74	6.91	-2.7	0.83	0.23	-1.3
	1	1	9.02	-	-	-	-
Tween 80	0	0	22.6	-	-	-	-
+	0.2	0.44	7.34	-4.1	0.28	0.46	-2.5
16-3-16	0.4	0.53	10.5	-1.9	0.65	0.59	-1.2
	0.6	0.62	9.29	-2.2	0.73	0.43	-1.3
	0.8	0.67	7.01	-3.7	0.68	0.19	-2.0
	1	1	12.3	-	-	-	-

Synergistic interactions are typically observed in mixed ionic and nonionic surfactant systems, and have been rationalized by Maeda et al. to be the result of the insertion of nonionic surfactant monomers between the ionic surfactant monomers in the mixed micelle, which results in an overall reduction of the electrostatic repulsive interactions between the headgroups.[8] As a result of these reduced repulsive interactions, a more stabilized micelle is formed.[8] This explanation likely accounts for the general synergism experienced in the mixed gemini/Tween systems, which is likely attributed to the insertion of the nonionic Tween monomers between the cationic gemini monomers in the mixed micelle. It is interesting to point out that some authors have indicated that the stabilization of mixed micelles composed of cationic surfactants and nonionic surfactants consisting of polyoxyethylene groups can be explained on the basis of the ion-dipole interactions occurring between the ether oxygens and the cationic headgroups.[9,10]

Although this is a possible contribution, the stabilization that occurs between the Tween surfactants and 16-3-16 is most likely consistent with the electrostatic stabilization that occurs from the insertion of the Tween monomers between the 16-3-16 monomers in the mixed micelle. There may, however, also be a stabilizing interaction between the dicationic headgroup of 16-3-16, and the polar ethylene oxide chain and sorbitan ring of the Tween surfactants.

The magnitudes of β ranged between -2.7 and -8.5 for the Tween 60/16-3-16 system, and between -1.9 and -4.1 for the Tween 80/16-3-16. These values are consistent with those typically observed in mixed *m-s-m* gemini and nonionic systems,[11-14] and are in good agreement with those generally observed for mixed ionic/nonionic systems.[15] For the Tween 20/16-3-16 system, the magnitude of β was found to deviate slightly around 0 with α_{Tween}, thus suggesting a near ideal mixing behaviour for this system. Such an observation has been observed before in the literature for mixed cationic *n*-alkylpyridinium bromide and nonionic nonylphenyl ethoxylate surfactant systems.[16]

Upon closer examination of the experimental results, it is clear that the magnitude of β is dependent upon the chemical structure of the Tween surfactant in the binary system. As shown in Figure 3.3.4, the Tween 60 surfactant was generally found to experience the strongest synergistic interaction with 16-3-16, followed by Tween 80 and Tween 20. The disparities in interaction between 16-3-16 and the Tween surfactants can be rationalized using Maeda's theory for mixed surfactant systems. According to Maeda, the transfer process of an ionic surfactant monomer to a nonionic micelle will consist of two distinct contributions, i) the interaction between the surfactant headgroups, and ii) the interaction between the hydrocarbon chains of the respective surfactants.[8] In our case, since the headgroups of the Tween surfactants are the same (see

Scheme 3.1.1), any differences in β can most likely be attributed to differences in the hydrocarbon tail of the Tween surfactant.

As shown in Figure 3.3.4, it is observed that for a fixed α_{Tween} value, the stability of the mixed micelle (i.e. magnitude of β) was found to decrease as the alkyl chain length of the Tween surfactant is decreased from 18 carbons (Tween 60/Tween 80) to 12 carbons (Tween 20). Furthermore, the stability of the mixed system was observed to generally increase as the unsaturation in the Tween alkyl tail is removed (Tween 60 versus Tween 80). This mixed micellar stability dependence on the alkyl chain length results from a more stabilized packing of the Tween 60/Tween 80 monomers with the 16-3-16 monomers in the mixed micelle, in comparison to that in the Tween 20/16-3-16 system. The alkyl tail lengths of Tween 60/Tween 80 only differ from 16-3-16 by 2 methylene units, which is in contrast to a difference of 4 methylene units in the Tween 20 and 16-3-16 system. Therefore, in the mixed Tween 60/16-3-16 and Tween 80/16-3-16 systems, there is likely an enhanced packing efficiency in the micelle, in comparison to the Tween 20/16-3-16 system. In fact, in the literature it has been observed that synergistic interactions between surfactants are enhanced when their alkyl tail lengths are matched;[17] this was rationalized by the findings of Shiao et al. who reported that there is an increased order, tighter packing and greater stability of the mixed micelle, when the length of adjacent hydrocarbon chains are the same.[18] With regard to the effect of the alkyl tail saturation, Tween 80 experiences a weaker synergistic interaction with 16-3-16 (in comparison to the Tween 60/16-3-16 system) as a result of the hampered packing efficiency of the alkyl chains in the micelle by the cis double bond present in the oleate tail of Tween 80.

It is important to note that in all cases, the magnitude of β was found to vary with the molar fraction of the Tween surfactant (see Figure 3.3.4). According to Rubingh's theory, the

interaction parameter should remain constant over the entire range of composition.[7] However, numerous studies, in particular, studies of mixed cationic and nonionic systems have found β to vary with composition.[19-22] As illustrated in Figure 3.3.4, there is no apparent trend in the magnitude of β with composition, which has been observed in the literature.[14,21,22]

3.4 Conclusions

Within this study, the nature and strength of the interactions between the 16-3-16 gemini surfactant and a homologous series of Tween surfactants was investigated. The results demonstrate a synergistic mixing behaviour between the cationic gemini and the nonionic Tween surfactants, which can be explained in terms of the decreased electrostatic repulsions between the headgroups due to the insertion of the Tween monomers between the cationic gemini monomers in the mixed micelle. The longer chain Tween 60 and Tween 80 surfactants were found to experience a stronger synergistic effect with 16-3-16, in comparison to the shorter chain Tween 20. Furthermore, the presence of the unsaturation in the alkyl tail of the Tween 80 surfactant was found to decrease the strength of the synergistic interaction with 16-3-16, in comparison to the Tween 60/16-3-16 system.

The results from this analysis are of interest for the applications of gemini surfactants in pharmaceutical preparations. As discussed in chapter 1, gemini surfactants possess numerous unique solution properties which make them advantageous for use in pharmaceutical formulations. These advantages coupled with the fact that gemini surfactants can potentially demonstrate strong synergistic mixing interactions with the commonly used Tween surfactants, further enhances the attractiveness of the use of gemini surfactants in pharmaceutical preparations. The synergistic interaction experienced between gemini and Tween surfactants can reduce the total amount of these surfactants required for a particular application, which in turn

reduces costs and environmental impact. Furthermore, the stabilized gemini/Tween mixed micelle formed may be of interest for the development of drug carrier systems.

Chapter 4

Conclusions

Within this work, two distinct projects were carried out to assess the pharmaceutical applications of gemini surfactants. The objective of the first project was to assess the applications of gemini surfactants as transfection agents for nonviral gene delivery by evaluating the physical stability characteristics of gemini surfactant-based lipoplex systems. Prior to this investigation, the physicochemical properties of the lipoplex system were evaluated by investigating the interactions between gemini surfactants and DNA, and between gemini surfactants and DOPE.

In chapter 2.1, the interaction between gemini surfactants and DNA was investigated using a combination of ITC, particle size, zeta potential and surface tension measurements. In the forward titration manner (i.e. gemini surfactant into DNA), surface tension results revealed that the CAC is dependent upon the alkyl tail length of the gemini surfactant but not on the structure of the spacer group (*m*-3-*m* vs. *m*-7-*m* vs. *m*-7NH-*m*). The 16-*s*-16 series of surfactants were always found to have a significantly smaller CAC in comparison to the 12-*s*-12 series, which is rationalized by the enhanced hydrophobic interactions in the 16-*s*-16/DNA complex. Complexation in the forward sequence was further evaluated using ITC, and the results obtained in our study were found to be in good agreement with those previously reported in the literature; that is, the enthalpy of binding between gemini surfactants and DNA is endothermic, and remains fairly constant until the reaction endpoint, where it drops abruptly to approximately 0 kJ/mol. In contrast, in the reverse titration manner, a combination of ITC, particle size and zeta potential measurements revealed that the mechanism of complexation between gemini surfactants and DNA likely involves a complex series of linked equilibria, where the system progresses from a “beads on a string” type interaction to a complete reorganization and the formation of neutral complexes, followed by flocculation and precipitation.

In chapter 2.2, the mixing interaction between gemini surfactants and DOPE was investigated using Clint's, Rubingh's and Motomura's theories for mixed micelle formation. It was observed that the mixing interaction between the 16-3-16, 16-7-16 and 16-7NH-16 gemini surfactants and DOPE is antagonistic in nature, which is rationalized in terms of the differences in the preferred aggregate structures of these two components. The strength of the antagonistic interaction was found to be dependent upon the spacer group, where 16-3-16 experienced the strongest antagonistic interaction with DOPE, followed by 16-7NH-16 and then 16-7-16. Furthermore, the strength of the antagonistic interaction was found to be dependent upon the solution composition, where the magnitude of β was observed to decrease as the gemini surfactant molar fraction in solution was increased. Future studies in this investigation could potentially involve examining the effect of the gemini surfactant alkyl tail length on the interaction properties with DOPE; as well as using TEM images to examine how the structure of the gemini/DOPE aggregate evolves as the molar ratio of these components are varied.

Chapter 2 was concluded by evaluating the physical stability characteristics of gemini surfactant-based DNA delivery systems. The physical stability analysis was carried out in accordance to the FDA's definition of the physical stability of liposome drug products, which is "a function of the integrity and size distribution of the lipid vesicles." The membrane integrity characteristics of the gemini surfactant-based lipoplexes were evaluated using DNase sensitivity assays, while the particle size distribution characteristics were evaluated using DLS and by performing zeta potential measurements. Our results revealed that the physical stability of gemini surfactant-based lipoplexes is limited by their membrane integrity characteristics, which can potentially be attributed to the dissociation of the lipoplex structure as a result of the antagonistic mixing interaction experienced between the micelle-forming gemini surfactants and

DOPE. Future studies can potentially involve examining the stability characteristics of lipoplex systems formulated with gemini surfactants that a have a preference to form vesicular aggregates. This can potentially result in a more favourable mixing interaction between the gemini surfactant and DOPE, and ultimately a more stabilized gemini/DOPE aggregate. In addition, future studies should involve examining the transfection efficiency of the gemini lipoplex as a function of storage time.

The second objective of this thesis was to investigate the interactions between gemini and Tween surfactants. As discussed in chapter 3, an understanding of the interactions between gemini surfactants and other surfactants typically found in pharmaceutical formulations is essential when assessing the applications of gemini surfactants for use in pharmaceutical preparations. The results from this analysis demonstrated a general synergistic mixing behavior between the cationic gemini and the nonionic Tween surfactants, which was explained in terms of the decreased electrostatic repulsive forces between the headgroups due to the insertion of the Tween monomers between the cationic gemini monomers in the mixed micelle. The strength of synergism between the gemini and Tween surfactants was found to be dependent upon on the alkyl tail length of the Tween surfactant, where both the 18 carbon Tween 60 and Tween 80 surfactants experienced a stronger synergistic interaction with 16-3-16, in comparison to the 12 carbon Tween 20. As well, the saturation of the Tween alkyl tail was found to have an effect of the interaction with 16-3-16, where the Tween 60 (no unsaturations) was found to have a stronger synergistic with 16-3-16, in comparison to Tween 80 which has an unsaturation in its alkyl tail. Future studies can potentially look at examining the effect of the chemical structure of the gemini surfactant (i.e. spacer and alkyl tail length) on the interaction properties with the Tween family of surfactants. As well, this project could be expanded by evaluating the

interactions between gemini surfactants and a variety of other family of surfactants commonly found in pharmaceutical formulations; some examples include the sorbitan ester (Span) and polyoxyethylated glycol monoether class of surfactants.

References

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